

A Matter of Life and Death:
Novel Ligands Mediating Cytotoxic Function and Revealing
Metabolically Stimulative Function of the Sigma-2 Receptor in
Human Cancer

Hilary Nicholson

A.B., Biochemistry, Colgate University, 2012

A.M., Molecular Pharmacology and Physiology, Brown University, 2015

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of
Philosophy in the Division of Biology and Medicine at Brown University

May 2016

© Copyright 2016 by Hilary Nicholson

This dissertation by Hilary Nicholson is accepted in its present form by the Division of Biology and Medicine as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date _____

Wayne D. Bowen, Ph.D., Advisor

Recommended to the Graduate Council

Date _____

John Marshall, Ph.D., Reader

Date _____

Rae Matsumoto, Ph.D., Reader (Touro University)

Date _____

Elena Oancea, Ph.D., Reader

Date _____

Anatoly Zhitkovich, Ph.D., Reader

Approved by the Graduate Council

Date _____

Peter Weber, Dean of the Graduate School

Hilary Nicholson

Brown University

Department of Molecular Pharmacology, Physiology, and Biotechnology

Box G, 171 Meeting St, Providence RI 02912

617-513-6837 (cell)/401-863-6137 (lab) hilary_nicholson@brown.edu

Education

Doctoral Candidate, **Brown University**, Providence RI (begun 9/2012, advanced to candidacy 12/2013)

- Molecular Pharmacology and Physiology, anticipated defense date March 2, 2015

Bachelor of Arts, Magna Cum Laude, **Colgate University**, Hamilton, NY (9/2008-5/2012)

- Biochemistry, high honors
- High Distinction in the Liberal Arts

Research Experience

Doctoral Research, Bowen Lab, Brown University, Providence, RI. 9/2012-present

- *Investigation of the function of the sigma-2 receptor in the modulation of cancer cell viability and metabolism.*

Research Associate, Eaton-Peabody Lab, Massachusetts Eye and Ear Infirmary (Harvard Affiliates), Boston, MA. 5/2011-8/2011

- *Development of an infrared-responsive auditory brainstem implant in a rat model.*

Student Researcher, Department of Chemistry, Colgate University, Hamilton, NY. 3/2011-5/2012

- *Synthesis and characterization of P48S/A49P, K51A, and A49 H. influenzae carbonic anhydrase mutants.*

Research Associate, Cell and Molecular Biology Lab, Sophia Gordon Cancer Center, Lahey Clinic, Burlington, MA.

5/2010-12/2010

- *Development of biomarker profile for progression of human bladder cancer for development of personalized treatment plan.*

Publications

Hilary Nicholson, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen, “Sigma-2 receptors play a role in cellular metabolism: Stimulation of glycolytic hallmarks by CM764 in human SK-N-SH neuroblastoma,” *Journal of Pharmacology and Experimental Therapeutics* 2015; published online before print 16 November 2015.

Hilary Nicholson, Anthony Comeau, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen, “Characterization of CM572, a selective irreversible partial agonist of the sigma-2 receptor with antitumor activity,” *Journal of Pharmacology and Experiment Therapeutics* 2015; 354(2):203-12.

Katherine M. Hoffmann, H. Rachael Million-Perez, Richard M. Merkhofer, Jr., **Hilary E. Nicholson** and Roger S. Rowlett, “Allosteric Reversion of Haemophilus influenzae beta-Carbonic Anhydrase via a Proline Shift,” *Biochemistry* 2015; 54(2):598-611.

In preparation:

Hilary Nicholson, Zongyi Liu, and Wayne D. Bowen, “Sigma-2 receptor is a novel target for selective treatment of human triple negative breast cancer,” expected submission March 2016.

Hilary Nicholson, Walid Alsharif, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen, “Structure Activity Relationship of sigma-2 receptor ligands and *in vitro* function in SK-N-SH neuroblastoma,” expected submission March 2016.

Honors and Awards

- Invited to present research at St. Jude Children’s Hospital Future Fellow Research Conference in Memphis, TN (6/2015)
- Selected as single institutional nominee for PhRMA Foundation Predoctoral Fellowship in Pharmacology/Toxicology by internal competition, Brown University (7/2014)
- Brown Chapter of Sigma Xi Prize, Brown University (5/2014)

- Class of 1997 Award for Academics, Community Commitment, and Leadership, Colgate University (5/2012)
- Turner Award for Best Undergraduate Honors Thesis, Colgate University (5/2012)
- Dean's Award for Academic Excellence, Colgate University (7 of 8 semesters)
- $\Phi\chi\Sigma$ Academic Honor Society, National (inducted Spring 2009)
- $\Gamma\Sigma\Lambda$ National Greek Honor Society, National (inducted Spring 2011)

Funding

- NIH IMSD Research Assistantship, NIGMS R25GM083270 (9/2014-present)
- Pharmacia Predoctoral Fellow (9/2013-8/2014)
- NIH T32 Predoctoral Fellow, NIGMS 5T32GM077995 (9/2012-8/2014)
- Summer Research Fellowship, Colgate University (5/2013-9/2013)

Invited Presentations

Chemistry Department Seminar, Colgate University, October 2014. "Crushing chemo: Exploitation of the sigma-2 receptor in chemotherapy across a variety of cancers." Hamilton, NY.

Abstracts and Poster Presentations

Hilary Nicholson and Wayne Bowen. The role of sigma-2 receptors in cellular metabolism: Stimulation of glycolytic hallmarks in SK-N-SH neuroblastoma. Poster presented at: 23rd Annual Lifespan Hospitals Research Symposium 2015; Providence, RI.

Cheri Liu, **Hilary Nicholson**, and Wayne Bowen. The sigma-2 receptor as a novel therapeutic target for triple negative breast cancer. Poster presented at: 23rd Annual Lifespan Hospitals Research Symposium 2015; Providence, RI.

Hilary Nicholson, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen. A potential neuroprotective function for the sigma-2 receptor. *Nanosymposium* presented by H. Nicholson at: Society for Neuroscience Annual Meeting 2015; Chicago, IL.

Zongyi Liu, **Hilary Nicholson**, Emily Savoca, and Wayne D. Bowen. Factors that affect sensitivity to sigma-2 receptor-mediated cell death in human SK-N-SH neuroblastoma. *Nanosymposium* presented by Z. Liu at: Society for Neuroscience Annual Meeting 2015; Chicago, IL.

Hilary Nicholson, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen. Secret of Sigma-2: Chemotherapeutic potential of sigma-2 receptor ligands in high risk neuroblastoma. Poster presented at: St. Jude Children's Hospital Future Fellow Research Conference 2015; Memphis, TN.

Hilary Nicholson, Walid Alsharif, Christopher R. McCurdy, and Wayne D. Bowen. Evaluation of structural changes in SN79-derived sigma-2 receptor modulators: effect on apoptotic efficacy in SK-N-SH neuroblastoma. Poster presented at: American Association for Cancer Research Annual Meeting 2015; Philadelphia, PA.

Zongyi Liu, **Hilary Nicholson**, and Wayne D. Bowen. Sigma-2 receptor-induced cell death: a novel approach to triple-negative breast cancer treatment. Poster presented at: American Association for Cancer Research Annual Meeting 2015; Philadelphia, PA.

Timothy Chou, Michaela Jacobs, **Hilary Nicholson**, and Wayne D. Bowen. Differential aggregation rates and therapeutic response of pancreatic cancer cell lines to sigma-2 receptor activation in 3D culture. Poster presented at: American Association for Cancer Research Annual Meeting 2015; Philadelphia, PA.

Hilary Nicholson, Nelly Waledji, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen. Metabolic stimulation: A novel function for the sigma-2 receptor with implications for neuroprotection. Poster presented at: 2nd Annual Mind Brain Research Day (2015); Providence, RI.

Hilary Nicholson, Nelly Weledji, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen. Metabolic stimulation by CM769: A novel function for the sigma-2 receptor. Poster presented at: Society for Neuroscience Annual Meeting 2014; Washington, D.C.

Hilary Nicholson, Pei Ling Chia, Anthony Comeau, Christophe Mesangeau, Christopher McCurdy, and Wayne Bowen. Characterization of CM-572 and CM-769: Novel irreversible modulators of sigma-2 receptor function. Poster presented at: American Association for Cancer Research Annual Meeting 2014; San Diego, CA.

Hilary Nicholson, Anthony Comeau, Christophe Mesangeau, Christopher McCurdy, and Wayne Bowen. Irreversible modulators of Sigma-2 receptor function: Isothiocyanate derivatives of SN-79. Poster presented at: Society for Neuroscience Annual Meeting 2013; San Diego, CA.

Hilary Nicholson, Anthony Comeau, Christophe Mesangeau, Christopher McCurdy, and Wayne Bowen. Development of selective irreversible antagonists for sigma-2 receptors. Poster presented at: American Association for Cancer Research Annual Meeting 2013; Washington, D.C. and Rhode Island Healthcare Showcase 2013; Providence, RI.

Donald Koroma, **Hilary Nicholson**, Kyle Totaro, Jason K. Sello, and Wayne D. Bowen. Examining the potential of proteasome inhibitors for curing cancer. Poster presented at: 7th Biology New England South Symposium; Boston, MA.

Roger S. Rowlett, Katherine M. Hoffmann, H. Rachael Million-Perez, Richard Merkhofer, and **Hilary E. Nicholson**. Allosteric reversion of *Haemophilus influenzae* β -carbonic anhydrase by a prolife shift variant. Poster presented at: Meeting of the American Crystallographic Association; Boston, MA.

Rohit Verma, Kenneth E. Hancock, Amilie A. Guex, Nedim Durakovic, **Hilary Nicholson**, Colette M. McKay, M. Christian Brown, and Daniel J. Lee. Responses of the inferior colliculus to optical stimulation of the cochlear nucleus. Poster presented at: Association for Research in Otolaryngology 35th Annual Midwinter Meeting 2012; San Diego, CA.

Teaching Experience

- **Instructor and Course Designer**: Summer@Brown course for high school students, School of Professional Studies, Brown University, July 2015. “Drug Discovery: Treating Human Disease through Medicine.”
- **Guest lecture**: BIOL1260: Physiological Pharmacology, Brown University, December 2014. “Cancer Pharmacology.”
- **Guest lecture**: Drug Discovery: Treating Human Disease through Medicine, Summer@Brown, Brown University, July 2014. “Cancer.”
- **Teaching Assistant**, Physiological Pharmacology, Professor John Marshall, Brown University, Providence, RI, Fall 2013.
- **Workshop Developer**, Basic Laboratory Skills Workshop, Brown University, Providence, RI, Fall 2013-Spring 2014.
- **Peer Tutor**, Center for Learning, Teaching, and Research, Colgate University, Hamilton, NY.
- **Laboratory Teaching Assistant**, General and Organic Chemistry, Department of Chemistry, Colgate University, Hamilton, NY.

Mentorship

- **Ashley Wu**, Brown University ScB Class of 2016, summer research, independent study, honors thesis (6/2015-present).
- **Vira Behnam Roudsari**, Brown University ScB Class of 2015, summer research, independent study, honors thesis (9/2012-5/2015). Current position: research associate at DocASAP.
- **Donald Koroma**, Brown University ScB Class of 2015, summer research, independent study, honors thesis (5/2013-5/2015). Current position: Brown University predoctoral student in Molecular Pharmacology and Physiology.
- **Nelly Weledji**, Brown University ScB Class of 2015, summer research, independent study, honors thesis (6/2013-5/2015), Ruth and William Silen, M.D. Third Place Award for Oral Presentation at

New England Science Symposium 2015. Current position: research assistant at Brigham and Women's Hospital.

- **Timothy Chou**, Brown University ScB Class of 2015, summer research, independent study, honors thesis (6/2013-5/2015). Current position: research assistant at Memorial Sloan-Kettering Cancer Center.
- **Michaela Jacobs**, Brown University ScB Class of 2015, summer research, independent study (6/2013-12/2013). Current position: Brown University Medical School student, class of 2019.
- **Pei Ling Chia**, Brown University ScB Class of 2015, summer research, independent study, honors thesis (9/2013-12/2014), Current position: research assistant at Singapore national laboratory.
- **Apurva Limaye**, Brown University ScM Class of 2015, Master's thesis research (9/2013-5/2015).

Professional Society Memberships

- American Association for Cancer Research (AACR, 2012-present)
- American Society of Clinical Oncology (ASCO, 2013-present)
- Society for Neuroscience (SfN, 2013-present)
- Sigma Xi (2014-present)
- American Association for the Advancement of Science (AAAS, 2014-present)
- American Society for Pharmacology and Experimental Therapeutics (APSET, 2014-present)

Service Activities

- **Regional Coordinator and Active Member**, ScienceCheerleader, society to promote science among young people and challenge stereotypes surrounding female scientists, Fall 2013-present.
- **Graduate Mentor**, Graduate-Undergraduate Mentorship Initiative, Brown University, Providence, RI, Fall 2013-present.
- **Departmental Representative and Nominations Committee Member**, Graduate Student Council, Brown University, Providence, RI, Fall 2012-Fall 2015.
- **Graduate Student Representative**, Brown University Graduate Council, Providence, RI, Fall 2013-Fall 2015.
- **Graduate Student Representative**, Honorary Degrees Advisory Committee, Brown University, Providence, RI, Fall 2014-Spring 2015.
- **Committee Member**, Board of Trustees Young Alumni Selection Committee, Brown University, Providence, RI, Spring 2015.
- **Executive Member**, College Curriculum Committee, Brown University, Providence, RI, Fall 2013-present.
- **Officer**, Colgate Society of Chemists, Hamilton, NY, Fall 2010-March 2011.
- **President**, Colgate Panhellenic Association, Hamilton, NY, Fall 2010-Fall 2011. VP Spring-Fall 2010. Greek Woman of the Year, 2010.

Abstract

The sigma-2 receptor is a pharmacologically-defined protein receptor present in a variety of tissues, and interest in its role in a wide array of diseases has been expanding since its discovery by Wayne Bowen in 1990. Expression is most highly upregulated in rapidly proliferating cancer cells, suggesting a role in cancer cell survival. Although the endogenous ligand has not yet been elucidated, a variety of synthetic agonists have been developed that induce apoptosis upon binding the sigma-2 receptor. This function, coupled with selectively high expression on rapidly proliferating cancer cells, makes the sigma-2 receptor an attractive target for the imaging and treatment of cancer.

One such agonist is CM572, an isothiocyanate derivative of the sigma-2 receptor-selective ligand SN79. CM572 irreversibly binds and activates sigma-2 receptors to induce apoptotic cell death at doses that do not cause significant levels of cytotoxicity in non-cancerous cells. Further, CM572 is able to reduce viability of highly aggressive cancers including triple negative breast and pancreatic cancer, suggesting a cell death mechanism that is not susceptible to conventional methods of developing drug resistance that are common in these cancers. Large scale implications for clinical use of CM572 include improved tumor imaging, more selective chemotherapy with reduced off-target adverse effects, and less frequent dosing regimens by virtue of its irreversible binding capability.

While CM572 shows great promise as a therapeutic agent, the structurally related SN79 derivative CM764 sheds light on novel aspects of sigma-2 receptor biology. Activation of sigma-2 receptors with CM764 leads to an increase in metabolic reduction of MTT and induction of several hallmarks of glycolysis, including increased HIF-1 α protein levels during normoxia and

increased expression of VEGF. This stimulation of metabolism was not coupled to proliferation, indicating a unique sigma-2 receptor-mediated potentially pro-survival benefit consistent with the observed upregulation of this receptor in rapidly proliferating tissues. In addition to the discovery of a novel sigma-2 receptor-mediated function, this study may represent a first glimpse into the endogenous mechanism of sigma-2 receptors. Together, these findings demonstrate the ability of sigma-2 receptors to mediate both cytotoxic and pro-survival functions in human cancers.

Acknowledgements

After the first thesis defense I ever went to, a group of people went out to dinner with the newly minted PhD and her boyfriend. During the meal, it was decided that the boyfriend deserved an “honorary PhD” for all of his help along the way (strictly speaking in non-research terms—his profession involved skydiving and no pharmacology to speak of). As I now finish my own PhD, I realize that such “honorary PhDs” are more deserved than I initially thought possible during that first celebratory dinner. And so, I’d like to hand out a few “honorary PhDs” to the people with whom I certainly share my own doctorate. This accomplishment is not mine alone, and could never have been completed that way.

The first honorary degree, although he is not wanting for accolades of his own, goes to Dr. Wayne D. Bowen. I came to you with overly ambitious ideas (to put it mildly) and an outline in mind for my time at Brown, which were wholly incompatible with each other. Over the last four years, you have helped me develop into the best scientist and mentor I can be, modeled after your own example. You never told me any project I proposed was out of reach (although I appreciate the much-needed perspective on several), and helped me work through problems I was trying very hard to circumvent. Intentionally or not, you taught me to advocate for myself and you prepared me for the realities of my life ahead, and there is no part of my present or future that have not been changed for the light you have shed on them.

The next set of honorary degrees will be awarded upon the other members of my defense committee, without whom I quite literally could not have finished my PhD. John Marshall, your advice and guidance on my doctoral work have been so valuable, and your humor and

perspective sometimes even more essential. Elena Oancea, thank you for the hard and soft skills you have taught me as well as the questions you have asked that have brought my research to a new level. Your advice on navigating the next step in my career was pivotal in my choosing the best move for both my career and my life. Anatoly Zhitkovich, I would not have had the understanding or courage to pursue some of the highest impact projects in this dissertation without your support and guidance as both a professor and committee member. I still can't tell you exactly what it is that draws us all to study this silly, uncooperative, and inexplicable receptor, but if I ever figure it out I'll let you know. Rae Matsumoto, I appreciate your encouragement of my research and career from Day 1 and the gift of presentation and confidence that came with the book you sent me. Getting the chance to see you at conferences was always a highlight of my trips.

Although not on my defense committee, I also have to extend gratitude to Julie Kauer for the advice, early morning words of wisdom, and encouragement that have allowed me to complete my PhD and have helped me choose the next step in my career path. Julie also gave me the opportunity to meet Abby Polter during my research rotation, which has had immeasurable impact on my life during my time at Brown. Abby needs no additional accolades or honorary degrees on her resume, but has pulled me out of more downward spirals and lifted me to such higher reaches than I had any right to expect. I appreciate your mentorship and friendship more than I can possibly say, and I know that I will continue to throughout my personal and professional future.

There are a number of honorary degrees I would like to award to the people who have facilitated my doctoral work behind the scenes, and who absolutely deserve to be recognized. Thank you to the McCurdy lab, particularly Christophe Mesangeau and Walid Alsharif, for the

ligands I have used throughout my projects. Thank you to Tun-Li Shen for your help running and explaining LC/MS while I mourned the ever-degrading compounds that turned out to be all the more exciting for their degradation. Thank you to Gerry and the team in the stockroom for helping me through all of my troubles obtaining what I needed and your humor along the way, and to the custodial staff at Biomed who always have kind words and smiling faces, whether it's at 7:00 am beginning my day or at midnight ending a long one. Thank you to Tatum Ponte for the endless support despite my computer illiteracy. Thank you to Jess Bello and Crystal Miller for facilitating my work in so many ways, as well as commiserating with me and adding humor to the most frustrating situations. Thank you to Andrew Campbell and Karen Ball and the IMSD program, Beth Harrington and the Division of Biomed, the NIH, and the MPP program for all of the help, guidance, and funding along this very winding road.

Many of the people to whom I would like to award honorary degrees already have them, but for the majority I may get to bestow their first (albeit perhaps not as official) doctorate. Cheri Liu, when you joined the Bowen lab it was the best gift I could have asked for. Through all the late-night pizza deliveries, presentation preparations, conference shenanigans, and breaks for tea time, my years in the lab simply would not have been the same without you. I am so grateful for your friendship over the years, and my successes have all leaned heavily on your support. To all of the undergraduate students who have come through the lab, thank you for letting me practice and develop my mentorship skills (sometimes at your expense, I apologize!) and thank you also for your friendship. Donald, Nelly, Vira, Tim, Ashley—you all helped truly turn our lab into a family and certainly the support extended far beyond covering each other when class schedules or overzealous experimenting created a need. Very special and sincere thanks to Roger Rowlett

and Colgate University for guiding me to and preparing me for this experience. It simply would not have been possible without you.

The number of people who have made it possible for me to complete my doctorate far exceeds what I can say in this section, but there are a few more I need to point out by name. Tracey, Zack, Alan, and Ynes—thank you for helping me maintain my passion for science and making new friends. You are what makes it so hard to be ending this chapter of both my dissertation and my career. Sveta, your friendship and our nights together are, simply put, what kept my sanity throughout the most difficult pieces of graduate school, and sharing both your and my successes made them all the better. Jen, Gillian, and Rachel, your constant presence in my life, even when there are miles and miles between us all, give me perspective on the things that matter most. I am so grateful and fortunate to have all of you in my life.

My final honorary degrees go to those that have seen the worst and the best that these four years have brought out in me, and the twenty before it. To my grandparents, this achievement belongs even more to you than to me. The life I have now would not be possible had you not been fueling the progress from the first moment. To my mother and my sister, two of the strongest and most inspiring women I know, it is not possible to explain how much of this accomplishment is credited to you. Thank you for every early morning walk-to-work phone call and every trip down here to have dinner with me when I needed it. Thank you, Ryan, for being there to catch me when I stumbled—I'm so glad to have you as a brother. To my whole family, I am so lucky to have a support system stronger than diamonds that allows me to reach for new heights knowing that I will be caught if I fall. And of course, an honorary degree for Max, who (by his own account) earned it several years before I did. Thank you for helping me move

forward when I was too tired to walk on my own, and for celebrating with me when the tiniest moments went right. I am so grateful for having you in my life.

I have learned all manner of things in graduate school, ranging from radioactive ligand competition binding assays to mentorship skills to how to make a 2 ½ pyramid with the Brown University Cheerleading Team (thank you for letting me play with you all for the last several years). But, the one thing that I have learned that is the most clear to me is to have courage. Through the experiments I have run, the new friends I have made, and the role models I have looked up to, I have learned to have the courage to try, to fail, and to succeed. During my time at Brown I have been challenged in every way I thought I could be and then pushed further, and the people who have walked the road with me have been the ones who have lent me their courage to continue forward. As said by A. A. Milne via Winnie the Pooh, “how lucky I am to have something that makes saying goodbye so hard.”

Table of Contents

Chapter 1: Introduction	1
1.1 Sigma Receptors	1
1.1.1 History of the Identification of Sigma Receptors	1
1.1.2 Sigma-1 Receptors	9
1.1.3 Sigma-2 Receptors	18
1.2 Sigma-2 Receptors and Cancer	29
Chapter 2: Characterization of CM572, a Selective Irreversible Partial Agonist of the Sigma-2 Receptor with Antitumor Activity.....	37
2.1 Preface.....	37
2.2 Abstract	40
2.3 Introduction.....	41
2.4 Materials and Methods.....	44
2.5 Results	48
2.6 Discussion	74
2.7 References	79
Chapter 3: Sigma-2 Receptor-Mediated Cell Death in Triple Negative Breast Cancer: Potential for Targeted Therapy	85
3.1 Preface.....	85
3.2 Abstract	87
3.3 Introduction.....	88

3.4 Materials and Methods.....	90
3.5 Results.....	94
3.6 Discussion.....	114
3.7 References.....	119

Chapter 4: Sigma-2 Receptors Play a Role in Cellular Metabolism: Stimulation of

Glycolytic Hallmarks by CM764 in Human SK-N-SH Neuroblastoma 126

4.1 Preface.....	126
4.3 Abstract.....	137
4.4 Introduction.....	138
4.5 Materials and Methods.....	141
4.6 Results.....	148
4.7 Discussion.....	179
4.8 Supplementary Figures	185
4.9 References.....	196

Chapter 5: Structure-activity relationship of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79) derivatives at sigma receptors 204

5.1 Preface.....	204
5.2 Abstract.....	206
5.3 Introduction.....	207
5.4 Results.....	210
5.5 Discussion.....	232
5.6 Materials and Methods.....	237
5.7 References.....	240

Chapter 6: General Conclusions and Discussion	245
Chapter 7: Future Directions.....	253
7.1 Multidrug Resistance	253
7.2 Hypoxia.....	259
7.3 Angiogenesis.....	260
7.4 Ceramide	263
7.5 Cloning the Sigma-2 Receptor.....	267
Chapter 8: Detailed Methodology	269
8.1 Cell Culture.....	269
8.1.1 General Cell Culture Reagents.....	269
8.1.2 HEK293 T/17 Human Embryonic Kidney Cells	270
8.1.3 HEM Human Epidermal Melanocyte Cells	270
8.1.4 HMEC Human Mammary Epithelial Cells.....	270
8.1.5 MCF-7 Human Breast Adenocarcinoma Cells.....	271
8.1.6 MG-63 Human Osteosarcoma Cells.....	272
8.1.7 PANC-1 Human Pancreatic Carcinoma Cells	272
8.1.8 SK-N-SH Human Neuroblastoma Cells.....	272
8.1.9 Triple Negative Breast Cancer Cells.....	273
8.4 Radioligand Binding Assays.....	273
8.4.1 Liver Membrane Preparation	273
8.4.2 Cell Line Membrane Preparation.....	275
8.4.3 Scatchard Analysis.....	276
8.4.4 Inferred Affinity (K _i) Determination by Competition Binding.....	278

8.4.5 Irreversible Binding Analysis	280
8.3 Cell Viability Assays	281
8.2.1 MTT Assay	281
8.2.2 LDH Assay	282
8.4 Calcium Release Assay	283
8.5 Western Blotting	285
8.5.1 Lysate Preparation.....	285
8.5.2 Protein Assay	285
8.5.3 Casting Gels.....	287
8.5.4 Western Blotting Protocol.....	287
8.5.5 Stripping Blots for Reprobing.....	289
8.6 Cell Proliferation Assays	289
8.6.1 CyQUANT Cell Proliferation Assay	289
8.6.2 BrdU Incorporation Cell Proliferation ELISA	290
8.6.3 DAPI Staining	292
8.7 Metabolic Factor Analysis Assays.....	293
8.7.1 NAD ⁺ /NADH Quantification Assay	293
8.7.2 ATP Quantification Assay.....	294
8.7.3 Reactive Oxygen Species Detection Assay.....	296
8.7.4 JC-1 Mitochondrial Potential Assay.....	297
References	299

List of Tables

CHAPTER 3: SIGMA-2 RECEPTOR-MEDIATED CELL DEATH IN TRIPLE NEGATIVE BREAST CANCER: POTENTIAL FOR TARGETED THERAPY

Table 1. Sigma-2 receptor expression in breast cancer and normal cell types.....95

Table 2. Cytotoxic potency of sigma-2 receptor ligands in breast tumor and normal cells.....102

CHAPTER 4: SIGMA-2 RECEPTORS PLAY A ROLE IN CELLULAR METABOLISM: STIMULATION OF GLYCOLYTIC HALLMARKS BY CM764 IN HUMAN SK-N-SH NEUROBLASTOMA

Table 1. Sigma receptor ligands and their affinities and selectivities.....160

CHAPTER 5: STRUCTURE-ACTIVITY RELATIONSHIP OF 6-ACETYL-3-(4-(4-(4- FLUOROPHENYL)PIPERAZIN-1-YL)BUTYL)BENZO[D]OXAZOL-2(3H)-ONE (SN79) DERIVATIVES AT SIGMA RECEPTORS

Table 1. Binding affinities of SN79 derivatives at sigma-1 and sigma-2 receptors.....211

List of Figures

CHAPTER 1: INTRODUCTION

Figure 1. Structures of significant ligands in the discovery and early development of sigma receptors.....	4
Figure 2. Photoaffinity labeling of guinea pig brain and PC12 cell membrane homogenates using [³ H]Az-DTG	7
Figure 3. Proposed tertiary structure of the mammalian sigma-1 receptor.....	11

CHAPTER 2: CHARACTERIZATION OF CM572, A SELECTIVE IRREVERSIBLE PARTIAL AGONIST OF THE SIGMA-2 RECEPTOR WITH ANTITUMOR ACTIVITY

Figure 1. Structures of SN79 and CM572.....	50
Figure 2. Affinity of CM572 at sigma-1 and sigma-2 receptors.....	51
Figure 3. Irreversible binding of CM572 at sigma-1 and sigma-2 receptors..	52
Figure 4. CM572-induced calcium response in SK-N-SH neuroblastoma.....	54
Figure 5. CM572 attenuation of CB-64D-induced calcium response.....	56
Figure 6. CM572-induced cytotoxicity in SK-N-SH neuroblastoma.	60
Figure 7. Comparison of effects of CM572 and siramesine upon acute or chronic exposure.....	64
Figure 8. CM572 attenuation of CB-64D-induced cytotoxicity in SK-N-SH neuroblastoma.	67
Figure 9. Effect of CM572 on Bid cleavage in SK-N-SH neuroblastoma.	70
Figure 10. Effect of CM572 across human tumor cell lines from different organ sites and comparison with effect in normal cell types.	73

CHAPTER 3: SIGMA-2 RECEPTOR-MEDIATED CELL DEATH IN TRIPLE NEGATIVE BREAST CANCER: POTENTIAL FOR TARGETED THERAPY

Figure 1: Structures of CM572 and SV119	98
Figure 2. Effect of sigma-2 receptor agonists on viability of triple negative breast cancer, non-triple negative breast cancer, and normal human mammary epithelial cells	100
Figure 3. Comparison of single-dose of sigma-2 agonists on viability of triple negative breast cancer, non-triple negative breast cancer, and normal human mammary epithelial cells.	104
Figure 4. Effect of sigma-2 receptor agonists on mitochondrial membrane potential in triple negative breast cancer cells.....	108
Figure 5. Effect of caspase inhibition on CM572 -induced cell death in TNBC cell lines.	110
Figure 6. Effect of caspase inhibition on SV119-induced cell death in TNBC cell lines.....	112

CHAPTER 4: SIGMA-2 RECEPTORS PLAY A ROLE IN CELLULAR METABOLISM: STIMULATION OF GLYCOLYTIC HALLMARKS BY CM764 IN HUMAN SK-N-SH NEUROBLASTOMA

Figure I. Structure of CM769.....	127
Figure II. Stability of 10 mM SN79 in DMSO stored at -20°C.	129
Figure III. Stability of 10 mM CM769 in DMSO stored at -20°C.....	130
Figure IV. Effect of degraded CM769 treatment on SK-N-SH neuroblastoma cell reduction of MTT..	132
Figure V. Effect of newly diluted CM769 treatment on SK-N-SH neuroblastoma cell viability.	134
Figure 1. Structures of SN79 and CM764.	149

Figure 2. CM764 binding at sigma-1 and sigma-2 receptors.....	150
Figure 3. Effect of CM764 treatment on MTT reduction in SK-N-SH neuroblastoma.....	152
Figure 4. Effect of CM764 treatment on DNA replication in SK-N-SH neuroblastoma.	154
Figure 5. Effect of sigma-2 receptor antagonists on CM764-induced MTT reduction in SK-N-SH neuroblastoma.	158
Figure 6. Effect of CM764 treatment on intracellular calcium in SK-N-SH neuroblastoma.. ...	162
Figure 7. Changes in NADH/NAD ⁺ in response to CM764 exposure.	164
Figure 8. Changes in ATP levels in response to CM764 exposure.	166
Figure 9. Effect of CM764 on reactive oxygen species levels in SK-N-SH neuroblastoma cells.	170
Figure 10. Effect of CM764 treatment on levels of HIF1 α in SK-N-SH neuroblastoma.	172
Figure 11. Effect of CM764 treatment on expression of VEGF in SK-N-SH neuroblastoma.. .	175
Figure 12. Effect of CM764 treatment across cell types of different tissues.....	178
Supplemental Figure 2: Pure CM764 from DMSO stock.....	187
Supplemental Figure 3: CM764 (30 μ M) incubated in culture media without cells for 0 h or 24 h	191
Supplemental Figure 4: CM764 (30 μ M) incubated in media with cells for 0 h or 24 h.....	195

CHAPTER 5: STRUCTURE-ACTIVITY RELATIONSHIP OF 6-ACETYL-3-(4-(4-(4-FLUOROPHENYL)PIPERAZIN-1-YL)BUTYL)BENZO[D]OXAZOL-2(3H)-ONE (SN79) DERIVATIVES AT SIGMA RECEPTORS

Figure 1. Effect of X-group substitutions on sigma-1 receptor binding affinity..	214
Figure 2. Effect of R-group substitutions on sigma-1 receptor binding affinity..	216

Figure 3. Effect of X-group substitutions on sigma-2 receptor binding affinity.....	219
Figure 4. Irreversible binding of isothiocyanate-substituted ligands at sigma-1 and sigma-2 receptors.....	221
Figure 5. Effect of isothiocyanate derivatives on cell viability of SK-N-SH neuroblastoma.....	224
Figure 6. Comparison of acute and continuous exposure to isothiocyanate substituted ligands on cell viability of SK-N-SH neuroblastoma.....	227
Figure 7. Ability of SN79 derivatives to increase reduction of MTT in SK-N-SH neuroblastoma.	231

CHAPTER 7: FUTURE DIRECTIONS

Figure 1. Effect of CM764 treatment on Nrf2 protein expression in SK-N-SH neuroblastoma cells.	257
Figure 2. Effect of CM764 on MTT reduction in HUVEC cells.	262

The following figures were not created by H. Nicholson and are credited as follows:

Chapter 1 Figure 2: Susan Hellewell

Chapter 1 Figure 3: Zhiping Wu

Chapter 2 Figure 3: Anthony Comeau

Chapter 3 Figure 2: created jointly with Cheri Liu

Chapter 3 Figures 4-6: Cheri Liu

Chapter 4 Figure 4: Anthony Comeau

Abbreviations

BD1047, N'-[2-(3,4-dichlorophenyl)ethyl]-N,N,N'-trimethylethane-1,2-diamine; BD1063, 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine; Bid, BH3-interacting domain death agonist; BSA, bovine serum albumin; CB-64D, (+)-1*R*,5*R*-(*E*)-8-Benzylidene-5-(3-hydroxyphenyl)-2-methylmorphan-7-one; CB-184, (+)-1*R*,5*R*-(*E*)-8-(3,4-dichlorobenzylidene)-5-(3-hydroxyphenyl)-2-methylmorphan-7-one; CM572, 3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[*d*]oxazol-2(3*H*)-one; CM764, 6-acetyl-3-(4-(4-(2-amino-4-fluorophenyl)piperazin-1-yl)butyl)benzo[*d*]oxazol-2(3*H*)-one; DAPI, 4',6-diamidino-2-phenylindole; DCFDA, 2',7'-dichlorofluorescein diacetate; DFO, deferoxamine; DMSO, dimethylsulfoxide; DTG, 1,3-di-*o*-tolylguanidine; EDTA, ethylenediaminetetraacetic acid; HBSS, Hank's Balanced Salt Solution; HEM, human primary melanocytes; HIF1 α , hypoxia-inducible factor 1 alpha; HMEC, human mammary epithelial cells; JC-1, tetraethylbenzimidazolylcarbocyanine iodide; LDH, lactate dehydrogenase; MEM, Minimal Essential Media; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; NE100, 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine; PBS, phosphate buffered saline; PGRMC1, progesterone receptor membrane component 1; RIPA buffer, radioimmunoprecipitation assay buffer; ROS, reactive oxygen species; RT, room temperature; SDS, sodium dodecasulfate; siramesine, 1'-{4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butyl}-3*H*-spiro[2-benzofuran-1,4'-piperidine]; SN79, (6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[*d*]oxazol-2(3*H*)-one); TBS, Tris buffered saline; TMB, tetramethylbenzidine; VEGF, vascular endothelial growth factor.

Chapter 1: Introduction

1.1 Sigma Receptors

1.1.1 History of the Identification of Sigma Receptors

In 1976, W. R. Martin and colleagues described three independent “syndromes” resultant from treatment of chronic spinal dogs with morphine congeners (Martin et al. 1976). Each “syndrome” was attributed to the interaction of a prototypic agonist with a discrete receptor, for which the receptor was then named with its corresponding Greek letter: morphine (μ receptor), ketocyclazocine (κ receptor), and SKF-10,047 (N-allylnormetazocine) (σ receptor). These independent responses were categorized by the physiological effects elicited by treatment with each ligand. Activation of the μ receptor was associated with “miosis, bradycardia, hypothermia, a general depression of the nociceptive responses, and indifference to environmental stimuli”. Agonism of the κ receptor with ketocyclazocine was found to cause overall sedative effects and depression of the flexor reflex, although no changes in skin twitch reflex or pulse were observed. In contrast to these two depressive phenotypes, activating the binding site that Martin and colleagues dubbed “ σ ” caused an increase in activity, including tachycardia, rapid breathing, pupil dilation, and mania.

The pharmacology of the μ and κ opioid receptor subtypes was impressively characterized through Martin’s studies of chronic spinal dogs, particularly when considering the evaluative tools available at the time. Without molecular biology or radioligand binding assays

available for the study of this system, Martin's group was nonetheless able to characterize the pharmacological profile of μ and κ opioid receptors to a level of accuracy that proved correct upon knockout and binding confirmations when these methods became available. Martin demonstrated that the effects of morphine and ketocyclazocine on the chronic spinal dogs were able to be attenuated by naltrexone, an antagonist now known to bind μ and κ opioid receptor subtypes, confirming that these ligands were agonists at his proposed receptor subtypes. The group also showed that ketocyclazocine treatment was not able to induce morphine-like effects in morphine-dependent dogs, demonstrating its subtype selectivity for the κ receptor. Further, continuous treatment of all three ligands resulted in tolerance, consistent with opioid receptor activity.

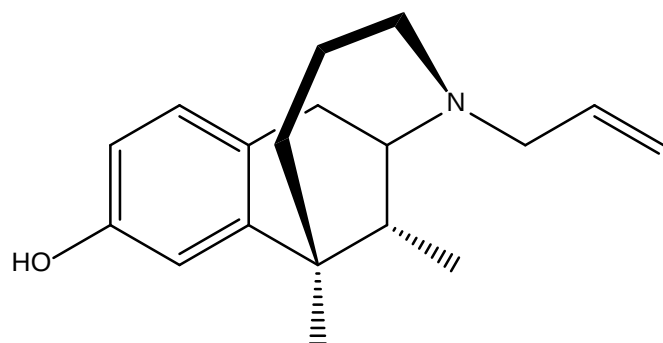
Although Martin was attributing physiological, whole-organism symptoms to presumed activity at the biochemical level, his interpretations and conclusions were strikingly accurate in many ways. The μ and κ receptors that were defined by the effects of morphine and ketocyclazocine, respectively, retained their names and are now part of a larger class of G-protein coupled receptors called the opioid receptors. They have been joined by δ and nociceptin receptors to make up the four major subtypes of opioid receptor, with some subtypes now being even further subcategorized to include micro-subtypes such as δ_1 and δ_2 . However, Martin's attribution of the manic effects of SKF-10,047 to a " σ opioid receptor" did not withstand the development of newer technologies, which revealed this overall response to be an amalgamation of effects elicited by activity at multiple receptors.

When isolated stereoisomers of the racemic ($\pm$)-SKF-10,047 became available, a clearer picture of Martin's " σ receptor" effects began to form. The (-) isomer was found to retain the ability to induce some of the " σ receptor" physiological effects of the racemic mixture, and this

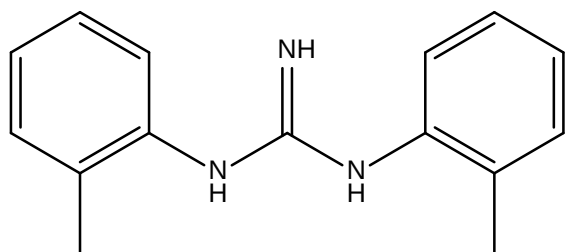
activity could be blocked by opioid receptor antagonists at the μ and κ subtypes including naltrexone (Khazan et al. 1984). Although (-)-SKF-10,0047 is now recognized not to be a full agonist of any opioid receptor subtype, antagonist or partial agonist activity at μ and κ opioid receptors could explain how naltrexone was able to block Martin's physiological " σ " effects. This observation accounts for the earlier results shown by Martin and his predecessors that ($\pm$)-SKF-10,047 acted as a morphine antagonist, and produced no analgesic effect of its own (Keats and Telford 1964; Pearl and Harris 1966). Indeed, in his landmark 1976 study in the chronic spinal dog, Martin did suggest that ($\pm$)-SKF-10,047 was a competitive antagonist at the μ receptor (Martin et al. 1976).

Rather, it was the (+) isomer (Figure 1A) that was responsible for the manic effects produced in the chronic spinal dog model, in such contrast to the morphine and ketocyclazocine treatments. This mania could not be attenuated by opioid receptor antagonists, and are now known not to be associated with opioid receptors at all (Khazan et al. 1984). These manic effects resembled those induced by binding of phencyclidine (PCP) to a binding pocket within the NMDA receptor, and through the development of [^3H](+)-SKF-10,047 it was determined that this ligand shared a direct binding site with phencyclidine (Zukin et al. 1984; Sircar et al. 1986; Mendelsohn et al. 1985). The combined activity of (-)-SKF-10,047 at μ and κ opioid receptors and (+)-SKF-10,047 at the PCP binding site within the NMDA receptor was able to account for the pharmacology and physiological effects Martin and colleagues observed in the chronic spinal dog.

A



B



C

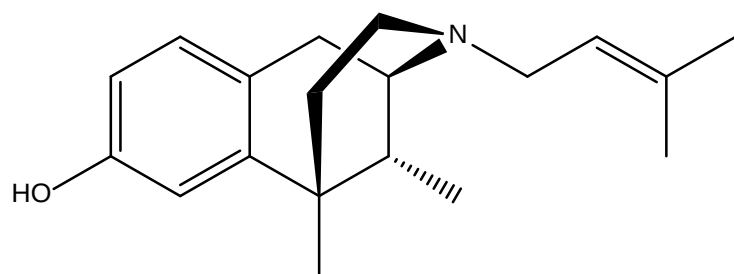


Figure 1. **Structures of significant ligands in the discovery and early development of sigma receptors.** A) (+)-N-allylnormetazocine ((+)-SKF-10,047). B) 1,3-di(2-tolyl)guanidine (DTG). C) (+)-pentazocine. All structures in this dissertation were created using ChemBioDraw Ultra 14.0 (CambridgeSoft, Cambridge, MA).

Binding of phencyclidine was not able to fully displace [^3H](+)-SKF-10,047 from rat brain membranes entirely, however (Wong et al. 1988). It was therefore assumed that (+)-SKF-10,047 must bind another distinct site. Although this final binding site had no functional relationship to Martin's initial opioid receptor subtypes, its affinity for (+)-SKF-10,047 prompted that it retain the name "sigma receptor". The pharmacological profile and activity of this binding site have since been extensively studied and continuously described and redefined, and it is now accepted as an independent binding site unrelated to the NMDA receptor or any opioid receptor. Early work from the laboratories of Tsung-Ping Su and William Tam on this sigma receptor showed reversed stereoselectivity from traditional opioid receptors, with the sigma receptor preferentially binding (+)-benzomorphans such as (+)-pentazocine and dextrallorphan as compared to their (-)-isomer and levorotatory counterparts (Su 1982). Further distinguishing this novel binding site from its opioid history, no affinity for naloxone or etorphine was observed using radioligand competition binding studies with [^3H]SKF-10,047 (Su 1982), but several psychologically active compounds did show affinity for the naloxone-insensitive sigma receptor including haloperidol, fluphenazine, and phencyclidine (Su 1982; Tam 1983; Tam and Cook 1984).

In the mid-1980's, a breakthrough in sigma receptor radioligand-based pharmacology came in the form of 1,3-di(2-[5- ^3H]tolyl)guanidine ([^3H]DTG) (Figure 1B) from the lab of Eckard Weber (Weber et al. 1986; Weber et al. 1987). This inexpensive plasticizing agent bound to a single binding site population in guinea pig brain membrane homogenates, representing the first selective sigma receptor ligand. This discovery allowed for more effective validation studies and pharmacological profiling of sigma receptors. The advent of [^3H](+)-pentazocine (Figure 1C), a close structural analog of (+)-SKF-10,047 that lacks PCP binding site affinity, followed

shortly after, again representing a sigma selective radioligand and increasing the pool of existing tools with which to study this novel receptor (de Costa et al. 1989; Bowen et al. 1993). These sigma-specific ligands were an improvement upon the use of [^3H](+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine (3PPP), which had been used for sigma receptor studies in the wake of (+)-SKF-10,047 and yet had significant activity as a presynaptic dopamine autoreceptor agonist (Hjorth et al. 1981). Now with viable selective ligands widely available, investigations of the sigma receptor became more prevalent and interest into the function and manipulation of this novel receptor grew.

The novel sigma-selective ligand di-o-tolylguanidine became of even greater importance to the sigma receptor field in 1990, when Hellewell and Bowen published a landmark paper demonstrating the use of [^3H]azido-di-o-tolylguanidine to photoaffinity label two binding sites with divisive characteristics (Figure 2) (Hellewell and Bowen 1990). To this point, many studies of sigma receptors were being carried out in guinea pig brain membrane homogenates, in which DTG bound to a 25 kDa protein (initially characterized as 29 kDa) that also showed high affinity binding with haloperidol and stereoselectivity for (+)-benzomorphans (Kavanaugh et al. 1988; Weber et al. 1986). Hellewell and Bowen demonstrated that in PC12 rat pheochromocytoma cell membranes, [^3H]Az-DTG was rather bound to two smaller 18 and 21 kDa proteins, and the 25 kDa binding site found in guinea pig brain membrane homogenates was largely absent in the PC12 cells.

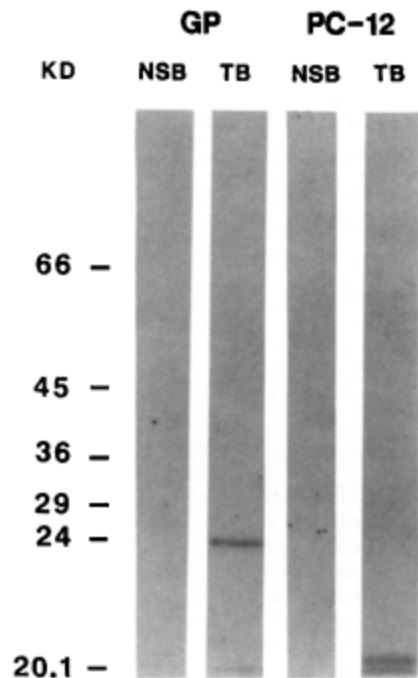


Figure 2. **Photoaffinity labeling of guinea pig brain and PC12 cell membrane homogenates using [^3H]Az-DTG.** Film exposures of [^3H]Az-DTG labeled membrane homogenates from guinea pig brain (GP) and rat pheochromocytoma cells (PC-12) show little non-specific binding (NSB) and two distinct labeled bands resultant from total binding (TB). The labeled site in guinea pig brain homogenate has a molecular weight of 25 kDa and corresponds to the sigma-1 receptor, which is in high density in this tissue. The labeled site in PC12 cell membrane homogenate has a distinctly lower molecular weight of 21.5 kDa and is pharmacologically distinct from the guinea pig brain site, and is now known as the sigma-2 receptor. This figure was the first evidence of sigma receptor subtypes with distinct sizes and pharmacological profiles. (Hellewell and Bowen 1990)

This smaller molecular weight PC12 cell membrane binding site retained high affinity for haloperidol, but showed reversed stereoselectivity from the guinea pig brain binding site, instead showing low preference for (+)-benzomorphans and even slight selectivity for the (-)-isomers. Through Scatchard analysis in PC12 cell membranes, it was determined that [³H]DTG was binding to the PC12 cell membranes with a single $K_d = 23.7 \pm 4.6$ nM and $B_{max} 2,025 \pm 660$ fmol/mg protein, with both values comparing well to the DTG binding site in guinea pig brain. Another sigma ligand that was used in the study, [³H](+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ([³H](+)-3-PPP), also retained comparable binding at the smaller PC12 binding site relative to the larger guinea pig brain binding site, demonstrating a clear pharmacological relationship between the two. This study presented the first evidence for sigma receptor subtypes, and led to the differentiation of the guinea pig brain binding site as the sigma-1 receptor and the PC12 cell binding site as the sigma-2 receptor subtype.

Both sigma receptor subtypes are expressed throughout many areas of the body. The brain, liver, gastrointestinal tract, blood cells, heart, and kidneys all express high levels of sigma receptors, with subtype distribution being variable. As a sigma-2-selective radioligand is not yet widely available, many autoradiographic and some binding studies do not differentiate between the sigma receptor subtypes, although sigma-1 receptor expression has been more concretely established due to the availability of selective ligands and the ability to use molecular biology to manipulate this cloned subtype. While the endogenous ligands and function for either sigma receptor subtype remain elusive, the widespread expression in many areas of the human body indicates important physiological roles for both sigma-1 and sigma-2 receptors.

1.1.2 Sigma-1 Receptors

In light of the elucidation of the two widely accepted sigma receptor subtypes, it is the sigma-1 receptor that is largely responsible for much of the pharmacological and functional results determined from early “sigma receptor” studies that did not differentiate between the subtypes. The radioligand [³H](+)-SKF-10,047 binds sigma-1 receptors with 29 ± 3 nM affinity but does not bind the sigma-2 subtype similarly (sigma-2 $K_i > 33,000$ nM) (Bowen et al. 1993). Thus, any early study results using [³H](+)-SKF-10,047 that could not be attributed to its activity at the PCP binding site within the NMDA receptor were likely a result of sigma-1 receptor binding without input from the sigma-2 subtype.

In 1996, two studies were published that each delineated the sequence of the mammalian sigma-1 receptor (Kekuda et al. 1996; Hanner et al. 1996). The 223-amino acid sequence that was elucidated from these studies initially from guinea pig by Hanner and colleagues and then from human tissue by the Kekuda lab, shares no homology with any known mammalian protein, yet is 90% identical (95% similar) across mammalian species (Su et al. 2010). The sequence of the sigma-1 receptor contains an endoplasmic reticulum retention signal (V. Ganapathy et al. 2007). Using multiple sequence-to-structure predictive bioinformatics programs, a tertiary structure for the sigma-1 receptor has been proposed (Wu et al. 2006) (Figure 3). The current model of the structure of the sigma-1 receptor includes two transmembrane domains, one additional membrane-interacting domain, and has both the N- and C-termini as well as a ligand binding domain on the long C-terminal tail in the cytosol. This model is consistent with an earlier study by Aydar et al. that proposed a two-transmembrane domain structure with cytosolic N- and C-termini based on accessibility of GFP tags, surface labeling with biotin, and

transmembrane segment homology analysis, but which did not propose any further secondary structure topography for the receptor (Aydar et al. 2002).

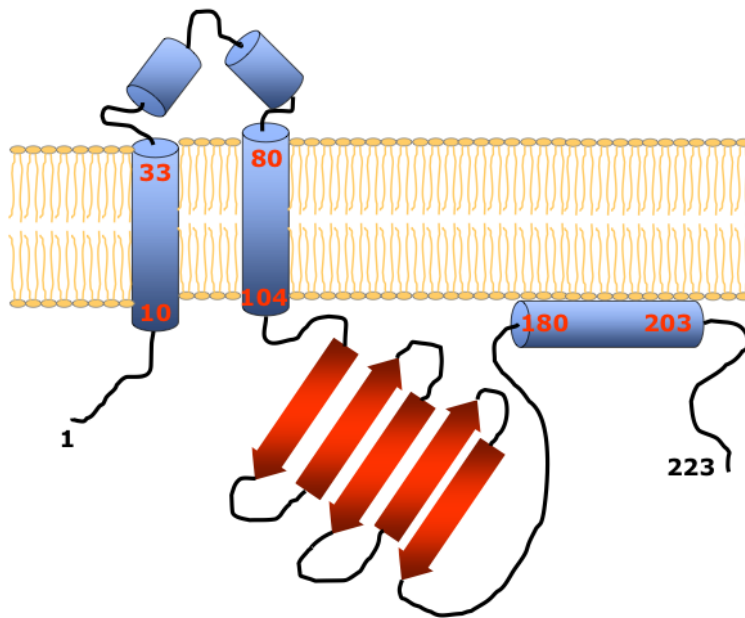


Figure 3. **Proposed tertiary structure of the mammalian sigma-1 receptor.** A series of bioinformatics programs was used to predict elements of secondary structure and transmembrane regions for the sigma-1 receptor based on amino acid sequence. This model posits a two-transmembrane domain structure, consistent with the predictive findings of an earlier study (Aydar et al. 2002). The alpha helix formed by residues 180-203 is not predicted to be sufficiently hydrophobic to be embedded within the membrane, but rather is likely associated with the membrane at the cytosolic face. Truncation of the sigma-1 receptor after the second transmembrane domain (Line k3) results in a non-functional protein, indicating that the ligand-binding domain is likely in the C-terminal region. (Wu et al. 2006)

Interestingly, the MCF-7 breast adenocarcinoma cell line does not endogenously express sigma-1 receptors (Vilner, John, et al. 1995). This observation has been exploited experimentally to examine the effect of induced expression of sigma-1 receptors in a null system, resulting in the Line 41 subclone cell line that overexpresses sigma-1 receptors (Wu and Bowen 2008). Line 41 cells proliferate at a faster rate than their parent MCF-7 cells, and are morphologically less rounded and appear to have extended pseudopodia unlike their circular parent counterpart (Wu and Bowen 2008). Further subclones based on the MCF-7 parent cell line include Line k3, which contains only residues 1-100 of the sigma-1 receptor (consisting of both transmembrane domains but no intracellular C-terminal tail) and Line sg101, which contains only residues 102-223 (consisting of the complete intracellular long C-terminal tail but neither transmembrane domain) (Wu and Bowen 2008). Naturally occurring variations on the 223-amino acid sigma-1 receptor are represented by two splice variants, one determined to be without the third of four exons and without traditional sigma-1 receptor binding activity in Jurkat cells (M. E. Ganapathy et al. 1999), and one lacking 47 base pairs in exon 2, which results in a frameshift mutation causing truncation of the protein that does not retain traditional sigma-1 receptor IP3R enhancement capability in mouse neuroblastoma (Shioda et al. 2012). The functional relevance of the splice variants has not yet fully been elucidated.

The existence of specific radioligand and antibody probes for sigma-1 receptors have made possible studies defining both the anatomical and subcellular localization of this receptor. Through the use of [³H](+)-SKF-10,047, [³H](+)-pentazocine, and additional tritiated sigma-1 receptor-selective ligands 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine (NE-100) and (+)-(3-(3-hydroxyphenyl)-N-n-propylpiperidine ((+)-3-PPP), sigma-1 receptors have been found in a variety of brain areas across several different species including guinea pig, rat,

mouse, cat, primate, and human (R. R. Matsumoto 2007). Brain areas of particularly enriched sigma-1 receptor density include the hypothalamus (Bouchard and Quirion 1997), hippocampus (Bouchard and Quirion 1997; McLean and Weber 1988; Gundlach et al. 1986), and several areas associated with the visual system (McLean and Weber 1988). Expression in these areas is consistent with emerging roles for sigma-1 receptors in hormone release, cognitive function, and vision, respectively (Eisenberg 1985; Ola et al. 2001; W.-F. Wang et al. 2002; Monnet 2007; Werling et al. 2007). Additional central nervous system areas enriched in sigma-1 receptors are the grey matter, ventral horn, and dorsal root ganglia of the spinal cord, consistent with a role in motor function and possible transmission of pain signals (Gundlach et al. 1986; Aanonsen and Seybold 1989; Pasternak et al. 1981). Non-CNS tissues expressing sigma-1 receptor include the heart, kidney, lung, T cell zones in the spleen, and some reproductive tissues. Understanding the specific function of sigma-1 receptors in these areas remains an ongoing area of study in the field, with some functions, such as calcium modulation, being well-described and others, such as a role in immunity, being merely speculative based on expression patterns (Novakova et al. 1995; Seth et al. 1998; S. A. Wolfe et al. 1997; Moebius et al. 1993; Kennedy and Henderson 1989; S. A. J. Wolfe et al. 1989).

Studies of sigma-1 receptors at the cellular and subcellular level have also contributed to a better understanding of the structure, function, and organellar localization of this complex receptor. The sigma-1 receptor is found in high density at endoplasmic reticulum membranes, a distribution that has been confirmed by many groups (Alonso et al. 2000; Cagnotto et al. 1994; Dussossoy et al. 1999; Hayashi and Su 2003b; McCann and Su 1990; Samovilova and Vinogradov 1992). This is consistent with the existence of an ER retention signal at the N-terminus of the sigma-1 receptor amino acid sequence as mentioned above. These results have

been confirmed using radioligand binding in differential centrifugation fractions, specific antibodies against the sigma-1 receptor, GFP-tagging of sigma-1 receptors, and immunohistochemistry studies. While many other subcellular areas have been reported to have sigma-1 receptors including the mitochondria and nucleus, only the ER has shown consistent expression across all studies.

An examination of the buoyant density of sigma-1 receptor binding sites measured by [^3H](+)-SKF-10,047 binding from a continuous sucrose gradient fractionation demonstrated that sigma-1 receptors are lighter than plasma membrane markers, suggesting that they exist in lipid rafts and confirming that they are predominantly located intracellularly, rather than at the extracellular plasma membrane (McCann and Su 1990). This data was corroborated by a study using NG108 cells and oligodendrocytes that demonstrated the existence of high levels of cholesterol, fatty acids, and neutral lipids in the membranes containing sigma-1 receptors (Hayashi and Su 2003a). It was further supported by a study using filipin and Nile red staining to mark free cholesterol and neutral lipids in sigma-1 receptor-containing globules using confocal microscopy to observe C-terminally-tagged yellow fluorescence protein sigma-1 receptors in NG108 cells (Hayashi and Su 2003b).

While immunohistochemistry, radioligand binding, and antibody tagging of sigma-1 receptors revealed valuable information about subcellular localization of this receptor, these techniques only allowed for observation of static positions. Subsequently, it has been revealed that the subcellular localization of sigma-1 receptors is dynamic. Morin-Surun and colleagues first demonstrated the ability of sigma-1 receptors to translocate by showing a shift in receptor localization from the endoplasmic reticulum membrane to the plasma membrane in response to treatment with (+)-pentazocine in adult guinea pig brainstem (Morin-Surun et al. 1999). Hayashi

and Su also observed sigma-1 receptor trafficking from the ER membrane, although the destination in their study of NG108 cells was the plasmalemma and nuclear membranes (Hayashi and Su 2007). In the same study, YFP-tagged sigma-1 receptors were used to demonstrate that treatment with (+)-pentazocine causes a decrease in sigma-1 receptor clustering in ER lipid rafts, and instead evenly distributes sigma-1 receptors throughout the ER network prior to translocation away from the ER. Interestingly, when the N-terminal ER retention signal is removed from sigma-1 receptors, they localize to cytosolic lipid droplets (Hayashi and Su 2003b).

In deference to its initial characterization as an opioid receptor, many endorphins, enkephalins, and individual amino acids were initially examined for sigma-1 receptor binding in an attempt to identify the endogenous ligand, but none were found to have significant affinity (Patterson et al. 1994). Due to their high density in central nervous system tissues, the possibility that the endogenous ligand for sigma-1 receptors is a neurosteroid has been raised multiple times since its discovery. Several steroids have been demonstrated to have moderate to high affinity for sigma-1 receptors by displacement of [³H](+)-SKF-10,047 including testosterone, pregnenolone sulfate, and desoxycorticosterone (Su et al. 1988). Notably, Tsung-Ping Su and colleagues demonstrated that progesterone displaces [³H](+)-SKF-10,047 in guinea pig brain with high affinity ($K_i=173-196$ nM) (Su et al. 1988). Confirming that this interaction is selective for sigma-1 receptors, the Su lab used [³H]progesterone to determine the affinity of (+)-pentazocine and (+)-SKF-10,047 at the progesterone binding site, resulting in an affinity of 63.9 ± 11.9 nM for (+)-pentazocine and 173 ± 26 nM for (+)-SKF-10,047 (McCann and Su 1991). These values correlate well with a sigma-1 receptor binding profile, and indicate that progesterone binds the sigma-1 receptor.

A similar study by the Vinogradov lab demonstrated that an extract from porcine liver could inhibit binding of [^3H](+)-SKF-10,047 in rat liver membrane homogenates, and further characterized the active component of the extract as a low molecular weight (<1000 Da) compound that was resistant to pronase digestion, indicating a non-protein product that was soluble in water and organic solvents (Nagornaia et al. 1988). An extrapolation of his earlier study, Tsung-Ping Su and his colleague Bruce Vaupel corroborated the Vinogradov lab's findings by characterizing a sigma-1 receptor endogenous ligand as a 485 Da non-protein compound that did not contain nitrogen (Su and Vaupel 1988). Support for a steroid as the endogenous ligand for the sigma-1 receptor is furthered by 67% amino acid sequence homology and subcellular localization to the yeast sterol C8-C7 isomerase, and several inhibitors for this enzyme potentially displace [^3H](+)-pentazocine binding (Moebius et al. 1997; Dussossoy et al. 1999) although the sigma-1 receptor does not possess sterol isomerase activity itself.

As discussed above, sigma-1 receptors appear to regulate a variety of functions in the brain. As such, it has been proposed that sigma-1 receptors may have multiple endogenous ligands. Indeed, in addition to progesterone, a series of studies demonstrated the potency of an endogenous protein product with high sigma-1 receptor affinity. Studies by Su et al. and independently by O'Donohue et al. demonstrated that extracts from guinea pig brain and porcine brain, respectively, could displace [^3H](+)-SKF-10,047 in a PCP-insensitive manner from brain tissue (Su et al. 1986; Contreras et al. 1987). In both studies, addition of a protein degrading enzyme (trypsin and pronase, respectively) largely eliminated [^3H](+)-SKF-10,047 displacement, indicating that the endogenous compound was a peptide. Further, the O'Donohue lab used absorbance spectra to demonstrate that the endogenous compound was likely to contain phenylalanine residues (Contreras et al. 1987). In 1993, Hudkins and colleagues demonstrated

that in addition to the traditional (+)-pentazocine binding site on sigma-1 receptors, an allosteric site existed that could modulate [³H](+)-pentazocine binding when occupied by phenytoin (Daven-Hudkins et al. 1993). Allosteric phenytoin binding was also able to shift the affinity of several canonical sigma receptor ligands from low- to high-affinity states, including that of (+)-3-PPP and (+)-SKF-10,047.

Functionally, sigma-1 receptors appear to regulate a variety of homeostatic functions in mammalian cells. A primary role is the tonic inhibition of inositol 1,4,5-triphosphate receptors (IP3R) through complexation with ankyrin (Hayashi et al. 2000; Hayashi and Su 2001; Su and Hayashi 2001). Upon treatment with a sigma-1 receptor agonist, the sigma-1/ankyrin complex releases IP3R, thus potentiating calcium release. This role of sigma-1 receptor function is consistent with the observation that (+)-pentazocine treatment causes a decrease in sigma-1 receptor lipid raft localization within 10 minutes, congruous with both a dissociation from IP3R and dynamic receptor localization. Another proposed role of sigma-1 receptors surrounds lipid raft formation and lipid transport. NG108 cells expressing non-functional YFP-tagged sigma-1 receptors contain fewer lipid rafts than their functional YFP-tagged counterparts (Hayashi and Su 2003b), while overexpression of functional sigma-1 receptors results in an increase in lipid raft number in PC-12 cells (Takebayashi et al. 2004).

Additional homeostatic regulatory roles for sigma-1 receptors include the regulation of potassium channels (Mavlyutov and Ruoho 2007; Aydar et al. 2002; Lupardus et al. 2000), calcium channels (Hayashi et al. 2000), and several neurotransmitter systems (Horan et al. 2002; Bermack and Debonnel 2005; Mtchedlishvili and Kapur 2003; Maurice et al. 2002). In higher level systems, a role for sigma-1 receptors has been implicated in recovery and improvement of learning and memory in conditions of drug-induced learning impairment and Alzheimer disease

(Maurice et al. 2001; Monnet and Maurice 2006; Maurice and Lockhart 1997). Additional roles in depression and anxiety (Ukai et al. 1998; Urani et al. 2004; Maurice 2007), schizophrenia (Cobos et al. 2008), analgesia (Ueda et al. 2001), and drugs of abuse and addiction (Nguyen et al. 2005; R. R. Matsumoto et al. 2001; R. R. Matsumoto et al. 2002; R. R. Matsumoto 2009) have been suggested and are gaining support in literature. The emerging clarity of roles for the sigma-1 receptor in major cognitive processes and synaptic plasticity demonstrate the necessity for further study of this unique target that may have vast therapeutic implications for mental disorders, cognitive processing, and neurological diseases.

1.1.3 Sigma-2 Receptors

Consistent with its later discovery, less is known about the sigma-2 receptor than about the sigma-1 subtype. As much of the early investigation of sigma receptors was done with (+)-SKF-10,047, which does not bind the sigma-2 subtype, the information elucidated from many of these studies can be confidently attributed to the sigma-1 receptor. The landmark 1990 publication from Hellewell and Bowen that first reported sigma receptor subtypes demonstrated the first sigma-2-binding ligand, di-o-tolyl-guanidine (Figure 2) (Hellewell and Bowen 1990). Since that time, extensive work by pioneers in the sigma-2 receptor field have expanded the number of tools available with which to study the sigma-2 receptor selectively, although significantly overlapping pharmacology with the sigma-1 subtype have complicated such efforts. Indeed, the widely used radioligand binding assay to measure sigma-2 receptors involves first masking sigma-1 receptors with (+)-pentazocine when measuring sigma-2 receptors using [³H]DTG (Vilner, John, et al. 1995).

Anatomical distribution of sigma-2 receptors has been delineated in several organs across various species. Rat and human brain have both been confirmed to have sigma-2 receptors using sigma-2-selective radioligand probes or non-subtype selective probes under sigma-2 receptor binding conditions (Bouchard and Quirion 1997; Sørby et al. 2002). Sigma-2 receptors are also present in rat liver, kidney, and heart, as well as porcine gastrointestinal tract, although the endogenous function of sigma-2 receptors in these organs is yet unknown (Harada et al. 1994; Hellewell et al. 1994; Novakova et al. 1995). Interestingly, sigma-2 receptor density is highly variable in the same organ across species, with human tissues having generally lower B_{max} values as compared to rodent tissues (Sørby et al. 2002).

Expression of sigma-2 receptors in cell lines has been studied with increasing interest since their discovery in 1990. These receptors have been found to be expressed in every tumor cell line investigated to date, regardless of the species from which the cell line has been derived (Vilner, John, et al. 1995). Receptor density appears to be variable within a high range of expression, spanning roughly 500-7,000 fmol/mg protein. To date, all tumor cell lines investigated also express sigma-1 receptors, with MCF-7 human breast adenocarcinoma cells being a notable exception. While Scatchard analysis of [³H]DTG binding sites in all cell lines investigated were linear, indicating a single binding site, differences in complete pharmacological profile suggest sigma-2 receptor heterogeneity across tumor cell types (Vilner, John, et al. 1995). Sigma-2 receptor density has also been demonstrated to be dynamic with proliferative status. In a study using Line 66 mouse mammary adenocarcinoma, actively proliferating cells expressed 10-fold higher sigma-2 receptor levels than quiescent cells (Mach et al. 1997). This correlation of sigma-2 receptor density with proliferative status was confirmed *in vivo* in a follow-up study of Line 66 tumors grown in female nude mice (Wheeler et al. 2000).

At the subcellular level, a 1996 study by Torrence-Campbell and Bowen demonstrated that sigma-2 receptors are more resistant to solubilization than their sigma-1 counterpart (Torrence-Campbell and Bowen 1996). This study raised the possibility that, like sigma-1 receptors, sigma-2 receptors might also reside in lipid rafts; however, the results indicated that sigma-2 receptors may reside more heavily in lipid rafts than sigma-1 receptors due to their higher retention in particulate fractions. Indeed, a follow-up study demonstrated colocalization of sigma-2 receptors with flotillin-2, a lipid raft marker (Gebreselassie and Bowen 2004). Binding of [^3H]-DTG in membrane preparations solubilized in 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer, a detergent in which membranes are generally solubilized but lipid rafts remain intact, demonstrated typical sigma-2 receptor characteristics.

Subsequently, the use of two-photon and confocal microscopy has made possible the visualization of sigma-2 receptor localization on a more discrete level. Similar to sigma-1 receptor distribution, sigma-2 receptors were found to colocalize with markers of mitochondria, lysosomes, endoplasmic reticulum, and plasma membrane by the use of fluorescent probes (Zeng et al. 2007). The presence of sigma-2 receptors on the plasma membrane has been expanded upon by a study using a series of ligands developed by Brian de Costa, which demonstrated that amine-containing ligands had less potent sigma-2 receptor activity at low pH values, in which the amine group would be protonated and therefore the ligand would be unable to easily cross the plasma membrane, as compared to high pH values, at which the amine would be deprotonated and the ligands would be more easily able to pass into the cytosol (Vilner, de Costa, et al. 1995). These results indicate that the ligand binding site for sigma-2 receptors at the plasma membrane

is likely located on the cytosolic side of the membrane, rather than on the extracellular surface, which would be readily accessible at all pH values.

Like that of the sigma-1 subtype, the endogenous ligand for the sigma-2 receptor has not yet clearly been determined. As such, the endogenous function of this receptor remains a mystery. However, sigma-2 receptor expression in the brain and other organs, which is particularly dense in rodent tissues, suggests a ubiquitous physiological role for these receptors. In its initial stages of discovery, the challenge of studying the sigma-2 receptor was compounded by a paucity of sigma-2 receptor-selective ligands. Early ligands such as DTG, (+)-3-PPP, and haloperidol do not differentiate well between sigma subtypes, and discriminating ligands such as (+)-pentazocine and (+)-SKF-10,047 bind only sigma-1 receptors. The development of sigma-2 selective ligands has been an ongoing challenge in the field, with only a few notable successes.

Although the endogenous ligand for the sigma-2 receptor has not yet been clearly elucidated, a series of studies by Connor and Chavkin in the early 1990's implicated ionic zinc as a natural binding product for the sigma-2 receptor. This group stimulated mossy fibers in rat hippocampal slices using focal electrical stimulation, and noted a resultant displacement of [³H]DTG from a haloperidol-sensitive site in the dentate region of the slice in a time-dependent manner (Connor and Chavkin 1991). This study indicated that a product released upon excitatory pathway stimulation initiated by granule cells displaced [³H]DTG, and as such may be an endogenous ligand for the [³H]DTG binding site. Upon further investigation of the substances released as a result of mossy fiber stimulation, Connor and Chavkin determined that ionic zinc (Zn²⁺) displaced [³H]DTG, but not [³H](+)-pentazocine, from rat brain membranes (IC₅₀=110 ± 3 μM for [³H]DTG displacement, IC₅₀=1.4 ± 0.05 mM for [³H](+)-pentazocine displacement) (Connor and Chavkin 1992). As [³H](+)-pentazocine was not displaced in these experiments, the

displacement of [^3H]DTG was attributed to removal from sigma-2 receptors. Chelation of Zn^{2+} using the specific zinc chelator metallothionein peptide 1 inhibited [^3H]DTG displacement, further indicating the specificity of this effect. Two initial possible explanations are plausible to explain these observations: 1) Zn^{2+} may bind directly to the [^3H]DTG binding site, or 2) Zn^{2+} may modulate the [^3H]DTG binding site, allosterically or directly, such that it is modified and no longer canonically binds [^3H]DTG.

While a widely accepted endogenous ligand for sigma-2 receptors has still yet to be elucidated, a variety of sigma-2 receptor-targeting synthetic ligands have led to various studies about possible functions that the sigma-2 receptor is capable of inducing. Siramesine, CB-64D, CB-184, and Brian de Costa's "BD" series of ligands (including BD737, BD1008, BD1047, and BD1063) have all been studied liberally in investigations of sigma-2 receptor activity (Vilner, de Costa, et al. 1995; Bowen et al. 1995; Ostenfeld et al. 2005). The Mach lab and the McCurdy lab have also both contributed significantly to the pool of sigma ligands available for study, and have often investigated the *in vitro*, and in some cases *in vivo*, activity of their ligands (Xu et al. 2005; Seminerio et al. 2012; Mesangeau et al. 2008; Kaushal et al. 2011; Kaushal et al. 2013).

Activation of sigma-2 receptors by synthetic ligands can induce a variety of functions *in vitro*, with a variety of outcomes on cellular function and viability. Perhaps the most immediate effect of sigma-2 receptor activation demonstrated to date is the initiation of a transient increase in cytosolic calcium (Vilner and Bowen 2000). This calcium is derived from thapsigargin-sensitive stores in the endoplasmic reticulum, consistent with the high density of sigma-2 receptors at the ER membrane. In SK-N-SH neuroblastoma cells, which express high levels of sigma-2 receptors, calcium levels peak 1-3 minutes after exposure to ligand and return to basal levels after roughly 5 minutes of exposure, indicating a fast and transient induction. If ligand is

removed within the first ten minutes of exposure, no changes in cell morphology or viability are observed, consistent with a non-toxic, perhaps stimulative role for this change in cytosolic calcium level. Interestingly, if exposure to agonist is sustained for 20 minutes, a slow second rise in cytosolic calcium is observed, likely originating from the mitochondria. This second calcium signal corresponds temporally to appearance of markers of cell death.

Another result of sigma-2 receptor activation by synthetic agonists that has been revealed is the ability to increase ceramide levels (Crawford et al. 2002; Bowen et al. 2000; Bowen et al. 2001). Depending on cell type and source of precursors, generation of ceramide can induce cell growth, proliferation, or death (Kolesnick and Krönke 1998; Ogretmen and Hannun 2004). In MCF-7/Adr, T47D, and SK-N-SH cancer cell lines, binding of sigma-2 receptor agonist CB-184 caused a ≥ 2.5 -fold increase in ceramide (Crawford et al. 2002; Bowen et al. 2000). This effect could be attenuated by sigma-2 receptor antagonism by AC927. Similarly, CB-184-induced activation of sigma-2 receptors in SKBr3 cells resulted in dose-dependent formation of sphingosylphosphorylcholine, another lipid-based cellular messenger that can induce pro-survival and pro-apoptotic pathways (Bowen et al. 2001). This induction also could be attenuated by AC927 binding, and is consistent with more recent findings demonstrating a loss of sphingomyelin, a precursor of hydrolysis-based formation of sphingosylphosphorylcholine, in response to sigma-2 receptor activation (Crawford et al. 2002). While the discrete role of sphingosylphosphorylcholine in cells has yet to be fully elucidated, it is likely that this response is related to the calcium transient induced by sigma-2 receptor activation, as sphingosylphosphorylcholine is known to interact with phospholipase C-coupled G protein coupled receptors, as well as endoplasmic reticulum-bound calcium gating molecules (Ge et al. 2011; Orlati et al. 1998; Bowen 2007).

A terminal functional readout for sigma-2 receptor activation by synthetic agonists is the reduction of cell viability. An early study of sigma receptor function investigated a series of sigma receptor-binding compounds for their effects on C6 glioma cell morphology and viability (Vilner, de Costa, et al. 1995). All sigma-2 receptor agonists induced withdrawal of cellular projections and rounding within three hours of exposure, which could be reversed if ligand was removed at that point (although at the time of publication the ligands were not yet known to share the characteristic of high affinity sigma-2 receptor binding). Upon continued exposure, a reduction in cell viability was observed. The exact pathways induced by sigma-2 receptor activation that lead to cell death are ambiguous and diverse, with exact stepwise readouts remaining inconsistent between ligands and cell types.

Functional assays for sigma-2 receptor activation confirmation remain controversial upstream of cell death, however certain steps appear to be sustained across a variety of cell types, although not universally. Activation of caspase-3 and DNA fragmentation have been both observed in response to sigma-2 receptor agonist treatment induced by at least 12 discrete ligands across several cell types from diverse origins including EMT-6 mouse breast cancer and MDA-MB-435 human melanoma (Zeng et al. 2014; Zeng et al. 2012). Activation of additional caspases has also been demonstrated in SK-N-SH human neuroblastoma and PANC-1 human pancreatic cancer, suggesting caspase involvement in sigma-2-induced apoptosis (X. Wang and Bowen 2006; X. Wang and Bowen 2009). However, complete dependence on caspase activity has not yet been demonstrated, as attempts to inhibit apoptosis in any cell line using subtype specific caspase inhibitors as well as the broad spectrum caspase inhibitor Z-VAD-FMK have been only partially effective at best (Zeng et al. 2012; X. Wang and Bowen 2006; X. Wang and Bowen 2009; Crawford and Bowen 2002).

Additional steps that have been demonstrated to occur in response to agonist activity at sigma-2 receptors include cleavage of full-length BH3 interacting-domain death agonist (Bid) into its pro-apoptotic fragment (X. Wang and Bowen 2006), induction of an immediate transient increase in cytosolic calcium (Vilner and Bowen 2000; Cassano et al. 2009), loss of lysosomal membrane integrity (Hornick et al. 2012; Ostenfled et al. 2005), oxidative stress (Ostenfeld et al. 2005; Hornick et al. 2012), and activation of cathepsins (Jonhede et al. 2010; Ostenfled et al. 2005; Hornick et al. 2012). Additionally, increased synthesis of the autophagy marker light chain 3 and changes in cyclin levels have been reported in response to sigma-2 receptor activation, suggesting non-apoptotic mechanisms of reducing cell viability that can be mediated by this receptor (Zeng et al. 2012). The variety of effects on the morphology, cell cycle progression, and viability of cells that appear to be mediated by sigma-2 receptors is extensive. This lack of consensus for functional readouts in advance of cell death that can be used to determine ligand classification increases the reliance of the field on pharmacological techniques. Ligands classified as sigma-2 receptor antagonists are largely determined by their ability to attenuate the activity of sigma-2 receptor agonists, and vice versa, presumably by competition for receptor occupancy.

In addition to reduced cell viability, the only step in sigma-2-induced cell death that appears to be conserved across all cell types investigated to date is the depolarization of mitochondria. This step in the variety of mechanisms of cell death that appear in sigma-2-mediated loss of cell viability is consistent with the observed results from many groups, as mitochondrial depolarization can occur both as an inducer and as a result of additional apoptotic and autophagy-inducing pathways (Elmore 2007). As a step in the classical intrinsic apoptotic pathway in sigma-2-mediated cell death resultant from activation with the sigma-2 receptor full

agonist CB-64D, mitochondrial depolarization in SK-N-SH human neuroblastoma cells promotes apoptosis through release of cytochrome c, endonuclease G, and apoptosis-inducing factor (AIF) from mitochondrial stores into the cytosol (X. Wang and Bowen 2006). EndoG and AIF then traffic to the nucleus, where they can disrupt DNA stability, while cytochrome c initiates caspase cascades through the activation of caspase-9 (Jiang and Wang 2004; Cande et al. 2002; Li et al. 2001; Heiskanen et al. 1999). Alternatively, such mechanisms as caspase-8/10 activation can lead to apoptosis by caspase cascade, while additionally inducing cleavage of Bid and therefore promoting mitochondrial depolarization as a secondary measure (Kroemer et al. 2007). Mitochondria can also depolarize in autophagic processes, again demonstrating the potential for convergence across a variety of sigma-2 receptor-induced mechanisms of cell death (Lemasters 2014; Gomes and Scorrano 2013).

Despite the progress that has been made in elucidating the distribution and function of the sigma-2 receptor, there are extensive challenges that make the study of this complex receptor difficult. The majority of sigma-2 receptor-binding ligands also demonstrate moderate to high affinity for the sigma-1 receptor subtype, thus making it difficult to examine effects mediated by the sigma-2 receptor without input from sigma-1 receptor activity. Additionally, the apparent intracellular location of this receptor means that ligands must be cell-permeable in order to bind the sigma-2 receptor, posing another barrier to simple ligand development. However, these two challenges are minor in comparison to the difficulties associated with investigating a receptor that is not cloned. Neither molecular biology techniques nor antibodies are available for sigma-2 receptor study, as a genetic sequence has yet to be determined and the protein structure has not yet been solved.

The sigma-2 receptor has not yet been cloned for a variety of reasons that range from facile to complex. A simple contributing factor stems from the roots of sigma receptor history, which lie in behavioral pharmacology. It is only in recent years that scientists from adjacent fields, including molecular biology, have become interested in sigma receptors. As such, historically there has not been significant effort from researchers with the required skillset to clone the sigma-2 receptor. The growing list of applications and functions that are emerging for the sigma-2 receptor will undoubtedly help change this fact, and will increase interest and research surrounding the protein. A second challenge to the cloning of the sigma-2 receptor is the lack of a homolog that could be used for homology cloning, as was successful in the cloning of many G-protein coupled receptors. The sigma-2 receptor has no known cousin in the mammalian genome, despite sharing part its name with the sigma-1 receptor.

If a sequence were to be elucidated for this protein, still there would not exist a cell line that does not already express sigma-2 receptors that could be used to determine gain-of-function. Transfection with the sigma-2 receptor sequence in a plasmid could lead to functional changes, however it would not be possible to determine whether such changes originated from an increase in sigma-2 receptor level or from increase in a different protein, which may or may not affect or mimic sigma-2 receptor function. This method is further hindered by a lack of consensus in the field concerning appropriate functional assays that indicate sigma-2 receptor activity. A final challenge that has traditionally plagued the field yet is now being overcome is the paucity of non-tritiated radioligands. Tritium is a weak β -emitter, and is thus not ideal for colony selection by indicating receptor presence. The recent development of iodinated radioligand probes targeting the sigma-2 receptor may aid in future attempts to clone this enigmatic protein.

In 2011, Mach and colleagues published a study linking the 21.5 kDa sigma-2 receptor binding site with the 25 kDa progesterone receptor membrane component-1 (PGRMC1). In this study the photoaffinity probe WC-21, developed by the Mach lab, was crosslinked to a protein that was then isolated (Xu et al. 2011). WC-21 had been shown previously to displace [^3H]RHM-1, a sigma-2-selective radioligand developed in the same lab. The crosslinked protein was sequenced and identified as PGRMC1. Binding of the sigma-2 receptor radioligand [^{125}I]RHM-4 in HeLa cells was displaced by PGRMC1 ligand AG205, and overexpression or siRNA silencing of PGRMC1 in HeLa cells resulted in an increase or decrease in [^{125}I]RHM-4 binding, respectively. These data indicate a clear relationship between the sigma-2 receptor and PGRMC1; however it is not entirely clear that the two are in fact the same protein. Differing molecular weights, opposing *in vitro* functions, and the inability of PGRMC1 ligand AG205 to displace [^3H]DTG suggest there may be a more complex relationship between PGRMC1 and sigma-2 receptors. Additional possibilities that could explain this relationship include binding of WC-21 to PGRMC1, indicating overlapping pharmacology of the two proteins, or complexation of PGRMC1 and sigma-2 receptors. It is difficult to assess the possibility of overlapping pharmacology due to a paucity of PGRMC1-selective radioligands for use in binding studies, however genetic studies have provided some insight into this relationship.

Indeed, the hypothesis that PGRMC1 and sigma-2 receptors are one in the same has received criticism in the intervening time since it was first proposed. Using CRISPR-Cas9 gene editing technology, Ruoho and colleagues demonstrated that cells in which the *PGRMC1* gene has been knocked out retain [^3H]DTG binding sites, indicating continued presence of sigma-2 receptors (Chu et al. 2015). It was therefore concluded that PGRMC1 and the sigma-2 receptor are derived from independent genes. Continuing to dispel the notion that PGRMC1 and the

sigma-2 receptor are one in the same, Abate and colleagues demonstrated that silencing *PGRMC1* with shRNA in MCF-7 cells did not decrease the Bmax of sigma-2 receptors indicated by [³H]DTG binding, nor did stable overexpression of *PGRMC1* result in an increase in [³H]DTG binding saturation level. While this study again discredits the hypothesis that the sigma-2 receptor and PGRMC1 are identical proteins, it still leaves room for the possibility of protein-protein interactions.

This idea was supported in a recent nanosymposium at the Society for Neuroscience Annual Meeting 2015, during which the corresponding author of the original associative study linking PGRMC1 and sigma-2 receptors, Dr. Robert Mach, showed as yet unpublished data indicating that WC-21 binds both the sigma-2 receptor and PGRMC1 simultaneously on opposite sides of the ligand. This revelation indicates an extremely close proximity of PGRMC1 and the sigma-2 receptor to allow for such dual interaction. The synthesis of a ligand similar to WC-21 that is able to crosslink both proteins simultaneously may be the key to the purification and cloning of the sigma-2 receptor, while the current results shed light on the first evidence of direct protein-protein interaction involving sigma-2 receptors.

1.2 Sigma-2 Receptors and Cancer

As the foundation of knowledge surrounding sigma-2 receptor biology has grown, so has interest in the implications of this receptor in disease states. The most evident application for sigma-2 receptor intervention is in oncology, where differential expression and induction of apoptosis can both be hypothesized to have benefit as diagnostic and chemotherapeutic tools. A major challenge to the development of selective and well-tolerated treatments for many cancers

is the dearth of targets that can effectively differentiate between tumor cells and non-cancerous tissue. As cancer is a disorder derived from normal cells within the body, typical protective measures such as the immune system may not detect such malignancies as they express normal cell markers.

In 2015, the National Cancer Institute estimated there would be 1.6 million new cancer diagnoses and over half a million deaths from cancer in the United States, with ~40% of the population contracting the disease in their lifetime (“Cancer Statistics” 2015). One major difficulty in treating cancer derives from the origin of the cancer itself. The first cancerous cell that gives birth to a tumor is, in its origin, a healthy normal cell. As such, the cell will express many of the same cell markers and employ the same required cellular machinery as the normal cells from which it is derived. This makes discriminating between cancerous cells and normal cells difficult, and targeting only cancerous cells a major challenge. As many tumor cells proliferate more rapidly than differentiated healthy cells, many conventional chemotherapeutic agents target this difference by interfering with DNA replication, mitosis, and cell cycle progression mechanisms. Examples of such drugs that are in clinical use include the antimetabolite 5’ fluorouracil that interferes with nucleotide synthesis, the topoisomerase II inhibitor etoposide, and the microtubule stabilizer paclitaxel. However, therapies of this nature often have adverse effects for non-cancerous cells when they do divide, and can also be highly detrimental to normal cells that have high turnover rates as in the gastrointestinal tract and bone marrow. Further, such therapies only allow for the targeting of proliferating cancer cells, while quiescent tumors are not affected.

In contrast to chemotherapeutic agents that act on cellular targets upon which cancer cells have a higher dependence than normal cells yet normal cells still employ, the sigma-2 receptor

holds promise as a target that is exclusive to cancer cells, whether by expression or function. As described above, sigma-2 receptor expression has been detected in every cancer cell line that has been examined to date. There are two levels of receptor upregulation that make sigma-2 receptor density particularly selective for cancer cells. First, cancer cell lines overexpress sigma-2 receptors as compared to the normal cell type from which the cancer originated (Bem et al. 1991). Second, the more rapidly proliferating and aggressive the cancer cells become, the more highly they express sigma-2 receptors (Mach et al. 1997). These two facets of sigma-2 receptor density indicate that, particularly for highly malignant cancers, sigma-2 receptor-targeting therapy may have a large therapeutic window based on ligand-target interaction probability alone.

Extending the potential for selective sigma-2 receptor-mediated therapy is the existence of sigma-2 receptor partial agonists, low doses of which have been shown to bind sigma-2 receptors but not activate them (Zeng et al. 2014). An analogy can be made between a low dose of ligand and a cell that expresses few sigma-2 receptors, with the corresponding position that cells expressing high densities of sigma-2 receptors would be more likely to have a high number of these sites bound and therefore become activated. This inference allows for the hypothesis that sigma-2 receptor partial agonists may not activate apoptosis in cells expressing a very low number of receptors, while cells highly expressing the sigma-2 receptor may have enough sites bound to become activated and induce cell death. Therefore, a partial agonist would likely target cancerous cells, particularly highly aggressive malignancies, while sparing noncancerous tissues that either do not express or express very low densities of sigma-2 receptors.

Due to their differential expression levels from normal cells to any cancer, whether quiescent or rapidly proliferating, the sigma-2 receptor has been employed in tumor imaging.

While several radioactive ligands have been developed for this use, the most successful thus far is [^{18}F]ISO-1, developed by the Mach lab. [^{18}F]ISO-1 has been used in rodent brain and breast cancer models as well as in human patients with lymphoma, head and neck cancer, and breast cancer to successfully image the location of the tumor (Mach et al. 2013). Generally, surrounding normal tissues exhibit negligible uptake of the radiotracers. Therapy planning has also benefited from the use of sigma-2 receptor probes for tumor imaging, as sigma-2 receptor density is well correlated with the P:Q ratio of solid tumors (Wheeler et al. 2000; Mach et al. 1997). Thus, highly effective imaging by sigma-2 receptor probes indicates a high P:Q ratio, and therefore a personalized treatment plan based on the proliferative status of the tumor can be developed.

By the same principle of differential receptor density between cancerous cells and healthy tissue that is exploited for tumor imaging, sigma-2 receptors have been targeted for drug delivery purposes. Conjugation of the sigma-2 receptor-targeting ligand SW-43 to a mimetic of the pro-apoptotic second mitochondria-derived activator of caspases (SMAC) was shown to potentiate ovarian cancer cell death and enhance selective targeting of the cancer cells (Garg et al. 2014). Similar effects were observed with sigma-2 receptor agonist SV119 conjugated to pro-apoptotic Bim or to the small molecule chemotherapeutic rapamycin, both cases in which selectivity and potency were enhanced using the conjugate as compared to either ligand alone (Spitzer et al. 2012). When conjugated to a sigma-2 receptor agonist, there exists the additional benefit of sigma-2 receptor-induced cell death to couple to cell death induced by the conjugated cytotoxic cargo being delivered to the cancer cells. Sigma-2 receptor ligands have also recently been conjugated to gold nanocages and delivered to human melanoma, breast cancer, and prostate cancer cell lines (Y. Wang et al. 2012). These nanocages have the potential to be loaded with a

wide variety of chemotherapeutic agents that do not readily pass through cell membranes. This can both enhance efficacy of the treatment as well as reduce off-target adverse effects by increasing cancer selectivity with targeted delivery.

Pertaining to cancer treatment, additional challenges surround mechanisms of chemotherapeutic resistance. The most commonly mutated tumor suppressor in cancer is p53, often dubbed the “guardian of the genome”. This tumor suppressor detects DNA damage, and can halt cell cycle progression to determine whether an attempt to repair the damage should be made or if the damage is too extensive and apoptosis should be induced. Mutations to p53 that render it nonfunctional negate the activity of many DNA-damaging chemotherapeutic agents that aim to target the higher DNA replicative rate of cancer cells as compared to normal cells. Sigma-2 receptors are expressed on both p53 mutant and wild-type cells, and efficacy and potency of sigma-2 receptor agonists to induce cell death do not appear to be different in cells with varied p53 status (Crawford and Bowen 2002). Additionally, these results indicate that sigma-2 receptor-induced cell death is not dependent on detection of DNA damage by p53, shedding light on a complex and elusive mechanism.

The activity of sigma-2 receptors is also sustained across cell lines with dysfunctional caspases, which are frequently mutated in cancers as a mechanism to avoid apoptosis (Ghavami et al. 2009). Silencing of specific caspases or loss of caspase processing systems have been demonstrated to increase chemotherapeutic resistance in human neuroblastoma and lymphoblastic leukemia, and loss of caspase-9 regulation results in highly resistant melanoma (Teitz et al. 2001; Prokop et al. 2000). Inhibition of caspase activity in MCF-7 cells using broad spectrum as well as specific caspase-1 inhibitors were able to limit the activity of common S-phase-active chemotherapeutic agents doxorubicin and dactinomycin, but sigma-2 receptor-

induced cell death was not attenuated in response to treatment with CB-64D or CB-184 (Crawford and Bowen 2002). This is consistent with the inability of caspase inhibitors to attenuate sigma-2 receptor-induced cell death in a variety of cell lines in which caspases have been shown to be activated when agonists of the sigma-2 receptor are applied (X. Wang and Bowen 2006; Zeng et al. 2012; Crawford and Bowen 2002). It is possible that an array of apoptotic mechanisms is induced synchronously in response to sigma-2 receptor activation, and therefore loss of a single element is unable to halt the progression of cell death. The diversity of apoptotic and autophagic steps observed in response to treatment of cells with a sigma-2 receptor agonist supports this idea. Sustained sigma-2 receptor activity in cancerous cells regardless of p53 status and functionality of caspases highlight the promise this receptor holds for chemotherapy, particularly where conventional chemotherapeutic agents are not or are no longer viable.

Another mechanism of resistance acquired by cancer cells in an effort to avoid induction of cell death is the upregulation of drug efflux pumps. Hydrophobic compounds that pass through cellular membranes may be toxic to cells, and therefore drug efflux pumps such as P-glycoprotein (also known as multidrug resistance protein 1 (MDR1)), encoded by the *ABCB1/MDR1* gene, can be beneficial to a healthy cell. Cancerous cells can exploit this protective mechanism by upregulating such pumps and causing chemotherapeutic agents to be pumped out of the cells before they can induce cell death. Indeed, even in p53 wild-type cells, upregulation of P-glycoprotein is sufficient to provide resistance to doxorubicin (Linn et al. 1996). Interestingly, a 10 μ M dose of CB-64D, which was below the threshold for detectable changes in cell morphology or viability, induced a 50% reduction in *MDR1* mRNA in C6 rat glioblastoma cells (Bowen et al. 1997). In SK-N-SH human neuroblastoma cells, the same dose

of CB-64D eliminated detectable *MDR1* expression. The effect could be blocked by sigma receptor antagonist BD1047, indicating its specificity. These data suggest that sigma-2-targeted therapy may be beneficial as combinatorial treatment with traditionally successful chemotherapeutics that are rendered ineffective by multi-drug resistance pump activity. Additionally, potency of CB-64D in MCF-7 cells, which are p53 wild-type and do not overexpress P-glycoprotein, was equal to that in MCF-7/Adr cells, which overexpress P-glycoprotein and have mutant p53 (Crawford and Bowen 2002). These results indicate that CB-64D and possibly related structures are not substrates for P-glycoprotein, and additionally sigma-2-mediated therapy can overcome multiple mechanisms of resistance simultaneously.

In light of this finding, an investigation of possible synergistic effects between P-glycoprotein substrate chemotherapeutic agent doxorubicin and sigma-2 receptor agonist CB-184 was undertaken (Crawford and Bowen 2002). Results demonstrated that combination therapy of CB-184 and doxorubicin exceeded the sum efficacy and potency of either treatment alone, indicating synergy. Further, p53-dependent actinomycin D, which is ineffective in p53-mutant MCF-7/Adr cells when used alone, was found to be highly potent when dosed in combination with CB-184. These results indicate that sigma-2 receptor-mediated therapy may help reduce chemotherapeutic resistance against traditional agents, as well as reveals the possible exploitation of the synergistic effects observed when conventional chemotherapeutics are dosed in combination with sigma-2 receptor-targeting agonists. This may allow for the use of lower doses of either agent, thus reducing exposure and the potential for the development of resistance mechanisms.

The promise that sigma-2 receptor targeting holds for cancer therapy is furthered by studies demonstrating the successful treatment of tumor xenograft animal models. In mice

bearing pancreatic cancer allografts, a single bolus dose of sigma-2 receptor agonist WC26 induced apoptotic cell death in ~50% of tumor cells (Kashiwagi et al. 2007). Blood chemistry panels from these mice indicated minimal peripheral toxicity, indicating the specificity of the treatment, and tissue samples from liver, lung, non-tumor pancreas, brain, kidney, and spleen demonstrated the preferential binding of the sigma-2 receptor ligand to tumor tissue. Lower dose administration of WC26 over a 5-day period slowed tumor growth, and survival of the 5-day treated mice was significantly improved over untreated animals for over 40 days following treatment.

Another study of pancreatic cancer xenograft mice treated with sigma-2 receptor agonists SV119, SW43, or siramesine showed the ability to reduce tumor volume equally to treatment with the commonly prescribed chemotherapeutic gemcitabine, while combination therapy using SW43 and gemcitabine resulted in maximal inhibition of tumor growth with minimal toxicity to surrounding and peripheral tissues (Hornick et al. 2010). Similar results were seen using combination therapy with SV119 and paclitaxel in an analogous model (Kashiwagi et al. 2009). Survival of ovarian cancer xenograft mice was also significantly improved and tumor burden diminished upon treatment with sigma-2 receptor agonist SW43 conjugated to SMAC (Garg et al. 2014). While sigma-2 receptor-targeted cancer therapy has not yet been tested in humans, sigma-2 receptor agonist siramesine has been examined in humans for anti-anxiety implications, and while not efficacious for this purpose, the ligand was well-tolerated by healthy patients in Phase I trials (Heading 2001). All of these results taken together provide a bright outlook for the use of sigma-2 receptors to diagnose and treat cancer.

Chapter 2: Characterization of CM572, a Selective Irreversible Partial Agonist of the Sigma-2 Receptor with Antitumor Activity

2.1 Preface

As there has yet to be a cancer cell line discovered that does not express sigma-2 receptors, and without a known sequence for the sigma-2 receptor gene, it is not possible at this time to examine a cancer cell system without sigma-2 receptors. A large amount of information about the physiological function of a particular factor can be gleaned from examining differences between systems that express the factor and systems that lack the factor, particularly in regards to endogenous role. Without molecular biology tools, a pharmacological approximation of a cancer cell system lacking input from sigma-2 receptors could be envisioned through application of an irreversible antagonist. Such a ligand would be expected to permanently block endogenous ligand binding and activation, therefore mimicking a system without sigma-2 receptor function. Alternatively, functional desensitization and subsequent receptor internalization can be achieved by continuous exposure to an agonist, with the end result being a system with few or no receptors left.

This study sought to characterize CM572, an isothiocyanate derivative of the well-characterized sigma-2 receptor-selective antagonist SN79 ($K_i=27$ nM at sigma-1 receptors and 7 nM at sigma-2 receptors) (Kaushal et al. 2013). We hypothesized that the substitution of the SN79 methyl ketone moiety on the heterocyclic ring structure with an isothiocyanate group (resulting in CM572) would impart irreversible binding capability through nucleophilic attack of

a lysine or cysteine in the binding pocket of the sigma-2 receptor. If the new ligand retained the antagonist properties of the parent compound, this would allow for approximation of a cancer cell system without input from sigma-2 receptors through irreversible blockade of the binding sites. SK-N-SH neuroblastoma cells were chosen for initial characterization of the ligand as these cells highly express both sigma receptor subtypes and have been shown to be sensitive to sigma receptor targeting ligands (Vilner and Bowen 2000; X. Wang and Bowen 2006; Vilner, John, et al. 1995).

The following portion of this chapter has been published:

Hilary Nicholson, Anthony Comeau, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen. 2015. "Characterization of CM572, a selective irreversible partial agonist of the sigma-2 receptor with antitumor activity." *Journal of Pharmacology and Experimental Therapeutics* 354(2): 203-12.

Authorship Contributions

Participated in research design: Nicholson, Bowen

Conducted experiments: Nicholson, Comeau

Contributed new reagents or analytic tools: Mesangeau, McCurdy

Performed data analysis: Nicholson, Comeau

Wrote or contributed to the writing of the manuscript: Nicholson, Bowen

ABBREVIATIONS: Bid, BH3-interacting domain death agonist; BSA, bovine serum albumin; CB-64D, (+)-1R,5R-(E)-8-Benzylidene-5-(3-hydroxyphenyl)-2-methylmorphan-7-one); CB-184, (+)-1R,5R-(E)-8-(3,4-dichlorobenzylidene)-5-(3-hydroxyphenyl)-2-methylmorphan-7-one); CM572, 3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[d]oxazol-2(3H)-one; DTG, 1,3-di-o-tolylguanidine; HEM, human primary melanocytes; HBSS, Hank's Buffered Salt Solution; HMEC, human mammary epithelial cells; MEM, Minimal Essential Media; MTT, 3-[4,5 dimethylthiazol-2-y]-2,5 diphenyltetrazolium bromide; PGRMC1, progesterone receptor membrane component 1; RIPA buffer, radioimmunoprecipitation assay buffer; RT, room temperature; siramesine, 1'-{4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butyl}-3*H*-spiro[2-benzofuran-1,4'-piperidine]; SN79, (6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one); TBS, Tris buffered saline.

2.2 Abstract

Sigma-2 receptors are promising therapeutic targets due to significant upregulation in tumor cells compared to normal tissue. Here we characterize CM572 (3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[d]oxazol-2(3*H*)-one) (sigma-1 $K_i \geq 10$ μ M, sigma-2 $K_i = 14.6 \pm 6.9$ nM), a novel isothiocyanate derivative of the putative sigma-2 antagonist, SN79 (6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3*H*)-one). CM572 binds irreversibly to sigma-2 receptors by virtue of the isothiocyanate moiety, but not to sigma-1. Studies in human SK-N-SH neuroblastoma cells revealed that CM572 induced an immediate, dose-dependent rise in cytosolic calcium concentration. A 24 h treatment of SK-N-SH cells with CM572 induced dose-dependent cell death, with an $EC_{50} = 7.6 \pm 1.7$ μ M. This effect was sustained over 24 h even after a 60 min pretreatment with CM572, followed by extensive washing to remove ligand, indicating an irreversible effect consistent with the irreversible binding data. Western blot analysis revealed that CM572 also induced cleavage activation of pro-apoptotic Bid. These data suggest irreversible agonist-like activity. Low concentrations of CM572 that were minimally effective were able to significantly attenuate the calcium signal and cell death induced by the sigma-2 agonist CB-64D ((+)-1*R*,5*R*-(*E*)-8-benzylidene-5-(3-hydroxyphenyl)-2-methylmorphan-7-one). CM572 was also cytotoxic against PANC-1 pancreatic and MCF-7 breast cancer cell lines. The cytotoxic activity of CM572 was selective for cancer cells over normal cells, being much less potent against primary human melanocytes and human mammary epithelial cells. Taken together, these data show that CM572 is a selective, irreversible sigma-2 receptor partial agonist. This novel irreversible ligand may further our understanding of the endogenous role of this receptor, in addition to having potential use in targeted cancer diagnosis and therapy.

2.3 Introduction

Sigma receptors are a class of membrane-bound receptors that have affinity for a wide variety of psychotropic agents. There are two subtypes of sigma receptor, denoted sigma-1 and sigma-2, which differ in their pharmacological profile (Hellewell and Bowen 1990; Hellewell et al. 1994). Both subtypes are found in the central nervous system and in peripheral tissues throughout the body. The sigma-1 receptor has been well characterized and functions as a ligand-regulated chaperone protein that modulates the function of other receptors, ion channels, and transporters and plays a role in promotion of cell survival (Hayashi and Su, 2007; Maurice and Su, 2009).

The sigma-2 receptor is highly expressed in a variety of cancer cell lines (Vilner et al., 1995b), and becomes even more highly upregulated when cancer cells are in a state of rapid proliferation (Mach et al., 1997; Al-Nalbusi et al., 1999; Wheeler et al., 2000). Ligands presumed to activate sigma-2 receptors induce apoptotic cell death in cancer cell lines (Vilner et al., 1995a; Crawford and Bowen, 2002; Ostensfeld et al. 2005; Bowen, 2007; Abate et al., 2012; Zeng et al. 2012; Mach et al., 2013). Because of these aspects, the sigma-2 receptor has received growing attention as a potential target in cancer therapy and efforts are in progress to elucidate the identity and function of this protein and to develop highly selective targeting ligands. Using a fluorescent photoaffinity probe, [125 I]RHM-4 as sigma-2 radioligand, and proteomic analysis, Mach and colleagues have demonstrated a relationship of sigma-2 receptors to progesterone receptor membrane component-1 (PGRMC1), which is also highly expressed in cancer cells and regulates cell growth and survival (Xu et al., 2011). This work highlights the importance of the development of unique tools with which to further study this receptor.

While antagonists for sigma-1 receptors are readily available, selective antagonists for sigma-2 receptors have not been well described. This is partially due to a paucity of selective sigma-2 receptor ligands and detailed knowledge about the functional roles of sigma-2 receptors. Sigma-2 receptor ligand classification has had several proposals for criteria defining an agonist, antagonist, or partial agonist. As mentioned above, compounds presumed to be sigma-2 agonists induce apoptotic cell death. Several aspects of the purported sigma-2 signaling mechanism have been used in an attempt to define agonists. For example, Mach and colleagues have used the criteria of caspase-3 activation and loss of cell viability in EMT-6 mouse breast cancer and MDA-MB-435 human melanoma cells to define putative sigma-2 agonists and antagonists that correlated with comparisons to siramesine (1'-{4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butyl}-3*H*-spiro[2-benzofuran-1,4'-piperidine]), a presumed sigma-2 full agonist (Ostenfeld et al., 2005; Zeng et al., 2012, 2014). Sigma-2 receptors mediate a rapid and transient calcium release from the endoplasmic reticulum of SK-N-SH neuroblastoma cells (Vilner and Bowen 2000; Cassano et al. 2009). We have characterized CB-64D and CB-184 as sigma-2 agonists, based on their ability to induce a calcium transient and apoptotic cell death in SK-N-SH neuroblastoma and breast tumor cell lines (Bowen et al. 1995; Crawford and Bowen 2002). We have also proposed that caspase-10-induced cleavage and activation of the proapoptotic Bcl-2 family protein Bid, mitochondrial depolarization, and subsequent release of mitochondrial apoptogenic factors are important steps in sigma-2-induced apoptosis in some cells (Hazelwood and Bowen, 2006; Wang and Bowen, 2006). CB-64D induces rapid Bid cleavage and mitochondrial release of endonuclease G and apoptosis inducing factor in SK-N-SH neuroblastoma cells, and effects are attenuated by a broad spectrum caspase inhibitor as well as a caspase-10-specific inhibitor (Wang and Bowen, 2006).

Despite these advances in classifying sigma-2 agonists, there has been little reported on direct characterization of antagonists in these assays. That is, direct demonstration that the activity of putative agonists can be attenuated by other sigma-2 ligands that do not exert an effect. The moderately sigma-2-selective ligand SN79 has been characterized as an antagonist of sigma-1/sigma-2 receptors (Kaushal et al. 2011; Kaushal et al. 2012). SN79 protects against neurotoxicity induced by high doses of methamphetamine, which binds to sigma receptors, while the sigma receptor agonist DTG enhanced the neurotoxic effect. In an approach to develop a functional antagonist of sigma-2 receptors, we characterized a series of isothiocyanate derivatives of SN79 (Comeau et al. 2012). The goal was to incorporate the isothiocyanate moiety in order to impart the ability to bind irreversibly to the receptor, thereby producing chronic, functional sigma-2 receptor blockade. One of these compounds, CM572 is further characterized here. Using effects on radioligand binding, calcium response, Bid cleavage, and cell viability, we characterize CM572 as a potent, irreversible, sigma-2 selective partial agonist.

2.4 Materials and Methods

Radioligand binding assay

Receptor binding assays were performed using rat liver membrane homogenates, as previously described with minor modifications (Hellewell et al. 1994). Briefly, incubations were carried out in 20 mM HEPES, pH 7.4 for 120 min at 25°C using 150 µg rat liver membrane protein. HEPES buffer was substituted for the normally used 50 mM Tris-HCl, pH 8.0 buffer in incubations with CM572 in order to avoid reaction of the isothiocyanate group with the amino group of Tris. Sigma-1 receptors were labeled with 3 nM [³H](+)-pentazocine (PerkinElmer, Waltham, MA). Sigma-2 receptors were labeled with 5 nM [³H]DTG (PerkinElmer, Waltham, MA) in the presence of 100 nM (+)-pentazocine to block sigma-1 receptors. Nonspecific binding was determined in the presence of 10 µM haloperidol. Assays were terminated by addition of 5 mL ice cold 10 mM Tris-HCl, pH 7.4 and filtration using a Brandel Cell Harvester (Brandel, Gaithersburg, MD). Filters were washed twice with 5 mL ice cold buffer. Filters were soaked in 0.5% polyethyleneimine for at least 30 min at 25°C prior to use. K_i values were determined by competition assays with data analysis using GraphPad Prism (GraphPad Software, La Jolla, CA).

Irreversible binding analysis

Membranes were incubated with the indicated concentration of CM572 for 60 min at 25°C in 20 mM HEPES, pH 7.4 at a protein concentration of 0.30 mg/mL. The preparation was then diluted to a protein concentration of 0.018 mg/mL with ice-cold buffer and centrifuged at 37,000 × g for 10 min. The pellet was resuspended to the original volume with ice-cold buffer and centrifuged again. Following resuspension to the original volume with 20 mM HEPES, pH

7.4, the preparation was allowed to incubate for 60 min at 25°C to allow for dissociation of any non-covalently bound isothiocyanate. The preparation was then subjected to centrifugation at $37,000 \times g$ for 10 min and resuspended to a protein concentration of 0.6 mg/mL in 50 mM Tris pH 8.0 and used directly in the radioligand assay described above. The control membranes were treated in the identical manner without exposure to CM572. This washout method was shown to effect complete dissociation of 500 nM SN79 from both sigma-1 and sigma-2 receptors (data not shown).

Cell culture

Human SK-N-SH neuroblastoma and MCF-7 breast adenocarcinoma (ATCC, Manassas, VA) cells were cultured in MEM (Gibco, Grand Island, NY) with 10 mg/L insulin and 10% fetal bovine serum at 37°C and 5% CO₂ in a humidified atmosphere. Human PANC-1 pancreatic epithelioid carcinoma (ATCC, Manassas, VA) cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum and 2 mM L-glutamine at 37°C and 5% CO₂ in a humidified atmosphere. Human melanocytes from neonatal foreskin (HEM) were a generous gift from Dr. Elena Oancea, Brown University, Providence, RI. HEM cells were cultured in Medium 254 (Life Technologies, Grand Island, NY) with 5% Human Melanocyte Growth Supplement 2 (Life Technologies, Grand Island, NY) at 37°C and 5% CO₂ in a humidified atmosphere. Normal pre-stasis human mammary epithelial cells (HMEC) were kindly provided by Dr. Martha Stampfer, Lawrence Berkeley National Laboratory, Berkeley, CA. HMEC cells were cultured in M87A+CT+X medium at 37°C and 5% CO₂ in a humidified atmosphere.

Cell viability assay

Cytotoxicity was measured using MTT assays (Trevigen, Gaithersburg, MD). Assays were performed in 96-well plates with 15,000 cells/well (SK-N-SH, HEM) or 10,000 cells/well (MCF-7, PANC-1, HMEC). Cells were allowed to attach overnight prior to dosing. After 24 h (SK-N-SH, HEM, PANC-1, HMEC) or 48 h (MCF-7, HMEC) incubation with ligand (total volume 100 μ L/well), 10 μ L MTT reagent was added and allowed to be metabolized for 3 h. Detergent was then added (100 μ L) and allowed to solubilize membranes and MTT crystals for 2 h. Absorbance was then measured at 570 nm. Dose-response curves were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

In experiments where the irreversible effect of ligands was examined, cells were exposed acutely to sigma ligand for the indicated period of time (30, 60, or 120 min) in Hank's Buffered Salt Solution (HBSS) (Gibco, Grand Island, NY). Wells were then washed twice with 100 μ L of culture medium to remove any unbound ligand. After addition of 100 μ L fresh normal culture medium, cells were allowed to incubate for 24 h and viability was assessed by MTT assay.

Calcium release assay

Calcium responses were measured using fura-2,AM ratiometric assay (Invitrogen, Grand Island, MD). Cells were allowed to attach and proliferate for 48 h after seeding at 20,000 cells/well. Media was then replaced with 2.47 μ M fura-2,AM and 0.065% pluoronic acid in HBSS and incubated for 60 min. Fura-2 solution was then removed and cells were washed three times with 100 μ L HBSS each prior to injection of ligand in HBSS for final total volume of 100 μ L (injection volumes did not exceed 30 μ L). Calcium changes indicated by ratio of fura-2

fluorescence were measured with excitation at 340 nm and 380 nm with emissions measured at 510 nm using a PerkinElmer Victor V platereader (PerkinElmer, Waltham, MA) in 96-well format.

Western blot

Cells were plated in 35 mm petri dishes at 500,000 cells/dish and were allowed to attach overnight prior to treatment. Samples were treated with the indicated dose of CM572 for the indicated time prior to lysis with RIPA buffer with protease and phosphatase inhibitor cocktail. Western blotting for Bid was performed using 15% acrylamide gel, transferred overnight at 40 V at 4°C onto nitrocellulose paper. Blot was then blocked for 1 h at RT shaking in 10% milk/0.1% Tween/TBS and washed three times with ~15 mL 0.1% Tween/TBS per wash. Blot was then incubated overnight with shaking at 4°C with 1:200 Bid antibody (Santa Cruz, Dallas, TX) in 10% milk/0.1% Tween/TBS followed by continued incubation shaking at RT for 1 h. Blot was then washed three times with ~15 mL 0.1% Tween/TBS per wash, incubated at RT with 1:5000 rabbit secondary antibody (Santa Cruz, Dallas, TX) in 10% milk/0.1% Tween/TBS for 1 h, washed three times with ~15 mL 0.1% Tween/TBS per wash, and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Waltham, MA) with directions as specified by manufacturer. Exposures were made using UltraCruz Autoradiography Film (Santa Cruz, Dallas, TX). For loading control, the same procedure was followed with anti α/β tubulin primary antibody (Cell Signaling, Danvers, MA) with incubation at 1:1000 in 5% BSA/0.1% Tween/TBS.

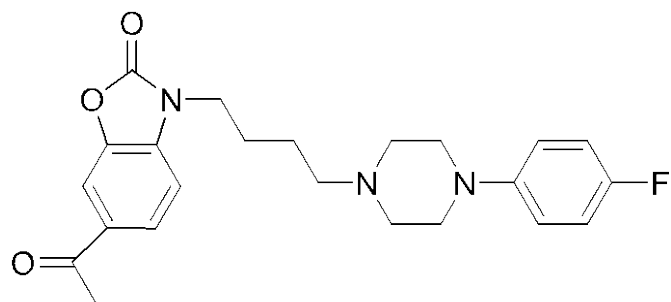
2.5 Results

Radioligand binding and examination of irreversibility in rat liver membranes

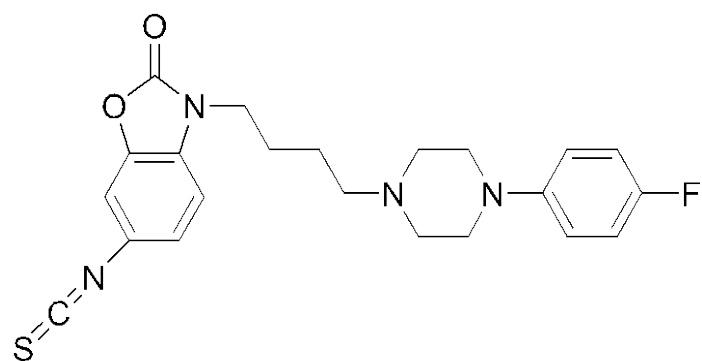
The structures of SN79 and CM572 are shown in Figure 1. Substitution of the methyl ketone moiety of SN79 with an isothiocyanate group results in CM572 (synthesis described previously) (McCurdy et al. 2014). Radioligand competition binding assays were performed in rat liver membranes to determine affinity of CM572 for sigma-1 and sigma-2 receptors. These results are shown in Figure 2. K_i values were determined to be ≥ 10 μ M at sigma-1 and 14.6 ± 6.9 nM at sigma-2, demonstrating a ≥ 700 -fold selectivity of the ligand for sigma-2 over sigma-1, compared to a ~ 4 -fold selectivity for SN79 ($K_i = 7$ nM and 27 nM at sigma-2 and sigma-1, respectively) (Kaushal et al. 2013). The increase in selectivity compared to the parent compound is due largely to the loss in sigma-1 receptor binding activity.

In order to examine whether introduction of the isothiocyanate moiety imparted irreversible binding capability, rat liver membranes were pretreated with various concentrations of CM572 followed by extensive washing to remove unbound compound. Sigma-1 and sigma-2 receptor binding activity (under standard conditions) was then assayed to determine recovery of binding sites. Any loss of receptor binding activity is taken to be the result of residual occupation of the receptor by irreversibly bound CM572. The results are shown in Figure 3. Pretreatment of membranes with CM572 for 60 min resulted in a dose-dependent loss of sigma-2 receptor binding, with an EC_{50} of approximately 30 nM. Less than 30% of sigma-2 binding sites remained at 100 nM, indicating that over 70% of the available sigma-2 sites were irreversibly bound by CM572. By contrast, there was no loss of sigma-1 receptor binding, even at the highest dose of 1000 nM. When membranes were incubated with 500 nM SN79 and subjected to the same procedure nearly total recovery of both sigma-1 and sigma-2 binding was observed, showing that

the conditions used are sufficient to remove a reversibly bound ligand of even higher binding affinity (data not shown). Thus, CM572 binds irreversibly to sigma-2 receptors in a highly selective manner.



SN79



CM572

Figure 1. **Structures of SN79 and CM572.** Substitution of the methylketone moiety of SN79 with an isothiocyanate group results in CM572. Synthesis of CM572 was described previously (McCurdy et al. 2014).

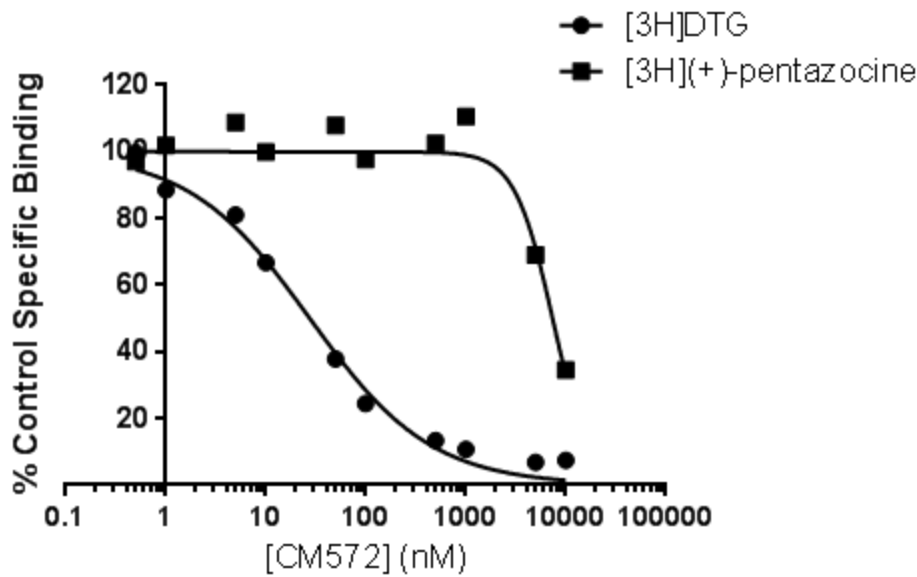


Figure 2. Affinity of CM572 at sigma-1 and sigma-2 receptors. Affinity of CM572 for sigma-1 and sigma-2 receptors was determined by competition binding in rat liver membranes. Assays were carried out as described in Methods, using [3H](+)-pentazocine to label sigma-1 receptors and [3H]DTG in presence of unlabeled (+)-pentazocine to label sigma-2 receptors. K_i values were determined by analysis of competition curves using GraphPad Prism 6 and radioligand K_d values previously determined ([3H](+)-pentazocine K_d , 7.5 nM; [3H]DTG K_d , 17.9 nM; Hellewell et al, 1994). The K_i value for CM572 at sigma-2 receptors was determined to be 14.6 ± 6.95 nM. At sigma-1 receptors, CM572 produced less than complete displacement, with 10 μ M inhibiting radioligand binding by approximately 50% ($K_i > 10$ μ M). Values for sigma-2 are the average of 4 independent experiments $\pm$ S.D., and values for sigma-1 are results of 2 independent experiments. Each experiment was carried out in duplicate. Figure shown is a composite of the averaged experiments.

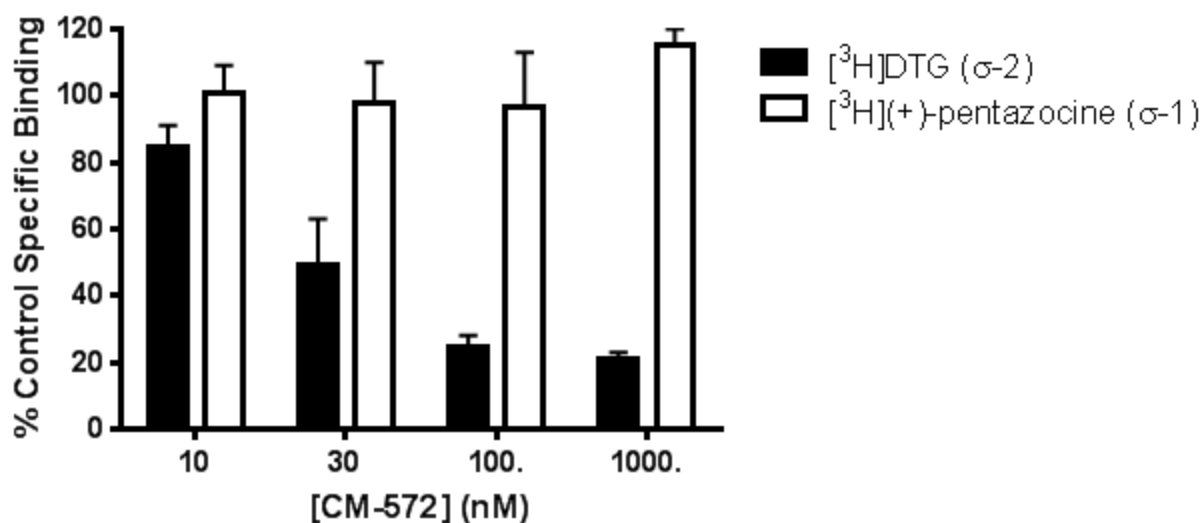


Figure 3. **Irreversible binding of CM572 at sigma-1 and sigma-2 receptors.** Rat liver membranes were exposed to the indicated concentrations of CM572, subjected to extensive washing to remove any unbound compound, and then analyzed for recovery of sigma-1 and sigma-2 receptor binding, as described in Methods. Complete recovery of sigma-1 receptor binding activity (as measured by recovery of [³H]-(+)-pentazocine binding) was observed at all doses of CM572. By contrast, loss of sigma-2 receptor binding activity (as measured by recovery of [³H]-DTG binding in the presence of (+)-pentazocine) was dose-dependent with CM572 pretreatment. Less than 30% of sigma-2 binding was recovered at and above 100 nM CM572, with an EC₅₀ value of ~30 nM. Results are expressed as percentage of total sigma-1 or sigma-2 binding recovered after pretreatment with CM572 at the indicated concentrations as compared to membranes that were not pretreated with CM572, but otherwise treated identically. Data presented is an average of 3 independent experiments ± S.E.M.

CM572-induced calcium response in SK-N-SH neuroblastoma

We next examined the effect of CM572 in cellular functional assays to determine the agonist or antagonist characteristics of the compound in human SK-N-SH neuroblastoma cells. To test for the immediate, transient calcium response characteristic of sigma-2 receptor activation, fura-2,AM ratiometric calcium release assays were performed at varying concentrations of CM572. Results are shown in Figure 4. At 3 μ M CM572, no calcium response was elicited in SK-N-SH neuroblastoma and at 10 μ M CM572 a very small signal was observed, whereas higher concentrations (30, 100 μ M) produced a robust dose-dependent calcium response. These data suggest that CM572 has agonist properties.

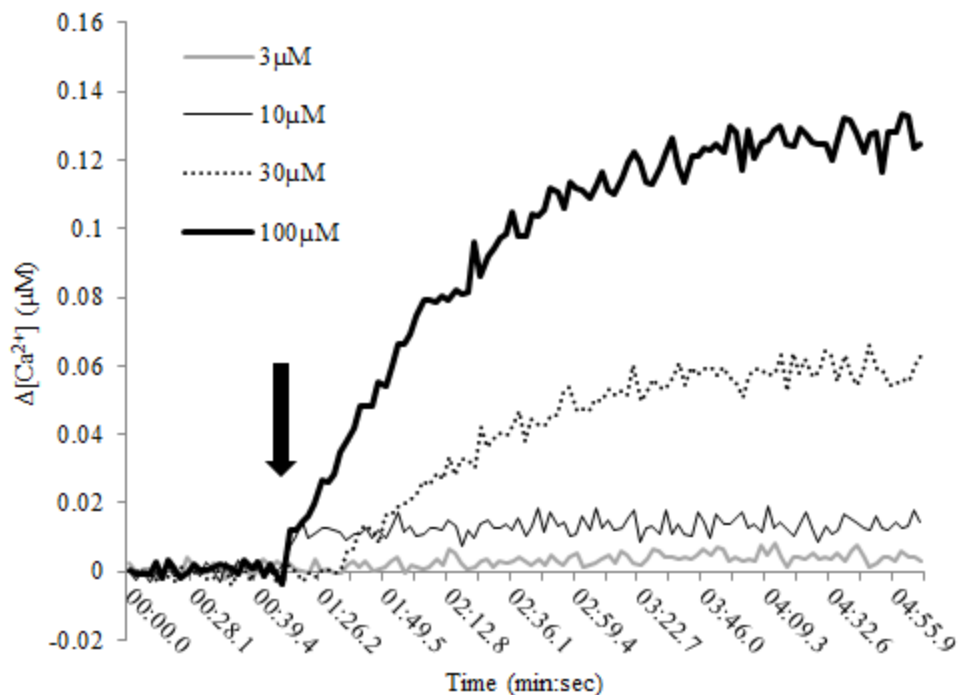


Figure 4. **CM572-induced calcium response in SK-N-SH neuroblastoma.** The effect of CM572 on calcium release was examined in Fura-2 loaded SK-N-SH neuroblastoma cells. Cells were loaded with Fura-2 and assays carried out as described in Methods. A 3 μM dose of CM572 did not elicit a calcium response, 10 μM CM572 elicited a very small calcium response, and concentrations at and above 30 μM induced calcium increases in a dose-dependent manner. Arrow indicates injection of CM572. Data is presented as change in calcium level above basal. Representative traces are shown from 8 replicate experiments for 3 and 10 μM doses and 3 replicates for 30 and 100 μM doses. Experiments were performed using 4 wells per condition.

CM572 attenuation of CB-64D-induced calcium response

CM572 is an analog of SN79, which is a sigma-2 receptor antagonist. Since low doses (3 and 10 μ M) of CM572 failed to produce a robust calcium response, we examined whether these doses might have antagonist actions against a known sigma-2 receptor agonist. CB-64D has previously been shown to produce a robust calcium signal in SK-N-SH neuroblastoma cells, consistent with its designation as a sigma-2 receptor agonist (Vilner and Bowen, 2000). CM572 was investigated for ability to block the calcium signal produced by CB-64D. The results are shown in Figure 5. Both 3 μ M and 10 μ M CM572 were able to significantly attenuate the calcium response induced by CB-64D when added simultaneously with 30 μ M CB-64D. As shown previously, 3 μ M CM572 alone showed no calcium response, but was able to significantly attenuate the CB-64D-induced response. CM572 at 10 μ M alone showed a small calcium response, but was able to nearly completely attenuate the response produced by 30 μ M CB-64D. Taken with the observation that higher concentrations of CM572 produce a robust calcium response, these data suggest that CM572 is a partial agonist at sigma-2 receptors.

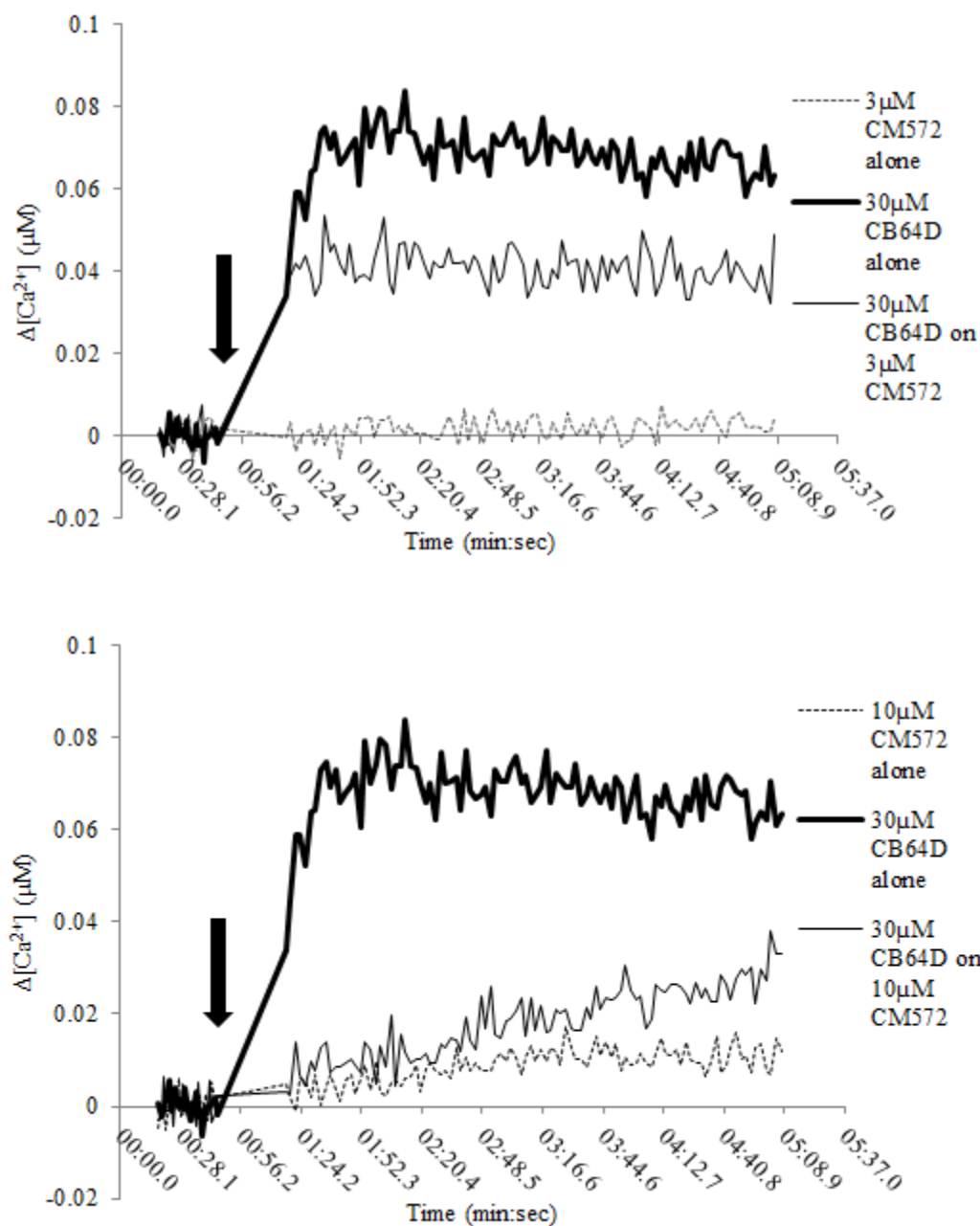


Figure 5. **CM572 attenuation of CB-64D-induced calcium response.** Concentrations of CM572 that were unable to produce a strong calcium response alone, 3 μM (top trace) and 10 μM (bottom trace), were able to attenuate the robust calcium response induced by 30 μM CB-64D, with 10 μM CM572 exhibiting much stronger attenuation. Arrow indicates injection of

CM572. Shown are representative traces from experiments that were repeated twice.

Experiments were performed using 4 wells per condition.

CM572-induced cytotoxicity in SK-N-SH neuroblastoma

A second criterion for agonist characterization of a sigma-2 ligand in SK-N-SH neuroblastoma is the ability to induce apoptotic cell death. MTT assays were performed to measure cell viability in response to CM572 at different doses with differing incubation times and conditions. The results are shown in Figure 6. The heavy solid line shows the effect of various doses of CM572 after a continuous 24 h exposure of cells to the compound in normal culture medium. Concentrations of CM572 up to and including 1 μ M did not induce cytotoxicity above 20%, whereas concentrations above 1 μ M induced robust loss of cell viability. The EC₅₀ for CM572-induced cell death was 7.6 ± 1.7 μ M.

Experiments were then carried out aimed at determining whether the irreversible binding demonstrated in rat liver membranes would manifest in the ability to induce cell death. Cells were treated for 30, 60, or 120 min with or without various concentrations of CM572 in HBSS, followed by extensive washing to remove any free compound from the system. Cells were then incubated for 24 h in ligand-free culture media and MTT assay was used to assess viability of remaining cells. The results are shown in Figure 6, indicated by the light solid line and broken lines. Washing out the CM572 after a brief exposure did not halt the cytotoxic effect of the compound. There was a general trend towards a rightward shift in the dose-response curve compared to the continuous exposure, but maximal effect was reached at the highest dose for all preincubation times. This indicates that CM572 is irreversibly bound during the preincubation and continues to activate the sigma-2 receptor after washout of unbound ligand to induce subsequent cell death. A preincubation time as short as 30 min was sufficient to produce this irreversible effect. Interestingly, in some experiments where cells were pretreated with concentrations of CM572 at or below 1 μ M (for example, 1 μ M preincubated for 60 min) there

appeared to be a slight stimulation of MTT reduction compared to control cells, resulting in negative “% Cytotoxicity” values.

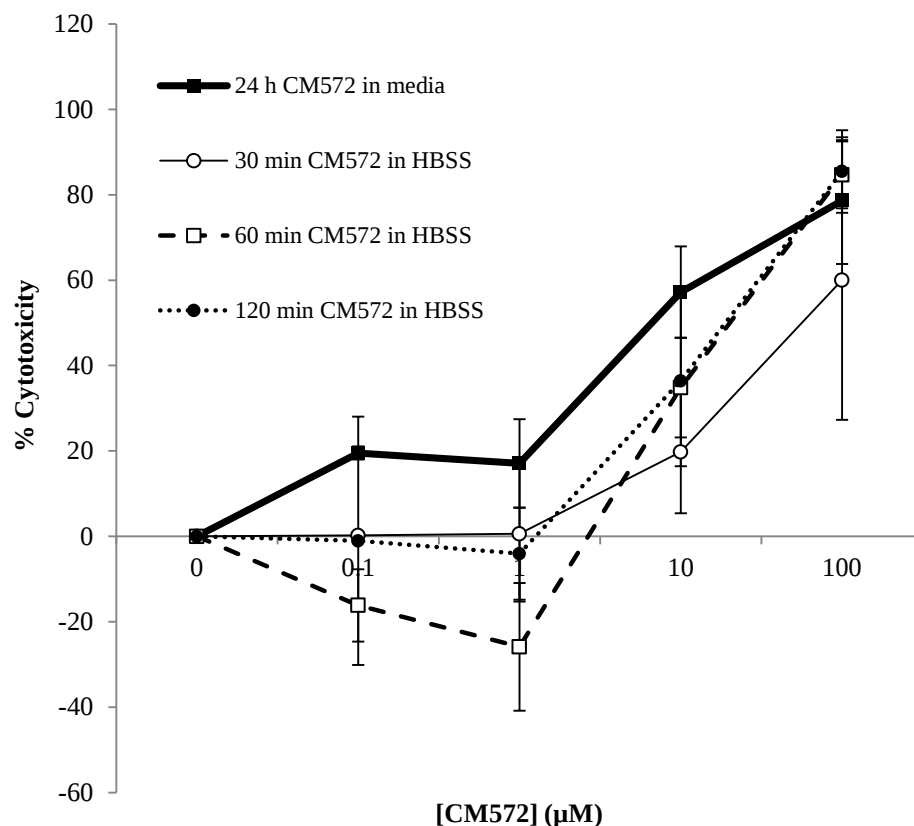


Figure 6. CM572-induced cytotoxicity in SK-N-SH neuroblastoma. SK-N-SH neuroblastoma cells were exposed to various doses of CM572 and cell viability examined by MTT assay, as described in Methods. Cells were either exposed to CM572 continuously for 24 h (heavy solid line) or exposed acutely for 30, 60, or 120 min, unbound compound removed, and cells allowed to incubate for 24 h in media without CM572 (light and dashed lines) as described in Methods. Controls were treated similarly, but without ligand. During the continuous 24 h incubation, CM572 induced cell death in a dose-dependent manner. CM572 also induced dose-dependent cell death 24 h after acute treatments followed by washing, with no statistically significant differences from 24 h treatment effects ($p > 0.05$ for all acute treatments as compared to continuous 24 h exposure at same dose, t-test). With acute CM572 treatment, no cell death was observed at or below 1 μM , resulting in a trend towards a rightward shift of the dose curve with

comparable maximal effect, compared to the continuous condition. Data shown for 24 h continuous and 30 min acute exposures are the average of two experiments, $\pm$ S.D. Data for 60 min and 120 min acute treatment are the average of three experiments, $\pm$ S.D. Acute exposures were performed in HBSS, though similar results were observed with media. Each experiment was performed using 5 wells for each condition.

To address whether the cytotoxicity resultant from washout studies was due to irreversible binding of CM572 to the sigma-2 receptor or alternatively to a commitment of cells to proceed to apoptosis during the pre-incubation timeframe, a comparative study was conducted with the sigma-2 agonist siramesine (MedChem Express, Princeton, NJ). Siramesine has high affinity and selectivity for sigma-2 receptors ($K_i=0.12$ nM and 17 nM at sigma-2 and sigma-1, respectively), and has been demonstrated to induce apoptosis in tumor cell lines (Ostenfeld et al. 2005; Cesen et al. 2013). Siramesine lacks an isothiocyanate group, and thus can only bind reversibly to sigma receptors. Results are shown in Figure 7. SK-N-SH neuroblastoma cells were treated with CM572 or siramesine for either 60 min, followed by washout and incubation for an additional 24 h, or with continuous exposure to compound for 24 h. With CM572, comparable levels of cell death were obtained with the pretreatment and washout as when the cells were exposed continuously for 24 h. For 10 μ M and 100 μ M CM572, the cell death resulting 24 h after the 60 min pre-exposure and drug removal was ~70% and ~100%, respectively, of that observed with the corresponding continuous 24 h exposure. By contrast, with siramesine, the 24 h cell death was markedly reduced when the cells were only pre-exposed for 60 min compared to the continuous exposure. For both concentrations of siramesine (15 and 25 μ M), the cell death resulting 24 h after the 60 min pre-exposure and drug removal was less than 15% of that observed with a continuous 24 h exposure. This indicates that the majority of the response elicited from a 60 min preincubation of cells with CM572 was likely due to irreversible ligand binding, rather than to an irreversible commitment to apoptosis initiated within 60 min of ligand treatment, since a 60 min treatment with siramesine achieved far from comparable levels of cell death compared to the 24 h continuous exposure.

A concern with irreversibly binding compounds that have reactive electrophilic moieties, such as isothiocyanate, is stability in the culture medium. There is the possibility that components in the MEM culture medium or serum might degrade the compound by reacting with the isothiocyanate group. However, CM572 appears to be relatively stable in culture media. We observed that when the acute pretreatments described in Figure 6 were carried out in normal culture medium instead of HBSS, the latent cytotoxicity was reduced by only about 25% (data not shown). Furthermore, for a 24 h continuous incubation of cells with 1, 3, or 10 μM CM572, there was no significant difference in the dose-dependent level of cell death observed whether the media contained 0, 2, 5, or 10% fetal bovine serum (3 experiments, each with 5 wells per condition). When a full 24 h dose-response curve was performed in media with 0% FBS, the EC_{50} value was $6.49 \pm 1.17 \mu\text{M}$ (average of 4 experiments, each with 5 wells per condition). This is comparable to the EC_{50} of 7.6 μM observed in 10% FBS (Figure 6). Thus it appears that CM572 is neither bound to nor degraded by serum to any large extent.

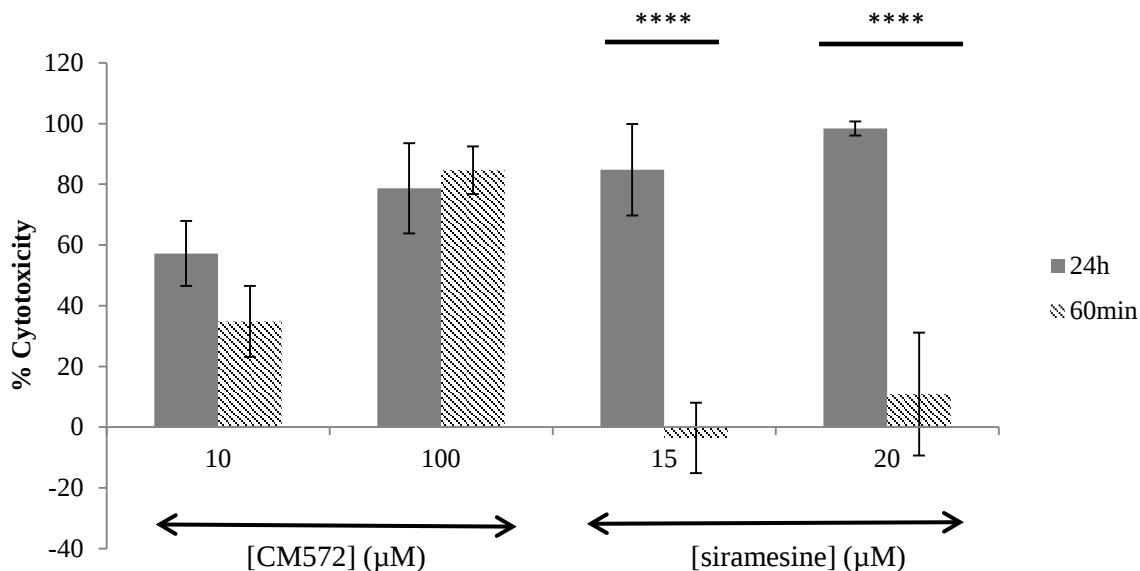


Figure 7. **Comparison of effects of CM572 and siramesine upon acute or chronic exposure.**

The cytotoxic effects of irreversibly binding CM572 and reversibly binding siramesine were compared using acute and chronic treatment conditions. Treatment conditions were as described in Methods and legend to Figure 6. Solid bars: Cells were exposed to the indicated concentration of sigma-2 ligand continuously for 24 h. Hashed bars: Cells were exposed to the indicated sigma-2 ligand for 60 min, followed by extensive washing to remove unbound ligand, and then incubated for 24 h. Cell viability was assessed by MTT assay at the end of the 24 h period. As previously shown in Figure 6, the cytotoxic effect of CM572 is more or less comparable when the 60 min acute exposure is compared to the 24 h continuous exposure ($p=0.06$ and $p=0.3$, t-test for difference between acute and 24 h treatment with 10 μM and 100 μM CM572, respectively). By contrast, at each dose of siramesine, only minimal cytotoxicity was observed after the 24 h period when the 60 min acute exposure was compared to the 24 h continuous exposure (**** $p=0.000003$ and $p=0.000006$, t-test for difference between acute and 24 h treatment with 15 and 20 μM siramesine, respectively). This indicates that the CM572-induced cytotoxicity after acute

treatment is due to irreversible binding, not irreversible commitment to apoptosis. Data are the averages of 3 experiments for CM572 and 5 experiments for siramesine, $\pm$ S.D. Each experiment was performed using 5 wells for each condition.

CM572 attenuation of CB-64D-induced cytotoxicity

The results observed in the calcium release assay (Figure 5) suggested that CM572 has antagonist properties (partial agonist), where minimally effective doses were able to attenuate the robust CB-64D-induced calcium response. To determine whether low-dose CM572 acts as a sigma-2 antagonist in cell viability assays, the ability to attenuate the apoptotic effect of CB-64D was examined. SK-N-SH neuroblastoma cells were pre-treated for 60 min with or without various concentrations of CM572, followed by extensive washing to remove compound. Cells were then exposed to 30 μ M CB-64D for 24 h and remaining cells determined using the MTT assay. Results are shown in Figure 8. All the doses of CM572 examined showed some ability to attenuate the cytotoxic effect of 30 μ M CB-64D. The effect was most pronounced at the low concentrations of CM572 (100 nM and 1 μ M). These concentrations alone had no detrimental effect on cell viability. Pretreatment of cells with both low doses of CM572 significantly attenuated the cytotoxic effect of 30 μ M CB-64D. At 10 μ M, CM572 pretreatment alone results in 36% cytotoxicity. However, when combined with 30 μ M CB-64D, cytotoxicity reached 74%, significantly less than the 100% cell kill produced by 30 μ M CB-64D alone showing that there is a less than additive effect of the combination. This is also observed to some extent at 100 μ M CM572, where alone it induces ~80% cytotoxicity and nearly the same value upon subsequent treatment with 30 μ M CB-64D. Therefore, CM572 appeared to prevent the maximal 100% cytotoxicity induced by the 30 μ M concentration of CB-64D alone, though the effect did not reach statistical significance. These results are consistent with CM572 acting as an irreversible partial agonist at sigma-2 receptors.

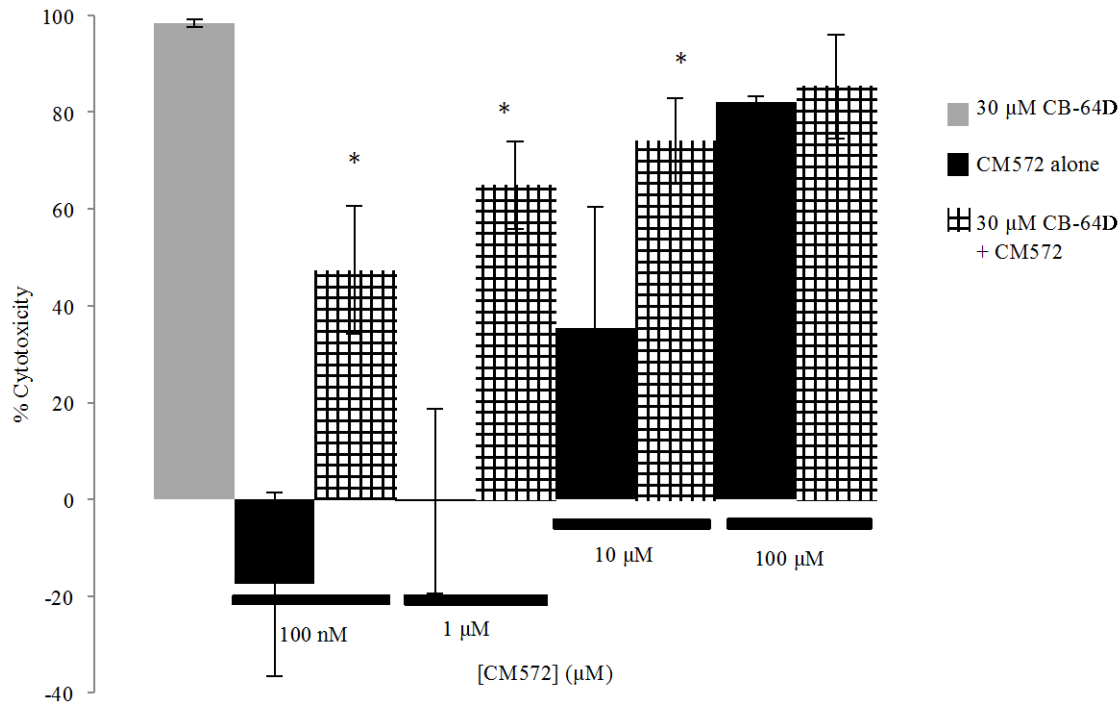


Figure 8. CM572 attenuation of CB-64D-induced cytotoxicity in SK-N-SH neuroblastoma.

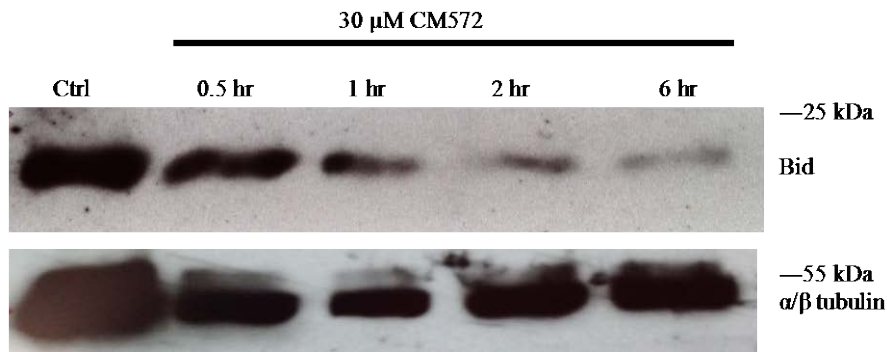
Cells were treated with the indicated concentration of CM572 for 60 min, followed by extensive washing to remove unbound ligand. Either vehicle (solid black bars) or 30 μM CB-64D (hatched bars) was then added to the wells, and cells incubated for an additional 24 hr. Results were compared to cells treated with 30 μM CB-64D alone (solid gray bar). The cell death induced by 30 μM alone was set to 100% (maximal cytotoxicity) and all results were compared to this value and expressed as percent of maximal cytotoxicity. CM572 (100 nM and 1 μM) did not induce cytotoxicity alone and were able to significantly attenuate cell death induced by CB-64D ($p=0.017$ and $p=0.018$, respectively, t-test for CM572 with CB-64D vs. effect of CB-64D alone), indicating antagonist properties. A higher dose of 10 μM CM572 induced significant cell death alone, but still appeared able to attenuate cytotoxicity of CB-64D, suggesting partial agonist activity ($p=0.032$, t-test for 10 μM CM572 with 30 μM CB-64D compared to 30 μM CB-64D

alone). At 100 μ M CM572, there was substantial cell death. When combined with 30 μ M CB-64D, the maximal cell death induced by 30 μ M CB-64D alone was unable to be achieved indicating CM572 antagonism, though the effect did not achieve statistical significance ($p=0.12$, t-test). Data is the average of two experiments, $\pm$ S.D. Each experiment was performed using 5 wells for each condition.

CM572-induced Bid cleavage

We have shown that sigma-2 agonists such as CB-64D induce caspase-10-dependent cleavage of the pro-apoptotic protein Bid, resulting in mitochondrial depolarization and release of mitochondrial pro-apoptotic factors (Wang and Bowen, 2006). Thus, a third criterion that was used to determine cellular function of CM572 in SK-N-SH neuroblastoma was ability to induce cleavage of Bid. Cells were treated with 30 μ M CM572 for various times up to 6 h and cytosolic levels of Bid determined via Western blotting. The results are shown in Figure 9. The level of full-length Bid (22 kDa) decreased with increasing exposure time to 30 μ M CM572, indicating cleavage to an active fragment. The effect is quite prominent by 2 h of treatment. This demonstrates that, like the apoptotic pathway induced by other sigma-2 agonists, Bid cleavage is a prominent element of CM572-induced apoptosis in SK-N-SH neuroblastoma. This further supports agonist properties for CM572 in the higher dose range of the ligand.

A



B

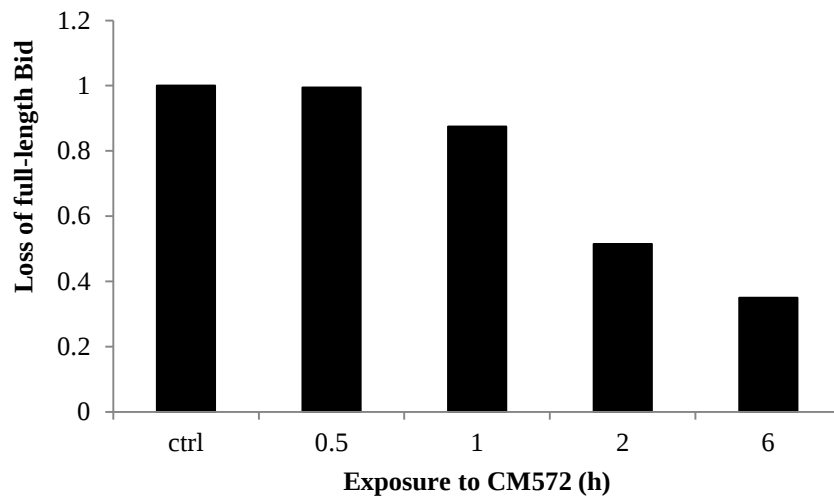


Figure 9. Effect of CM572 on Bid cleavage in SK-N-SH neuroblastoma. SK-N-SH cells were treated with 30 μ M CM572 for the indicated times. Control cells were treated without CM572 (media only) for 24 h. Cells were then extracted and subjected to Western blotting for Bid as described in Methods. Panel A: A 30 μ M treatment of CM572 induced cleavage of Bid as indicated by decreased levels of full-length Bid (22 kDa) with increasing incubation times. α/β tubulin (55 kDa) was used as a loading control. Experiment was repeated twice with similar results. A representative Western blot is shown. Panel B: Quantitation of Western blot data.

The OD ratios of Bid to tubulin were determined for each time point, and the ratio in the control set to 1. Ratios are expressed relative to control ratio. Data shown is the average of two experiments.

Effect of CM572 in other cancer types and in normal cells

The effect of CM572 on cell viability was determined in two additional tumor cell lines, human PANC-1 pancreatic epithelioid carcinoma and human MCF-7 breast adenocarcinoma. CM572 was also examined in two types of normal cells from different organ systems, human melanocytes from neonatal foreskin (HEM) and human mammary epithelial cells (HMEC). Cells were exposed to various concentrations of CM572 and cell viability examined. Figure 10 shows results for 3 and 10 μM CM572, CM572 induced marked cell death in all three of the tumor cell lines. However, there was considerably less cell death observed with the two normal cell types over this concentration range when compared to the tumor cells. CM572 showed high selectivity at 3 and 10 μM , where there was no effect on the viability of HEM cells and minimal effect on viability of HMEC cells compared to the tumor cell lines. For a same tissue comparison of a full dose-response curve, the 48 h EC_{50} for CM572 in MCF-7 breast tumor cells vs. normal HMEC cells was $4.9 \pm 1.17 \mu\text{M}$ vs. $32.3 \pm 1.7 \mu\text{M}$, respectively, showing a 6.4-fold selectivity for the tumor cells (average of 2 experiments $\pm$ S.D., 5 wells for each point). Therefore, CM572 appears to have a wide-ranging effect against various cancer types known to express sigma-2 receptors and exhibits a selective cytotoxic effect against cancer cells over normal cells.

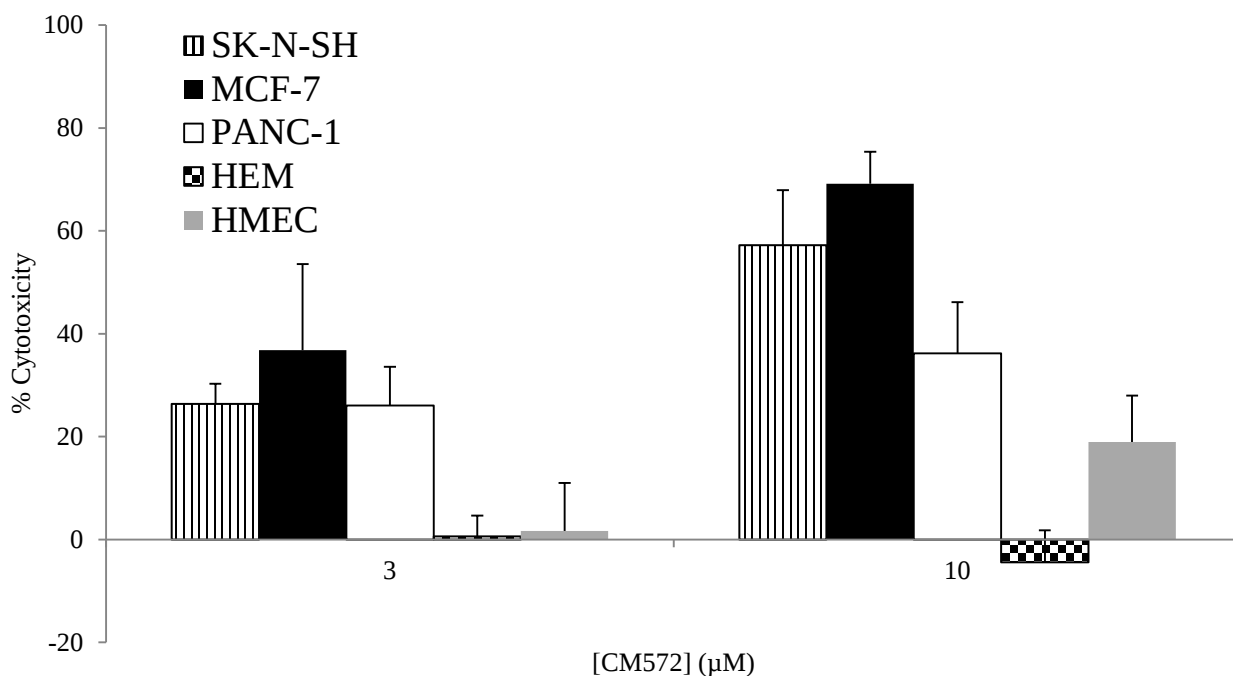


Figure 10. Effect of CM572 across human tumor cell lines from different organ sites and comparison with effect in normal cell types. Panel A: CM572 was incubated with MCF-7 breast adenocarcinoma, PANC-1 pancreatic epithelioid carcinoma, normal human melanocytes from neonatal foreskin (HEM), and normal human mammary epithelial cells (HMEC) at the indicated concentrations of CM572 for 24 h (PANC-1, HEM, HMEC) or 48 h (MCF-7). Panel B: SK-N-SH neuroblastoma was incubated with 10 μ M CM572 for 24 h. Cell viability was determined after the incubation period using the MTT assay as described in Methods. Data are the averages of 3 or 4 experiments, $\pm$ S.D. Each experiment was performed using 5 wells for each condition.

2.6 Discussion

CM572 bound with high affinity and selectivity to sigma-2 receptors, compared to sigma-1, exhibiting ≥ 700 -fold selectivity (Figure 2). Studies in rat liver membranes demonstrated that binding to sigma-2 receptors is irreversible, as indicated by failure to recover sigma-2 binding activity in pretreated membranes after extensive washing to remove any unbound ligand (Figure 3). This effect was dose-dependent. The 30 nM EC_{50} for this effect is consistent with its affinity at sigma-2 receptors ($K_i = 14.6$ nM). This suggests that once bound in the sigma-2 receptor binding pocket, CM572 is oriented in such a manner that a suitable receptor amine or thiol group is in proper configuration to attack the isothiocyanate moiety resulting in covalent binding. The failure to bind irreversibly to sigma-1 receptors can be attributed to the low affinity ($K_i > 10$ μ M).

CM572 induced a rise in cytosolic calcium levels in fura-2-loaded cells in a dose-dependent manner (Figure 4). Calcium release was quite robust at 30 and 100 μ M, indicating agonist-like properties. This result was surprising given that the parent compound SN79 has previously been shown to have only a small effect on calcium release even at high concentrations, consistent with its role as an antagonist (Garcia 2012). Lower doses of CM572 (3 μ M and 10 μ M) induced no or minimal release of calcium. These doses of CM572 that had minimal effect alone were able to attenuate the robust calcium signal induced by CB-64D, a compound characterized as a sigma-2 agonist (Figure 5). It should be noted that SN79 also attenuates CB-64D-induced calcium release, consistent with its characterization as a sigma-2 antagonist (Garcia 2012). Thus, unlike SN79, CM572 appears to behave as a partial agonist.

CM572 also exhibited the ability to induce dose-dependent cell death (Figure 6), a characteristic of sigma-2 agonists. The EC_{50} value of 7.6 μ M is comparable to that of CB-64D in

these cells ($EC_{50} = 5 \mu M$). Again, this is in contrast to the actions of the parent compound SN79, which has no effect on cell viability even at high concentrations (Garcia 2012). The cytotoxic potency of CM572 was unaffected by the presence or absence of serum protein. Thus, CM572 appears to be relatively stable in culture medium, despite the presence of the reactive isothiocyanate group.

Since CM572 becomes irreversibly bound to sigma-2 receptors (Figure 3), we surmised that cells pretreated with the compound would continue to die even after any free, non-irreversibly bound ligand was removed. Cells pretreated for 30, 60, or 120 min with various doses of CM572 continued to die over a period of 24 h, even after the compound was washed free of the system after the acute exposure (Figure 6). While there was some loss of potency, indicated by the trend towards a rightward shift in the dose curve for the pretreatment condition, the same maximal level of cell death could be achieved compared to the 24 h continuous exposure condition.

This above result strongly suggests that CM572 is binding covalently to sigma-2 receptors in live cells and continues to activate the receptor in an irreversible manner, such that cell death occurs even though there is no free ligand in the system. However, an alternative interpretation could be that during the pretreatment period of 30-120 min, the cells become committed to apoptosis and then go on to die after the ligand is removed from the system. In order to test this, we compared the effect of CM572 to those of the potent putative sigma-2 receptor agonist siramesine, which lacks ability to bind irreversibly (Figure 7). Siramesine induced dose-dependent cell death in a continuous 24 h exposure to cells. However, unlike cells pretreated acutely with CM572, cells pretreated with siramesine for 60 min and then washed were largely still viable 24 h later (Figure 7). This shows that a 60 min exposure of SK-N-SH

cells to a potent sigma-2 agonist is not sufficient to induce commitment to apoptosis. A similar result was previously observed with a 3 h treatment of C6 glioma cells with either reduced haloperidol or BD737, followed by washing. The treatment with 300 μ M of either ligand resulted in extensive cell rounding. But, when the ligand was removed the cells survived and returned to near normal morphology (Vilner et al. 1995a). These results support the notion that the effect observed with acute CM572 treatment is due to irreversible binding to and activation of sigma-2 receptors.

As low doses of CM572 induced minimal cytotoxic effect and given the observations on calcium release, we examined whether low doses of CM572 might block the cytotoxic effect of CB-64D (Figure 8). In these experiments, cells were treated acutely for 60 min with various doses of CM572 and then washed to remove any unbound ligand. The effect of CB-64D was then observed over a 24 h period. Concentrations of CM572 that were not cytotoxic alone (100 nM and 1 μ M) significantly attenuated the effect of 30 μ M CB-64D. At higher doses CM572 shows significant cytotoxicity alone, but was still able to attenuate the cytotoxic effect of CB-64D, not allowing it to induce maximal cell death. Thus, as with the results observed in calcium release assays, CM572 behaves as an irreversible partial agonist at sigma-2 receptors.

The final functional effect observed was on Bid cleavage. We have shown previously that sigma-2 receptor agonists such as CB-64D and haloperidol induce cleavage of the pro-apoptotic protein Bid in the cytosol (Wang and Bowen 2006). As has been observed with CB-64D, 30 μ M CM572 induced robust loss of intact Bid in the cytosol, beginning at 1 h with extensive loss by 6 h (Figure 9). This would thus classify CM572 as having agonist properties at this dose in this assay.

Since sigma-2 receptors are known to be upregulated in tumor cell lines from many different organ sites, we investigated the effect of CM572 on breast and pancreatic tumor cell lines in addition to the neuroblastoma line. CM572 induced cell death in both of these additional cell lines (Figure 10). An important characteristic to note is the relative selectivity of the cytotoxic effect of CM572 for cancer cells over normal cells. CM572 exhibited much less cytotoxic activity in normal human melanocytes and normal mammary epithelial cells compared to the tumor cell lines (Figure 10). When the sensitivity of normal breast cells to CM572 was compared to that of MCF-7 breast tumor cells over a 48 h dose-response treatment period, the tumor cells were found to be 6.4 times more sensitive to the cytotoxic effect of CM572. This is consistent with a previous finding that breast tumor biopsy tissue expressed a much higher density of sigma receptors compared to surrounding normal tissue (John et al., 1996). This tumor selectivity likely also applies to melanoma vs. normal melanocytes. CM572 had no effect on the viability of HEMs at concentration up to 10 μ M. Sigma-2 receptors are highly expressed in human melanoma cells (John et al., 1993; Vilner et al., 1995b). While we have not investigated the effect of CM572 in a melanoma cell line, there are several studies by others that demonstrate cytotoxic effects of sigma-2 agonists on melanoma in vitro. The non-selective sigma-2 receptor agonist, haloperidol, induced apoptosis in mouse B16 and human SK-MEL-28 melanoma cells, while the highly selective sigma-2 agonists SV119, WC-26, RHM-138, and siramesine all induced apoptosis in human MDA-MB-435 melanoma cells (Nordenberg et al., 2005; Zeng et al., 2012, 2014). Thus it is reasonable to assume that CM572 would also induce apoptosis in melanoma cells. The difference in efficacy between tumor cells and non-tumor cells suggests potential therapeutic promise for CM572 or related ligands in treatment of cancers. The

irreversible nature of the sigma-2 receptor interaction may allow novel treatment paradigms where less frequent dosing could be possible, reducing adverse side effects.

Taken together, these data show that CM572 is a potent and selective partial agonist at sigma-2 receptors. It binds irreversibly to the receptor via the isothiocyanate moiety and thus retains the ability to continuously activate the receptor even after free ligand is removed from the system. This finding was unexpected, given the properties of the parent compound, SN79, which lacks significant agonist activity (Garcia 2012). Introduction of the isothiocyanate moiety in place of the methylketone in the heterocyclic ring of SN79 appears to not only impart irreversible binding activity, but also to shift the profile to impart more agonist activity. It is not clear at this time whether this change in efficacy is due to the irreversible binding component or to some change in the electronic properties of the heterocyclic ring due to the presence of the isothiocyanate moiety. Studies of reversibly binding SN79 analogs with modifications in this position may help to resolve this issue. The unique properties of CM572 give it potential as an agent for cancer therapy as well as a novel tool with which to study functional blockade of sigma-2 receptors.

2.7 References

- Abate C, Perrone R, and Berardi F (2012) Classes of sigma-2 receptor ligands: structure affinity relationship (SAfiR) studies and antiproliferative activity. *Curr Pharm Des* **18**: 938-949.
- Al-Nalbusi I, Mach RH, Wang LM, Wallen CA, Keng PC, Sten K, Childers SR, and Wheeler KT (1999) Effect of ploidy, recruitment, environmental factors, and tamoxifen treatment on the expression of sigma-2 receptors in proliferating and quiescent tumor cells. *British J. Cancer* **81**, 925-933.
- Bowen WD (2007) Sigma-2 Receptors: Regulation of Cell Growth and Implications for Cancer Diagnosis and Therapeutics, in *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications* (Mastumoto R, Su TP, and Bowen WD, eds) pp 215-235, Springer, New York.
- Bowen WD, Bertha CM, Vilner BJ and Rice KC (1995) CB-64D and CB-184: ligands with high sigma 2 receptor affinity and subtype selectivity. *Eur J Pharmacol* **278**(3): 257-260.
- Cassano G, Gasparre G, Niso M, Contino M, Scalera V and Colabufo NA (2009) F281, synthetic agonist of the sigma-2 receptor, induces Ca²⁺ efflux from the endoplasmic reticulum and mitochondria in SK-N-SH cells. *Cell Calcium* **45**(4): 340-345.
- Cesen MH, Repnik U, Turk V and Turk B (2013) Siramesine triggers cell death through destabilisation of mitochondria, but not lysosomes. *Cell Death & Disease* **4**, e818; doi:10.1038/cddis.2013.361.
- Comeau A, Mesangeau C, McCurdy CR and Bowen WD (2012) Development of selective irreversible affinity ligands for sigma-2 receptors. Program No. 61.13. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

- Crawford KW and Bowen WD (2002) Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines. *Cancer Res* **62**(1): 313-322.
- Garcia DR (2012) Sigma-2 receptor-mediated cytotoxicity and calcium signaling: Evidence for bifurcating pathways. PhD thesis, Brown University.
- Hayashi T and Su TP (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca²⁺ signaling and cell survival. *Cell* **131**: 596-610.
- Hazelwood S and Bowen WD (2006) Sigma-2 receptor-mediated apoptosis in human SK-N-SH neuroblastoma cells: Role of lipid rafts, caspases, and mitochondrial depolarization. *Proceedings of the American Association for Cancer Research* 47: #4932.
(http://cancerres.aacrjournals.org/content/66/8_Supplement/1158.3)
- Hellewell SB and Bowen WD (1990) A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Res* **527**(2): 244-253.
- Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W and Bowen WD (1994) Rat liver and kidney contain high densities of sigma 1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur J Pharmacol* **268**(1): 9-18.
- John CS, Bowen WD, Saga T, Kinuya S, Vilner BJ, Baumgold J, Paik CH, Reba RC, Neumann RD, Varma VM, and McAfee JG (1993) A malignant melanoma imaging agent: Synthesis, characterization, *in vitro* binding and biodistribution of iodine-125-(2-piperidinylaminoethyl)4-iodobenzamide. *J. Nucl. Med.* **34**: 2169-2175.

- John CS., Vilner BJ, Schwartz AM, and Bowen WD (1996) Characterization of sigma receptor binding sites in human biopsied solid breast tumors. *J. Nucl. Med.* **37**: 267P.
- Kaushal N, Robson MJ, Vinnakota H, Narayanan S, Avery BA, McCurdy CR and Matsumoto RR (2011) Synthesis and pharmacological evaluation of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79), a cocaine antagonist, in rodents. *AAPS J* **13**(3): 336-346.
- Kaushal N, Seminerio MJ, Robson MJ, McCurdy CR and Matsumoto RR (2012) Pharmacological evaluation of SN79, a sigma (sigma) receptor ligand, against methamphetamine-induced neurotoxicity in vivo. *Eur Neuropsychopharmacol* **23**(8): 960-971.
- Mach RH, Zeng C, and Hawkins WG (2013) The sigma-2 receptor: A novel protein for the imaging and treatment of cancer. *J Med Chem* **56**: 7137-7160.
- Maurice T and Su TP (2009) The pharmacology of sigma-1 receptors. *Pharmacol Ther* **124**(2): 195-206.
- McCurdy CR, Mesangeau C, Matsumoto RR, Poupaert JH, Avery BA and Abdelazeem AHA (2014) Highly selective sigma receptor ligands. United States of America. **Patent # US8686008 B2.**
- Nordenberg J, Perlmutter I, Lavie G, Beery E, Uziel O, Morgenstern C, Fenig E, Weizman A (2005) Anti-proliferative activity of haloperidol in B16 mouse and human SK-MEL-28 melanoma cell lines. *Int. J. Oncol.* **27**(4): 1097-1103.
- Ostenfeld MS, Fehrenbacher N, Hoyer-Hansen M, Thomsen C, Farkas T and Jaattela M (2005) Effective tumor cell death by sigma-2 receptor ligand siramesine involves lysosomal leakage and oxidative stress. *Cancer Res* **65**(19): 8975-8983.

- Vilner BJ and Bowen WD (2000) Modulation of cellular calcium by sigma-2 receptors: release from intracellular stores in human SK-N-SH neuroblastoma cells. *J Pharmacol Exp Ther* **292**(3): 900-911.
- Vilner BJ, de Costa BR and Bowen WD (1995a) Cytotoxic effects of sigma ligands: sigma receptor-mediated alterations in cellular morphology and viability. *J Neurosci* **15**: 117-134.
- Vilner BJ, John CS and Bowen WD (1995b) Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Res* **55**(2): 408-413.
- Wang X and Bowen WD (2006) Sigma-2 receptors mediate apoptosis in SK-N-SH neuroblastoma cells via caspase-10-dependent Bid cleavage and mitochondrial release of endonuclease G and apoptosis-inducing factor. Program No. 90.1. 2006 Neuroscience Meeting Planner. Atlanta, GA: Society for Neuroscience, 2006. Online.
[\(http://www.abstractsonline.com/viewer/viewAbstractPrintFriendly.asp?CKey={47A7954A-EF5D-42D3-AC67-F079DDBD0568}&SKey={F4F9D514-92C8-456B-87E3-EF5F21660429}&MKey={D1974E76-28AF-4C1C-8AE8-4F73B56247A7}&AKey={3A7DC0B9-D787-44AA-BD08-FA7BB2FE9004}}](http://www.abstractsonline.com/viewer/viewAbstractPrintFriendly.asp?CKey={47A7954A-EF5D-42D3-AC67-F079DDBD0568}&SKey={F4F9D514-92C8-456B-87E3-EF5F21660429}&MKey={D1974E76-28AF-4C1C-8AE8-4F73B56247A7}&AKey={3A7DC0B9-D787-44AA-BD08-FA7BB2FE9004}})
- Wheeler KT, Wang LM, Wallen CA, Childers SR, Cline JM, Keng PC and Mach RH (2000) Sigma-2 receptors as a biomarker of proliferation in solid tumours. *British Journal of Cancer* **82**(6): 1223-1232.
- Xu JB, Zeng CB, Chu WH, Pan FH, Rothfuss JM, Zhang FJ, Tu ZD, Zhou D, Zeng DX, Vangveravong S, Johnston F, Spitzer D, Chang KC, Hotchkiss RS, Hawkins WG, Wheeler KT and Mach RH (2011) Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site. *Nat Commun* **2**: 380, DOI: 10.1038/ncomms1386.

Zeng C, Rothfuss J, Zhang J, Chu W, Vangveravong S, Tu Z, Pan F, Chang KC, Hotchkiss R and Mach RH (2012) Sigma-2 ligands induce tumour cell death by multiple signalling pathways. *Br J Cancer* **106**(4): 693-701.

Zeng C, Rothfuss JM, Zhang J, Vangveravong S, Chu W, Li S, Tu Z, Xu J and Mach RH (2014) Functional assays to define agonists and antagonists of the sigma-2 receptor. *Anal Biochem* **448**: 68-74.

Footnotes:

*This work was supported by the National Institutes of Health National Institute of General Medical Sciences T32 Predoctoral Pharmacology Training Grant [1-T32 GM077995-01A2] (HN); National Institutes of Health National Institute of General Medical Sciences R25 Initiative for Maximizing Student Development Grant [R25 GM083270] (HN); Brown Pharmacia Pre-doctoral Fellowship (HN); National Institutes of Health National Institute on Drug Abuse Postdoctoral T32 Training Grant [5T32DA016184-09] (AC); National Institutes of Health National Institute on Drug Abuse Grant [R01 DA023205] (CM, CRM); National Institutes of Health National Institute of General Medical Sciences Grant [P20 GM104932] (CM, CRM); and the Upjohn Professorship in Pharmacology, Brown University (WDB)

This work has been previously presented in part at the American Association for Cancer Research Annual Meeting 2013 (2242) and at the Society for Neuroscience Annual Meeting 2013 (63.03).

Chapter 3: Sigma-2 Receptor-Mediated Cell Death in Triple Negative Breast Cancer: Potential for Targeted Therapy

3.1 Preface

Promising results demonstrating the potential of CM572 as a highly cancer-selective chemotherapeutic agent confirmed that sigma-2 receptor-mediated therapy might provide a novel avenue for selective treatment of cancer (see Chapter 2). The novel facet of irreversible binding adds further benefit to this potential therapy lending possibility to a less frequent dosing regimen, as CM572 would not be cleared from the body until the cell to which it is bound dies. Results showing high potency for killing MCF-7 breast adenocarcinoma cells at doses that were largely not harmful to non-cancerous primary human mammary epithelial cells (HMEC) suggested that the breast may be an ideal target tissue for further evaluation of the efficacy of CM572 against cancers for which there is currently no targeted therapy. Triple negative breast cancer is an ideal disease in which to examine potential use of CM572 and other sigma-2 receptor-mediated therapy, as it has been studied with increasing frequency in recent years yet few advances have been made in its successful targeted treatment.

Lacking the traditional targets for breast cancer therapy, triple negative breast cancer does not express progesterone or estrogen receptors, nor does it overexpress HER2 growth factor receptors. Several immortal cancer cell lines representing different classes of triple negative breast cancer are commercially available and have been characterized fairly extensively, with predictable patterns of protein expression and response rates to conventional chemotherapeutics.

In patients, triple negative breast cancer typically grows very fast and is highly aggressive, and therefore detection often occurs at later stages than for non-triple negative breast cancer. Between mammograms, a triple negative breast cancer tumor can originate and progress to a late-stage metastasized cancer. Survival rates depend highly upon stage of detection and race of the patient and are therefore difficult to summarize for the disease as a whole, however researchers do agree that this disease is one of the most deadly cancers among women. This study sought to investigate the potential use of CM572 and another well-characterized but less potent sigma-2 receptor agonist SV119 in the selective treatment of triple negative breast cancer, a type of cancer that is highly difficult to treat and in which sigma-2 receptor expression and function has not yet been extensively investigated.

3.2 Abstract

Triple negative breast cancer (TNBC) is a particularly aggressive form of cancer, with the lowest 5-year survival rate and poorest prognosis of all forms of breast cancer. Lack of estrogen, progesterone, and overexpressed HER2 receptors, the typical targets of hormonal therapy, coupled with an increased incidence of multidrug resistance make TNBC difficult to treat. Sigma-2 receptors are highly expressed in cancer cells compared to normal tissues and induce programmed cell death when activated, providing a novel therapeutic target. Here, we demonstrate the expression of sigma-2 receptors in TNBC cell lines representing three of the six TNBC subclasses: MDA-MB-468 ductal carcinoma (BL1 basal-like), MDA-MB-231 invasive ductal carcinoma (MSL mesenchymal-like), and MDA-MB-453 adenocarcinoma (luminal androgen receptor-like). Presence of sigma-2 receptors as determined by [³H]DTG binding ranged was detected in all three TNBC cell lines, whereas normal pre-stasis human mammary epithelial cells (HMEC) showed no significant specific [³H]DTG binding, indicating the selectivity of the sigma-2 receptor as a chemotherapeutic target. The sigma-2 receptor agonists CM572 (3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[d]oxazol-2(3H)-one) and SV119 (9-azabicyclo[3.3.1]nonan-3-yl-2-methoxy-5-methylphenylcarbamate) induced cell death in all three TNBC cell types with potencies comparable to that in the non-TNBC breast adenocarcinoma cell line, MCF-7 (EC_{50} = 1.05-4.99 μ M and 19.98-24.58 μ M for CM572 and SV119, respectively). This indicates that resistance mechanisms typically posing a challenge to TNBC treatment do not inhibit sigma-2 receptor agonist activity. The mechanism of cell death is caspase-nonessential and includes mitochondrial depolarization. HMEC cells were markedly more resistant to sigma-2 agonists, consistent with negligible expression of sigma-2 receptors. The data indicate that the sigma-2 receptor is a viable target for treatment of TNBC.

3.3 Introduction

Triple negative breast cancer (TNBC) is a deadly form of cancer that lacks the three common targets for hormonal therapy: estrogen receptors, progesterone receptors, and overexpressed HER2 growth factor receptors. TNBC typically strikes patients at a younger age than non-triple negative breast cancer, and disproportionately affects African-American and Hispanic women (Boyle, 2012). Patients with triple negative breast cancer have a 5-year survival rate of 30% with high likelihood of recurrence in the first three years after treatment, highlighting the unmet need for effective therapies. Unfortunately, incidences of TNBC have seen a steady increase in the last several decades with more than one in every ten breast cancer diagnoses being classified as triple negative, and this increased incidence has occurred without a concurrent increase in available effective therapies for this disease (Boyle, 2012). The missing receptor targets render traditionally successful breast cancer treatments such as Tamoxifen, Herceptin, and aromatase inhibitors ineffective, leaving only non-targeted treatments with high toxicities (Andre and Zielinski, 2012). An additional complication is the high activity of multidrug resistance proteins, which render many chemotherapeutics that are successful in other cancers ineffective in TNBC (Ferreira et al., 2005).

Sigma receptors, classified as sigma-1 and sigma-2 (Hellewell and Bowen, 1990; Hellewell et al., 1994), are involved in cell proliferation and survival. Sigma-1 receptors enhance survival via several mechanisms by acting as a ligand-regulated chaperone (Hayashi and Su, 2007). Sigma-2 receptors, alternatively, have been associated with inducing cell death and have received recent attention for their potential use in the treatment of multiple cancers (Crawford and Bowen, 2002; Zeng et al., 2012). Sigma-2 receptors are upregulated in a wide variety of both rodent and human cancer cell lines (Hellewell and Bowen, 1990; Vilner et al.,

1995). In addition, the sigma-2 receptor becomes even more highly upregulated during states of rapid proliferation (Al-Nabulsi et al., 1999; Mach et al., 1997; Wheeler et al., 2000). These levels are much higher than are found in normal tissues. Upon activation, sigma-2 receptors are known to induce programmed cell death through multiple signaling pathways (Crawford and Bowen, 2002; Zeng et al., 2012). Furthermore, at least some sigma-2 receptor ligands have also been shown to evade multidrug resistance transport. For example, in MCF-7/Adr cells, which overexpress P-glycoprotein and are resistant to Doxorubicin, no difference in the cytotoxic EC₅₀ of CB-64D, a sigma-2 receptor agonist, was observed when compared to wild type MCF-7 cells (Crawford and Bowen, 2002). Sigma-2 agonists have also been shown to down-regulate expression of P-glycoprotein (Bowen et al., 1997; Azzariti et al., 2006). These observations, taken in conjunction with their upregulation in cancer tissues as compared to non-cancerous tissue, indicates the sigma-2 receptor as an attractive target for the treatment of deadly cancers for which there are currently few or no successful therapies. Here we examine the effect of two selective sigma-2 receptor ligands in triple negative breast tumor cells and show that sigma-2 agonists may show promise in therapy for this aggressive form of breast cancer.

3.4 Materials and Methods

Cell culture

The triple negative breast tumor cell lines MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells and MCF-7 breast adenocarcinoma cells were obtained from ATCC (Manassas, VA). Cells were cultured in MEM (Gibco, Grand Island, NY) with 10% fetal bovine serum and 10 mg/L insulin at 37°C at 5% CO₂ in a humidified atmosphere. Cells were not allowed to exceed 70% confluency prior to splitting. Human mammary epithelial cells (normal pre-stasis) (HMEC) were kindly provided by Dr. Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA) and were cultured in M87A+CT+X medium at 37°C and 5% CO₂ in a humidified atmosphere (Stampfer et al., 2013).

Membrane preparation

Cells were allowed to grow to 70% confluency and then detached using 2.5 mM EDTA/DPBS and spun down at 223 x g for 5 minutes. Supernatant was discarded and pellet was resuspended in ice-cold 10 mM Tris pH 7.4 containing 0.32 M sucrose and protease inhibitor cocktail (Pierce, Rockford, IL) at a density of 1×10^7 cells/mL. The mixture was then homogenized with 8 hand-driven strokes in a Teflon-glass homogenizer and then centrifuged at 105,000 x g for 1 h at 4°C. The supernatant was discarded and pellet was resuspended in ice cold 10 mM Tris pH 7.4 to a final concentration of ~15-20 mg protein/mL. Final protein concentration was determined by BCA assay according to manufacturer's specifications (Pierce, Rockford, IL). Membranes were stored at -80°C until use.

Radioligand binding assay

Radioligand binding assays were performed as previously described (Vilner et al., 1995) with slight modifications. Briefly, sigma-1 receptors were labeled using 3 nM [^3H](+)-pentazocine (specific activity 33.9 Ci/mmol). Sigma-2 receptors were labeled using 5 or 10 nM [^3H]di-o-tolylguanidine ([^3H]DTG) (specific activity 47.6 Ci/mmol), in the presence of 100 nM (+)-pentazocine to mask sigma-1 receptors. Incubations were carried out in 50 mM Tris-HCl, pH 8.0 for 120 min with 500 μg protein for each sample. Non-specific binding was determined in presence of 10 μM haloperidol. Assays were terminated by filtration over polyethyleneimine-treated glass-fiber filters, as previously described. Filters were counted in Ecoscint H (National Diagnostics, Atlanta, GA). Data analysis was performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA).

Cell viability assay

Cell viability assessment was done by MTT cell viability assay (Trevigen, Gaithersburg, MD) in 96-well plate format. Cells were plated at 7,000 cells/well (MDA-MB-231, MDA-MB-453, MDA-MB-468, HMEC) or 10,000 cells/well (MCF-7) and allowed to attach overnight prior to dosing. Cells were incubated with ligand (0.1-100 μM CM572 or 1-100 μM SV119) for 24 h (total volume 100 μL /well), then solution in well was removed and fresh media and ligand were added at the same concentrations as for the first 24 h period. Cells were then incubated in the fresh media with ligand for an additional 24 h (total incubation time with ligand was 48 h). Controls were treated in the same manner, but without addition of ligands. For caspase inhibition experiments, cells were pre-treated for 1 h with Z-VAD-FMK (100 μM) (EMD Millipore,

Tauton, MA). At the end of the incubation period, 10 μ L MTT reagent was added. MTT reagent was allowed to be metabolized for 3 h prior to the addition of detergent, which was then allowed to solubilize MTT formazan crystals and cell membranes for 2 h. Absorbance was then measured at 570 nm. EC₅₀ values were determined using GraphPad Prism 6 software (GraphPad, La Jolla, CA).

Mitochondrial depolarization assay

Changes in the mitochondrial membrane potential were measured using the fluorescent dye tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Abcam, Cambridge, MA). The JC-1 dye exists in two forms (monomer or aggregate) and accumulates in healthy mitochondria in its aggregate form, dependent on the mitochondrial potential. Mitochondrial depolarization is indicated by a decrease in the aggregate/monomer ratio. Cells were plated at 10,000 cells/well and allowed to attach overnight prior to incubation with 10 μ M JC-1 for 15 minutes, followed by 2 washes with PBS. Cells were then dosed with ligand (3-30 μ M CM572 or 30-100 μ M SV119) for 24-40 h (total volume 100 μ L/well). MDA-MB-231 cells were pre-treated for 1 h and throughout the duration of the experiment with 70 μ M verapamil hydrochloride (Sigma Aldrich, St. Louis, MO) to prevent efflux of JC-1 dye. Fluorescence was measured at 485 nm (excitation) and 595 nm (aggregate)/535 nm (monomer) (emission).

Sigma-2 receptor ligands

CM572 (3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[d]oxazol-2(3H)-one) was synthesized in the laboratory of Dr. Christopher McCurdy, School of Pharmacy, University of Mississippi. SV119 (9-azabicyclo[3.3.1]nonan-3-yl-2-methoxy-5-

methylphenylcarbamate) was the kind gift of Dr. Robert Mach, Department of Radiology, University of Pennsylvania. Structures are shown in Figure 2.

3.5 Results

Analysis of sigma-2 receptor binding in triple negative breast cancer cell lines

TNBC is a heterogeneous disease. Using gene expression profiling from 3,247 primary human breast cancers, Lehmann and colleagues found 587 cases of TNBC and then grouped TNBC into 6 subtypes (Lehmann et al., 2011). These were designated basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). Cell line models representative of each of these 6 classes were then identified and correspondingly grouped. For this study we used triple negative lines representative of three of these TNBC classes, from three different sites of origin: MDA-MB-468 ductal carcinoma (BL1 basal-like), MDA-MB-231 invasive ductal carcinoma (MSL mesenchymal-like), and MDA-MB-453 adenocarcinoma (luminal androgen receptor-like).

In order to determine the viability of sigma-2 receptors as targets for treatment of triple negative breast cancer, the presence of sigma-2 receptors was examined in the three TNBC cell lines (MDA-MB-231, MDA-MB-453, MDA-MB-468). Detection of [³H]DTG binding revealed that all three TNBC cell lines express sigma-2 receptors. Specific binding is presented in Table 1.

Table 1:

Specific binding of sigma-2 receptors in breast cancer and normal cell types.

Cell type	% Specific Binding	p-value
MDA-MB-231	74	0.00042
MDA-MB-453	78	0.0082
MDA-MB-468	69	0.021
MCF-7	69	0.032
HMEC	No significant binding	0.44

All triple negative breast cancer cell types investigated showed expression of sigma-2 receptors. The p-value resultant from a t-test measuring the difference between [³H]DTG total binding and [³H]DTG binding in the presence of 10 µM haloperidol is presented for each cell line. Using 10 nM [³H]DTG and 300 µg of membrane protein, only very low level of binding was detected in HMEC cells that did not reach significance. Specific binding values for all cell lines are the average of two independent experiments, ± SD. All experiments were carried out in duplicate.

Sigma-2 receptor expression in normal human mammary epithelial cells (HMEC) was also examined. [^3H]DTG (10 nM) binding did not reach statistical significance in HMEC cells, suggesting either lack of sigma-2 receptor expression or a K_d for [^3H]DTG too high to give significant receptor occupation at this radioligand concentration. The presence of sigma-2 receptors in TNBC cells and the relative absence in normal breast cells suggests that sigma-2 receptors are a viable target and that sigma-2 agonists should have a selective cytotoxic effect on the TNBC cells over normal cells.

While not analyzed in detail, we also investigated sigma-1 receptor binding in TNBC cells. The amount of [^3H](+)-pentazocine bound at a concentration of 3 nM was determined using two different membrane protein concentrations and the values averaged. All three TNBC cell lines expressed sigma-1 receptor binding activity.

Effect of sigma-2 receptor ligands on viability of triple negative breast cancer cell types, MCF-7, and HMEC

The effect of two selective sigma-2 receptor ligands on cell viability was examined. Their structures are shown in Figure 1. SV119 (sigma-1 K_i = 1418 nM; sigma-2 K_i = 5.2 nM), has been characterized as a sigma-2 receptor agonist (Zeng et al., 2014). CM572 (sigma-1 K_i > 10 μM ; sigma-2 K_i = 14.6 nM) has been classified as an irreversible, sigma-2 receptor partial agonist (Nicholson et al., 2015). The isothiocyanate moiety was shown to impart selective irreversible binding to the sigma-2 receptor, with a cytotoxic effect in SK-N-SH neuroblastoma cells that persists after a brief exposure to cells followed by ligand removal from the system.

Both ligands were chosen for their highly sigma-2 selective binding properties and recent characterization in the literature showing agonist activity.

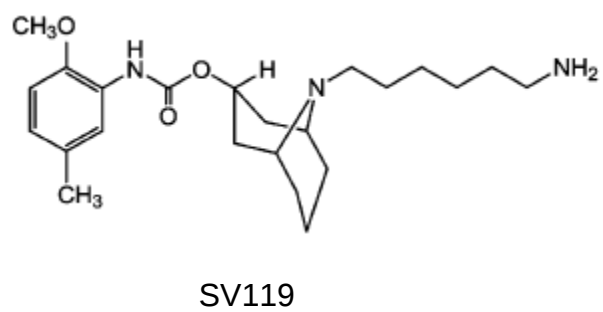
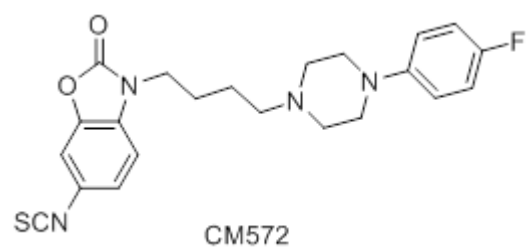
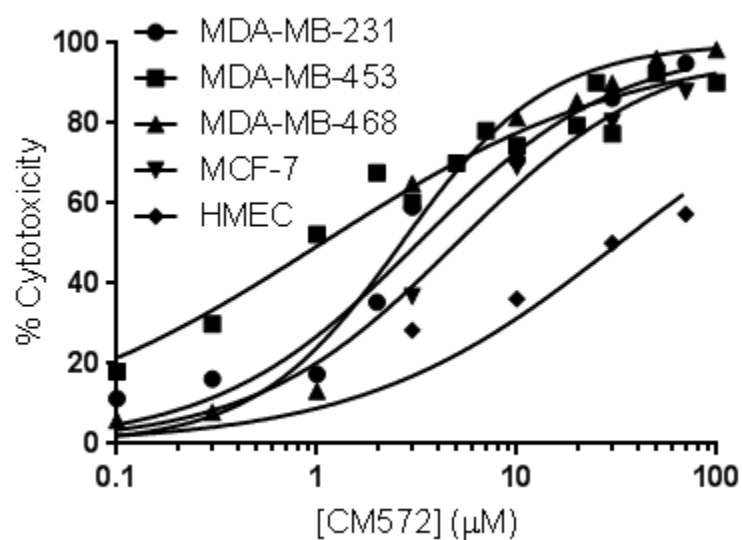


Figure 1: Structures of CM572 and SV119

Results in breast tumor cells are shown in Figure 2 and summarized in Table 2. Both ligands induced dose-dependent cell death in all three TNBC cell lines, with maximal cell death occurring at the highest dose. The potency across TNBC cell lines for each respective ligand was comparable, with EC₅₀ values ranging 1.05 – 3.19 μ M for CM572 and 19.98 – 24.58 μ M for SV119. Interestingly, the potencies of the compounds in the three TNBC cell lines were comparable to their potencies in the non-triple negative MCF-7 cells (Figure 3, Table 2). This indicates that the mechanisms governing the aggressiveness and resistance of TNBC cells to chemotherapeutic agents do not impair the activity of sigma-2 agonists, resulting in sigma-2 agonists being unable to discriminate TNBC cells from non-TNBC cells.

A



B

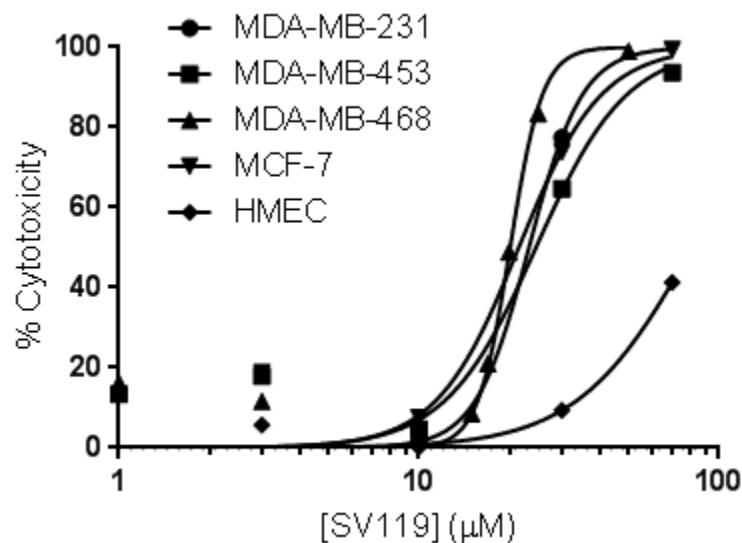


Figure 2. **Effect of sigma-2 receptor agonists on viability of triple negative breast cancer, non-triple negative breast cancer, and normal human mammary epithelial cells.** Cells were incubated with the indicated concentrations of CM572 or SV119 for 48 h, with one change of media as described in Methods. Viability was determined using the MTT assay. CM572 (panel

A) and SV119 (panel B) induced dose-dependent cell death in all three TNBC cell lines and the non-TNBC MCF-7 cell line, both compounds producing maximal cell kill. Both compounds had low potency and efficacy in normal human mammary epithelial cells, failing to induce maximal cell kill at the highest concentration used (70 μ M). All values are compiled from at least three independent experiments, with each condition having five replicate wells per experiment. Error bars were omitted for clarity. EC₅₀ values are shown in Table 2.

Table 2:

Cytotoxic potency of sigma-2 receptor ligands in breast tumor and normal cells.

Cell type	CM572 EC₅₀ (μM)	SV119 EC₅₀ (μM)
MDA-MB-231	3.19 ± 1.18	23.29 ± 1.24
MDA-MB-453	1.05 ± 1.18	24.58 ± 1.22
MDA-MB-468	2.64 ± 1.17	19.98 ± 1.09
MCF-7	4.99 ± 1.17	21.54 ± 1.26
HMEC	~ 30	>70

Data from Figure 2 were analyzed using GraphPad Prism 6 to determine EC₅₀ values. Sigma-2 receptor ligands CM572 and SV119 show potent ability to induce cell death in breast tumor cell lines. Triple negative breast cancer cell lines MDA-MB-231, MDA-MB-453, and MDA-MB-468 showed comparable EC₅₀ values for CM572 and SV119 as in MCF-7 cells (no statistically significant differences). Values are the averages of at least three experiments ± S.D., with each experiment carried out using five replicate wells for each point. By contrast, no accurate EC₅₀ value could be determined in normal HMEC cells, as maximal cell kill could not be reached at concentrations within the limit of solubility of the compounds. The values shown for HMEC were approximated from the 50% point on the graphs.

By contrast, normal HMEC cells were considerably less sensitive to the cytotoxic effect of both sigma-2 agonists. While 30 μ M SV119 and CM572 killed approximately 10% and 50% of HMEC cells, respectively, neither ligand was able to kill 100% of HMEC cells at the highest dose of 70 μ M over the 48 h period. This is unlike the TNBC cell lines, where 70 μ M of either ligand kills nearly 100% of cells in the wells. This data is clearly represented by comparison of a single dose of CM572 (Figure 3A) or SV119 (Figure 3B), where the difference in efficacy of each ligand in cancerous and non-cancerous cells is striking. Thus CM572 and SV119 exhibit a relatively selective cytotoxic effect on TNBC cells over normal breast cells.

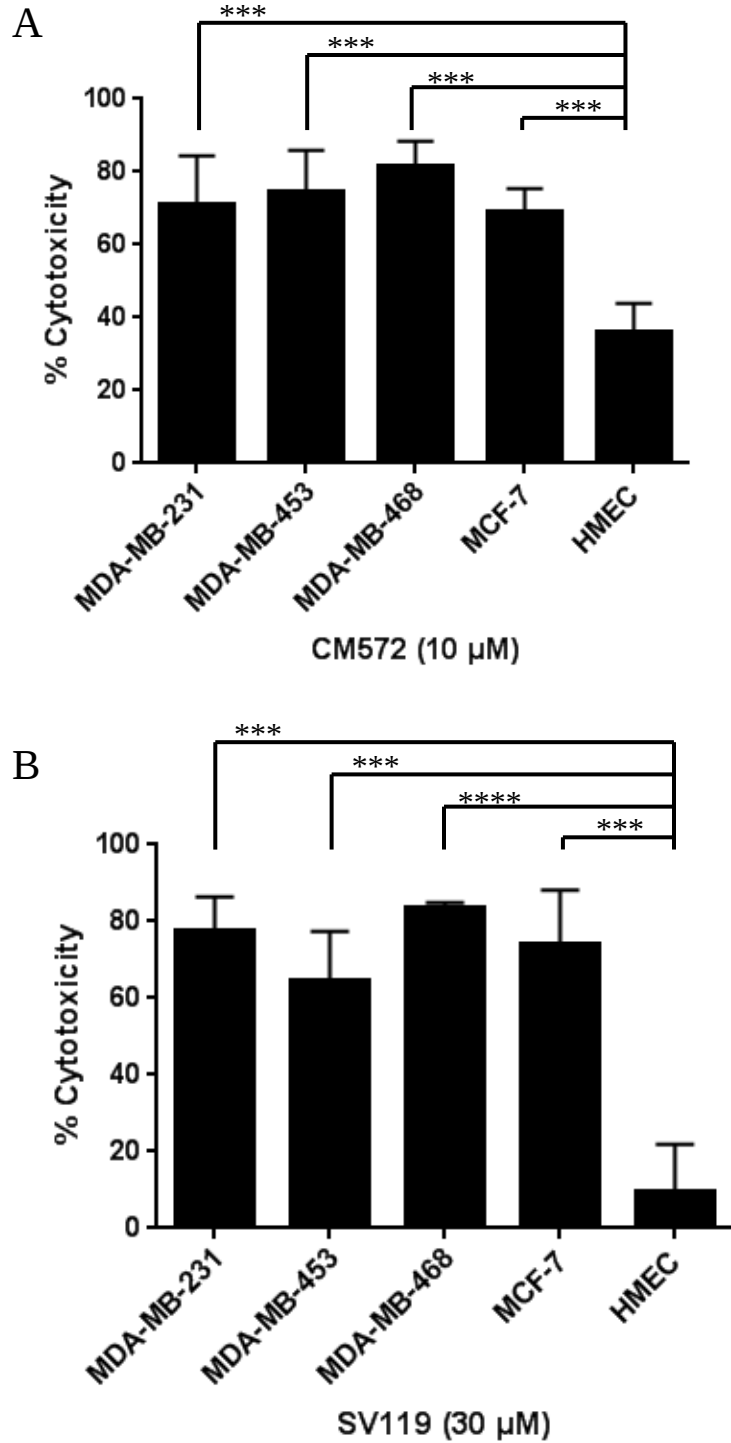


Figure 3. **Comparison of single-dose of sigma-2 agonists on viability of triple negative breast cancer, non-triple negative breast cancer, and normal human mammary epithelial cells.** Cells were incubated with 10 μ M CM572 (panel A) or 30 μ M SV119 (panel B) for 48 h

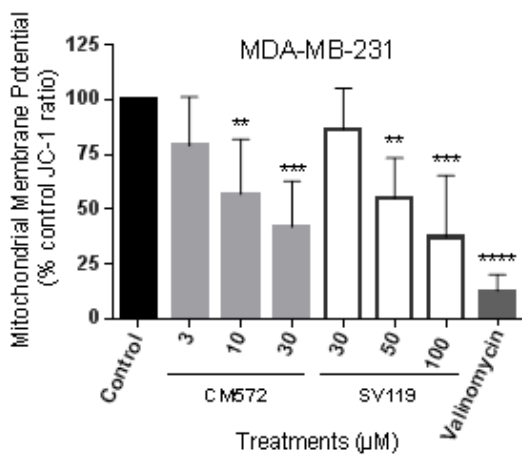
prior to determination of viable cells using the MTT assay. Both sigma-2 agonists were able to induce significant amounts of cell death in all three triple negative breast cancer cell lines and in non-triple negative MCF-7 breast adenocarcinoma cells with comparable efficacy at the indicated concentration. This efficacy was dramatically reduced for both sigma-2 agonists in normal human mammary epithelial cells. All values are compiled from at least four independent experiments for CM572 and at least three independent experiments for SV119, with each experiment performed with five replicates per condition. Error bars represent S.D. *** $p < 0.001$, **** $p < 0.0001$ for Dunnett's test for multiple comparisons of each efficacy of sigma-2 ligands in each cancerous cell line compared to efficacy in normal human mammary epithelial cells (HMEC), post-hoc from one-way ANOVA (overall $p < 0.0001$).

The dose-response curves for CM572 in the breast tumor cell lines were markedly more shallow compared to those produced by SV119. This likely reflects the irreversible binding of CM572, since the ligand-receptor interaction would not exhibit equilibrium kinetics as would SV119, a reversibly binding ligand. The same type of shallow dose curve was observed in SK-N-SH neuroblastoma cells (Nicholson et al., 2015).

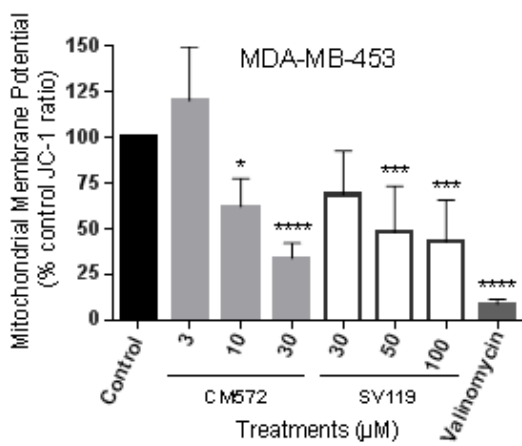
Role of mitochondrial depolarization and caspase activity in sigma-2 induced TNBC cell death

A variety of apoptotic pathways have been described resultant from sigma-2 receptor activation by an agonist. One step in the sigma-2 receptor-induced cell death pathway that has been previously demonstrated in neuroblastoma and pancreatic cancer cell lines is mitochondrial depolarization (Hazelwood and Bowen, 2006; Wang, 2009). In this study, mitochondrial membrane potential was measured using JC-1 dye in response to treatment with sigma-2 receptor agonists, with a decrease in JC-1 aggregate:monomer ratio representing a decrease in mitochondrial membrane potential. Results are shown in Figure 4. Consistent with previous studies in other cell lines, treatment with either CM572 or SV119 across all three subtypes of TNBC resulted in statistically significant and dose-dependent decreases in JC-1 aggregate/monomer ratio representing depolarization of the mitochondria and a loss of mitochondrial membrane potential. CM572 was more potent than SV119 at inducing mitochondrial depolarization, consistent with the relative potencies at inducing cell death (Table 2).

A



B



C

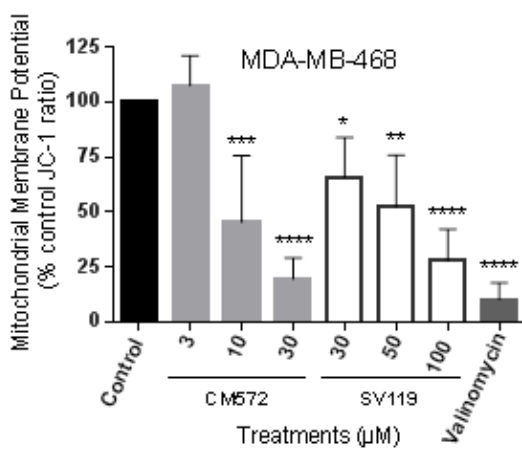


Figure 4. Effect of sigma-2 receptor agonists on mitochondrial membrane potential in triple negative breast cancer cells. MDA-MB-231 cells (panel A), MDA-MB-453 cells (panel B), and MDA-MB-468 cells (panel C) were treated for 40, 24, and 24 h, respectively, with 3-30 μ M CM572, 30-100 μ M SV119, or 75 μ M valinomycin (positive control) and changes in mitochondrial membrane potential measured using the JC-1 assay as described in Methods.. Results were normalized to the untreated control condition, which was set to 100%. CM572 and SV119 induced dose-dependent depolarization in all three TNBC cell lines. All values are an average from at least three independent experiments for each ligand $\pm$ S.D., with each experiment performed with three replicates per condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ for Dunnett's post hoc test for multiple comparisons of each treatment condition compared to untreated control cells ($p < 0.0001$ from one-way ANOVA for panels A-C). All conditions in MDA-M231 cells (panel A) included 70 μ M verapamil hydrochloride in order to effectively stain with JC-1 (due to P-glycoprotein overexpression, of which JC-1 is a substrate).

Caspase dependence has been demonstrated to be variable in response to sigma-2 receptor activation with both caspase-dependent and caspase-independent forms of programmed cell death being observed (Crawford and Bowen, 2002; Wang and Bowen, 2009; Zeng et al., 2012). The role of caspases was assessed by determining the effect of the broad-spectrum caspase inhibitor Z-VAV-FMK on the ability of sigma-2 receptor agonists to induce cell death in each of the three TNBC cell lines. Results are shown in Figures 5 and 6. Pretreatment of cells with the broad-spectrum caspase inhibitor Z-VAD-FMK failed to protect against CM572- (Figure 5) or SV119- (Figure 6) induced cell death across all three TNBC cell types. This indicates that the mechanism of sigma-2 receptor-induced cell death in TNBC cells is not dependent on activation of caspases, and any caspase activation that might occur would be non-essential.

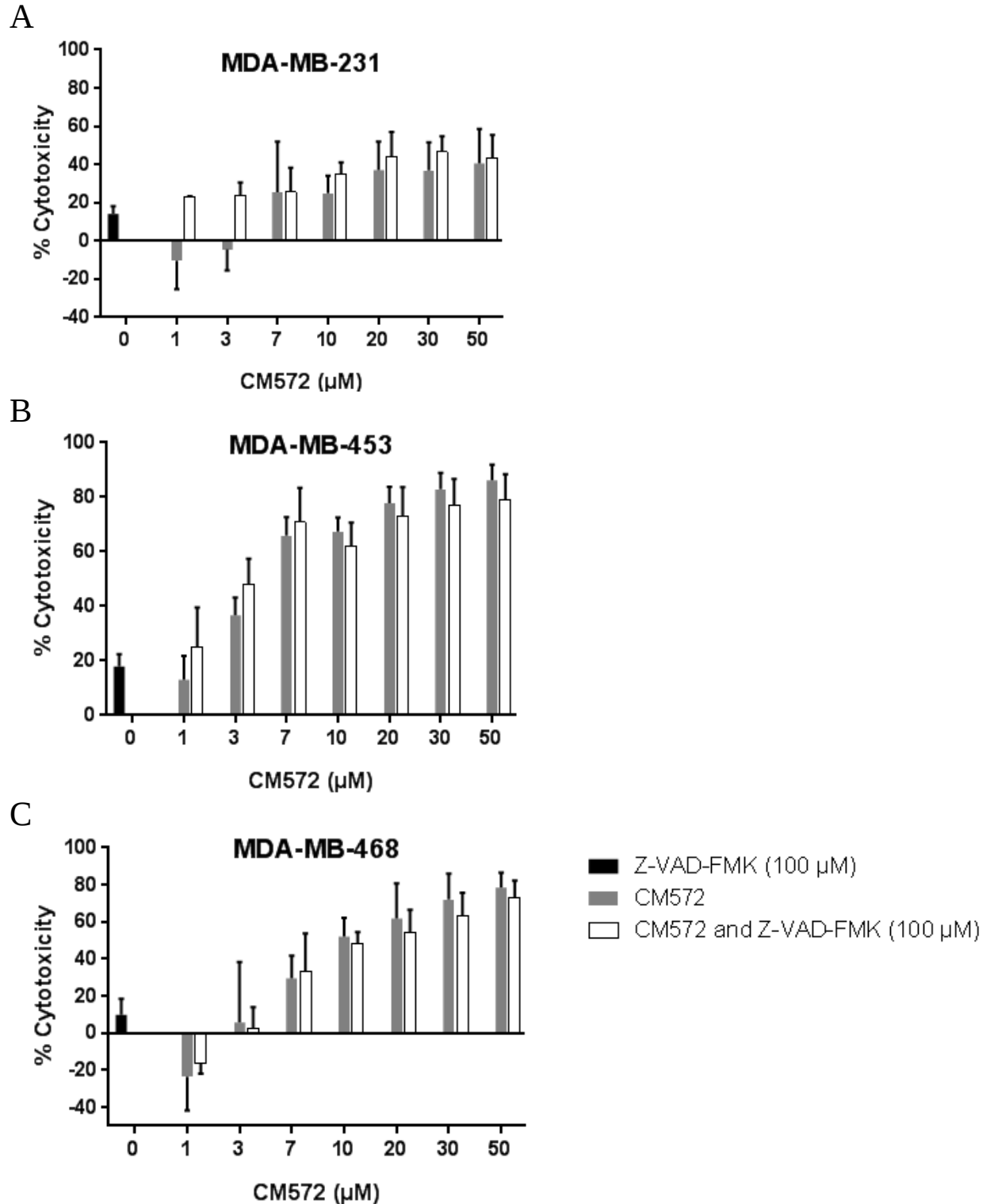
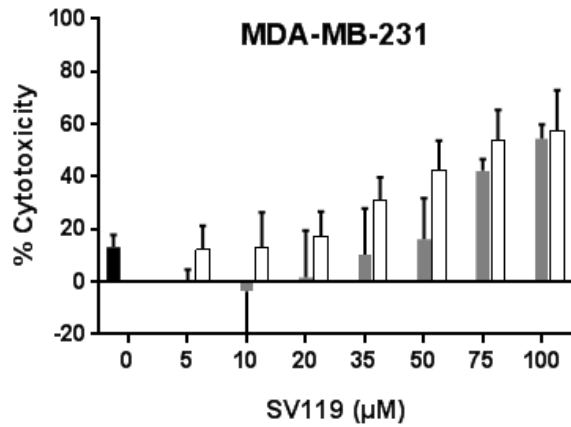


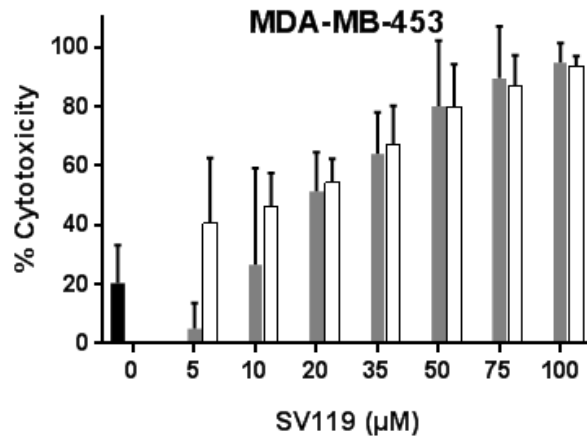
Figure 5. Effect of caspase inhibition on CM572 -induced cell death in TNBC cell lines. TNBC cells were treated with 1-50 μM CM572 alone or in combination with Z-VAD-

FMK (100 μ M). Cells were pre-treated for 1 h with pan-caspase inhibitor Z-VAD-FMK (for pan-caspase inhibition conditions), then treated with sigma-2 receptor ligand and/or inhibitor for 24 h prior to measurement of cytotoxicity using the MTT assay. Treatment with the pan-caspase inhibitor did not significantly attenuate cell death caused by CM572 in MDA-MB-231 (panel A), MDA-MB-453 (panel B), or MDA-MB-468 cells (panel C).. All values are from three independent experiments $\pm$ S.D., with five replicates per condition. Two-tailed, unpaired t-tests were used to compare CM572 alone vs. CM572 with pan-caspase inhibitor; no significant differences were found.

A



B



C

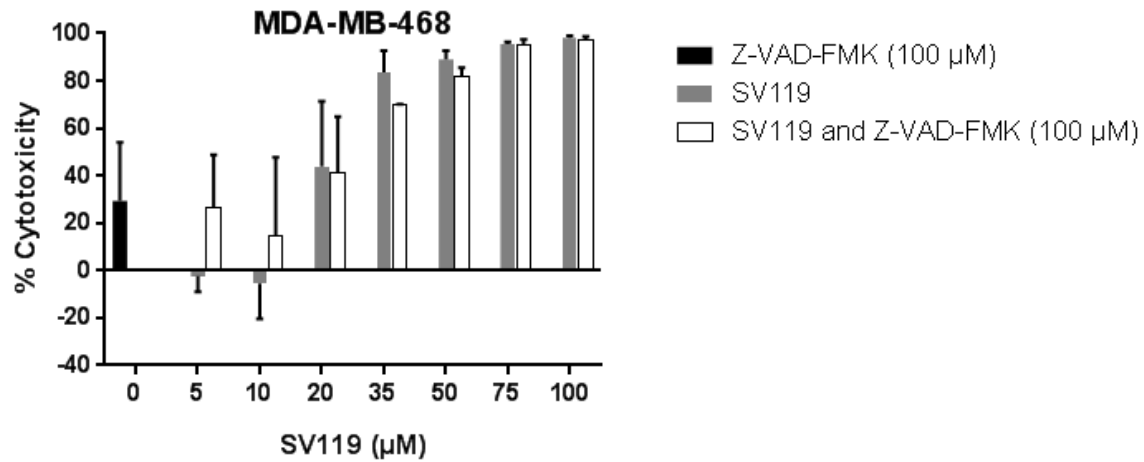


Figure 6. Effect of caspase inhibition on SV119-induced cell death in TNBC cell lines.

TNBC cells were treated with 5-100 μM SV119 alone or in combination with Z-VAD-FMK

(100 μ M) as described in the legend for Figure 5. Treatment with the pan-caspase inhibitor did not significantly attenuate cell death caused by SV119 in MDA-MB-231 (panel A), MDA-MB-453 (panel B), or MDA-MB-468 cells (panel C).. All values are from three independent experiments $\pm$ S.D., with five replicates per condition. Two-tailed, unpaired t-tests were used to compare SV119 alone vs. SV119 with pan-caspase inhibitor; no significant differences were found.

3.6 Discussion

This study confirmed sigma-2 receptor expression in MDA-MB-231, MDA-MB-453, and MDA-MB-468 TNBC cells, cell lines that represent three of the six subclasses of TNBC. A very low level of [^3H]DTG binding activity was detected in normal breast epithelial cells (HMEC). However the difference between total binding and non-specific binding (as detected using 10 μM haloperidol) did not reach statistical significance. This suggests that sigma-2 receptor expression in non-cancerous HMEC cells is dramatically lower than in TNBC and other breast cancer cells, if present at all. This is consistent with previous findings that patient biopsies from breast tumor tissue had high levels of sigma-2 receptors, while surrounding healthy breast tissue did not express detectable levels (John et al., 1996).

TNBC cells also expressed sigma-1 receptors. MCF-7 cells do not express detectable sigma-1 receptor binding (Vilner et al., 1995; Wu and Bowen, 2008). Evidence suggests that the sigma-1 receptor plays a role in enhancing cell proliferation and survival (Aydar et al., 2006; Wu and Bowen, 2008). However, its presence or absence does not seem to affect the ability of selective sigma-2 agonists to induce cell death (see below).

Both sigma-2 receptor ligands, CM572 and SV119, potently induced cell death in all three TNBC cell lines (Figure 2, Table 2). CM572 was considerably more potent than SV119 across all cell lines. Furthermore, the potency of CM572 and SV119 in TNBC cells was comparable to their potency in MCF-7 cells. By contrast, both ligands were considerably less cytotoxic in HMEC cells, showing significant selectivity of the sigma-2 agonists for TNBC cells over normal breast cells (Figure 2, Table 2). These results are further demonstrated by comparison of a single dose of each agonist (10 μM CM572, 30 μM SV119) in all five cell

types, demonstrating no statistically significant differences between TNBC and non-TNBC cancer cells, but with dramatic differences from HMEC cells (Figure 3).

The reduced potency of CM572 and SV119 in HMEC cells is consistent with the apparent absence of sigma-2 receptors as indicated by low to negligible specific [^3H]DTG binding. It should be noted, however, that the cells were not completely resistant to sigma-2 agonists. This could be due to a low level of sigma-2 expression that was not detectable through our methods. It is possible that some sigma-2 receptors are expressed in HMEC cells but with a K_d that does not favor detection of [^3H]DTG binding at the concentrations used. It should be noted that, while we did not examine sigma-2 binding in these cells, CM572 was even less efficacious in normal human epidermal melanocytes (HEM's) (Nicholson et al., 2015). For example in HEM's, CM572 exhibited no significant cytotoxicity at 10 μM (Nicholson et al., 2015). Thus, targeting sigma-2 receptors appears to show exceptional selectivity for cancer cells over normal cells. This could be due to lack of significant sigma-2 receptor expression, though this needs to be confirmed.

Treatment of all three TNBC cell types with the sigma-2 agonists resulted in a loss of mitochondrial membrane potential as demonstrated by a decrease in JC-1 aggregate/monomer ratio (Figure 4). The effect is dose-dependent, with CM572 having higher potency than SV119. This relationship mirrors the relative potencies of these two ligands to induce cell death, suggesting that mitochondrial depolarization plays a major role in the cell death mechanism. Mitochondrial depolarization is also consistent with effects of sigma-2 agonists on neuroblastoma and pancreatic carcinoma cell lines (Hazelwood and Bowen, 2006; Wang and Bowen, 2009).

The role of caspases was examined with the broad-spectrum caspase inhibitor, Z-VAD-FMK. Z-VAD-FMK failed to protect any of the TNBC cell lines from cell death induced by either CM572 or SV119 (Figures 5 and 6). This indicates that caspase activation is not an essential component of the cell death mechanism. This caspase-independence is consistent with observations in other breast tumor cell lines (Crawford and Bowen, 2002). Indeed, MCF-7 cells do not endogenously express caspase-3, and yet apoptosis is induced in these cells in response to sigma-2 receptor activation (Crawford and Bowen, 2002). It should be noted that using assays directly measuring caspase enzymatic activity, sigma-2 receptor agonists have been shown to activate caspase-3 in breast tumor and melanoma cell lines (Zeng et al., 2014). However, in view of sigma-2 receptor-induced mitochondrial depolarization, the failure of caspase inhibitors to protect breast tumor cells suggests that caspase-independent apoptogenic factors may be released along with those that activate caspases (Wang and Bowen, 2009). Interestingly, using [¹²⁵I]RHM-4 as radioligand, Mach and coworkers have also recently shown the expression of sigma-2 receptors in MDA-MB-231 cells (Makvandi et al., 2015). They demonstrated that sigma-2 receptor internalization could be used to deliver a small molecule mimetic of second mitochondria-derived activator of caspase (SMAC) when conjugated to a sigma-2 receptor ligand. This resulted in activation of caspases 3/7, consistent with the action of the SMAC mimetic on caspase activity.

The potency and efficacy of sigma-2 receptor ligands CM572 and SV119 was comparable in TNBC and MCF-7 cells, despite differences in aggressiveness and drug resistance of these cells (Figure 2, Table 2). This observation is of particular interest. This shows that common mechanisms driving proliferation and resistance that exist in TNBC but not in susceptible breast cancer cell lines (such as MCF-7 cells) do not impair the potency and efficacy

of sigma-2 receptor ligands to induce cell death. This suggests that sigma-2 agonists may activate a type of “Achilles’ heel” pathway common in TNBC and less aggressive cells that result in equal vulnerability to induced cell death. Another mechanism of drug resistance common in TNBC is inhibition of apoptosis (Longley and Johnston, 2005; O'Driscoll and Clynes, 2006). Sigma-2 receptors have been shown to activate several different apoptotic pathways, and thus their cytotoxic effect is unlikely to be rendered ineffective by a single factor or pathway becoming inhibited (Crawford and Bowen, 2002; Wang and Bowen, 2006; Zeng et al., 2012).

In addition to lacking estrogen receptors, progesterone receptors, and HER2 overexpression, TNBC cell lines often have overactive or overexpressed elements contributing to multidrug resistance. P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP) family members, and breast cancer-resistance protein (BCRP) have all been shown to contribute to resistance of triple negative breast cancer cell lines to traditional chemotherapeutics (Andre and Zielinski, 2012; Doyle et al., 1998; Ferreira et al., 2005a; Martin et al., 2014). MDA-MB-231, MDA-MB-453, MDA-MB-468, and MCF-7 cell lines show variation between expression levels of Pgp, MRP1, and BCRP. For example, MDA-MB-231 and MDA-MB-453 overexpress Pgp, while MDA-MB-468 exhibits no detectable expression (Blumenthal et al., 2001; Ferreira et al., 2005a; O'Driscoll and Clynes, 2006). MDA-MB-231 and MDA-MB-468 express both MRP1 and BCRP, while MDA-MB-453 does not (Deng et al., 2013; Doyle and Ross, 2003; Ferreira et al., 2005b; Li et al., 2013; Lv et al., 2014). In fact, we found that verapamil, a drug efflux pump inhibitor, had to be added to MDA-MB-231 cells for the mitochondrial depolarization assay in order for JC-1, an efflux pump substrate, to be retained in the cells (Figure 4). However, the varied expression level of these transporters did not alter the potency

or efficacy of CM572 and SV119, as the individual compounds were comparably potent across cell types regardless of multidrug resistance profile. A similar observation was made for CB-64D and CB-184, which had equal potency in MCF-7 cells and MCF-7/Adr-, the latter of which overexpresses P-glycoprotein (Crawford and Bowen, 2002). This suggests that these sigma-2 receptor ligands are not substrates for these drug efflux pumps and thus avoid the challenge that multidrug resistance associated transporters pose to traditional chemotherapeutics. In fact, there is evidence that suggests that Pgp overexpression enhances the ability of sigma-2 receptor ligands to induce cell death (Abate et al., 2015). Of additional note is that CM572 exhibits irreversible binding capability imparted by the isothiocyanate moiety (Nicholson et al., 2015). This facet of CM572 would prevent this compound from being pumped out by any transporter once it has covalently bound the sigma-2 receptor.

Taken together, the results reported here suggest that sigma-2 receptor-targeted therapeutics are potentially useful for treatment of triple negative breast cancer. Sigma-2 receptor agonists show good selectivity for cancer cells over normal cells. The cell death mechanism involves mitochondrial depolarization and is not caspase-dependent. Detailed delineation of the mechanism of sigma-2 receptor mediated cell death in TNBC cells may reveal additional components of the pathway that can be targeted. Notably, CM572, an irreversibly binding sigma-2 receptor partial agonist, may prove to be a potent and effective therapeutic against TNBC with unique advantages due to its irreversible interaction with the receptor.

3.7 References

- Abate, C., Pati, M.L., Contino, M., Colabufo, N.A., Perrone, R., Niso, M., Berardi, F., 2015. From mixed sigma-2 receptor/P-glycoprotein targeting agents to selective P-glycoprotein modulators: Small structural changes address the mechanism of interaction at the efflux pump. *European Journal of Medicinal Chemistry* 89, 606-615.
- Al-Nabulsi, I., Mach, R.H., Wang, L.M., Wallen, C.A., Keng, P.C., Sten, K., Childers, S.R., Wheeler, K.T., 1999. Effect of ploidy, recruitment, environmental factors, and tamoxifen treatment on the expression of sigma-2 receptors in proliferating and quiescent tumour cells. *British Journal of Cancer* 81, 925-933.
- Andre, F., Zielinski, C.C., 2012. Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. *Annals of Oncology* 23 Suppl 6, vi46-51.
- Azzariti, A., Colabufo, N.A., Berardi, F., Porcelli, L., Niso, M., Simone, G.M., Perrone, R., Paradiso, A., 2006. Cyclohexylpiperazine derivative PB28, a sigma2 agonist and sigma1 antagonist receptor, inhibits cell growth, modulates P-glycoprotein, and synergizes with anthracyclines in breast cancer. *Molecular Cancer Therapeutics* 5, 1807-1816.
- Blumenthal, R.D., Waskewich, C., Goldenberg, D.M., Lew, W., Flefle, C., Burton, J., 2001. Chronotherapy and chronotoxicity of the cyclooxygenase-2 inhibitor, celecoxib, in athymic mice bearing human breast cancer xenografts. *Clinical Cancer Research* 7, 3178-3185.
- Bowen, W.D., Jin, B., Blann, E., Vilner, B.J., Lyn-Cook, B.D., 1997. Sigma receptor ligands modulate expression of the multidrug resistance gene in human and rodent brain tumor cell lines. *Proceedings of the American Association for Cancer Research* 38: 479, #3206.

- Boyle, P., 2012. Triple-negative breast cancer: epidemiological considerations and recommendations. *Annals of Oncology* 23 Suppl 6, vi7-12.
- Crawford, K.W., Bowen, W.D., 2002. Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines. *Cancer Research* 62, 313-322.
- Deng, Z.J., Morton, S.W., Ben-Akiva, E., Dreaden, E.C., Shopsowitz, K.E., Hammond, P.T., 2013. Layer-by-Layer Nanoparticles for Systemic Codelivery of an Anticancer Drug and siRNA for Potential Triple-Negative Breast Cancer Treatment. *ACS Nano* 7, 9571-9584.
- Doyle, L.A., Ross, D.D., 2003. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* 22, 7340-7358.
- Doyle, L.A., Yang, W., Abruzzo, L.V., Krogmann, T., Gao, Y., Rishi, A.K., Ross, D.D., 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 95, 15665-15670.
- Ferreira, M.J., Gyemant, N., Madureira, A.M., Tanaka, M., Koos, K., Didziapetris, R., Molnar, J., 2005a. The effects of jatrophone derivatives on the reversion of MDR1- and MRP-mediated multidrug resistance in the MDA-MB-231 (HTB-26) cell line. *Anticancer Research* 25, 4173-4178.
- Ferreira, M.J.U., Gyemant, N., Madureira, A.M., Tanaka, M., Koos, K., Didziapetris, R., Molnar, J., 2005b. The effects of jatrophone derivatives on the reversion of MDRI- and MRP-mediated multidrug resistance in the MDA-MB-231 (HTB-26) cell line. *Anticancer Research* 25, 4173-4178.
- Hazelwood, S. and Bowen, W.D., 2006. Sigma-2 receptor-mediated apoptosis in human SK-N-SH neuroblastoma cells: Role of lipid rafts, caspases, and mitochondrial depolarization.

Proceedings of the American Association for Cancer Research 47: #4932.

(http://cancerres.aacrjournals.org/content/66/8_Supplement/1158.3)

Hellewell, S.B. and Bowen, W.D., 1990. A sigma-like binding site in rat pheochromocytoma (PC12) cells: Decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that in guinea pig brain. *Brain Research* 527: 244-253.

Hellewell, S.B., Bruce, A., Feinstein, G., Orringer, J., Williams, W., Bowen, W.D., 1994. Rat liver and kidney contain high densities of sigma 1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. *European Journal of Pharmacology* 268, 9-18.

John, C.S., Vilner, B.J., Schwartz, A.M., Bowen, W.D., 1996. Characterization of sigma receptor binding sites in human biopsied solid tumors [abstract], *Journal of Nuclear Medicine*.

Lehmann, B.D., Bauer, J.A., Chen, X., Sanders, M.E., Chakravarthy, A.B., Shyr, Y., Pietenpol, J.A., 2011. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of Clinical Investigation* 121, 2750-2767.

Li, W.W., Jia, M., Qin, X.M., Hu, J., Zhang, X.F., Zhou, G.Y., 2013. Harmful effect of ER beta on BCRP-mediated drug resistance and cell proliferation in ER alpha/PR-negative breast cancer. *FEBS Journal* 280, 6128-6140.

Longley, D.B., Johnston, P.G., 2005. Molecular mechanisms of drug resistance. *Journal of Pathology* 205, 275-292.

- Lv, X., Pang, X., Jin, X., Song, Y., Li, H., 2014. B-catenin knockdown enhances the effects of fluorouracil in the breast cancer cell line MDA-MB-468. *Biomedical Reports* 2, 910-914.
- Mach, R.H., Smith, C.R., al-Nabulsi, I., Whirrett, B.R., Childers, S.R., Wheeler, K.T., 1997. Sigma 2 receptors as potential biomarkers of proliferation in breast cancer. *Cancer Research* 57, 156-161.
- Makvandi, M., Tilahun, E.D., Lieberman, B.P., Anderson, R.-C., Zeng, C., Xu, K., Hou, C., McDonald, E.S., Pryma, D.A., Mach, R.H., 2015. The sigma-2 receptor as a therapeutic target for drug delivery in triple negative breast cancer. *Biochemical and Biophysical Research Communications* 467(4), 1070-1075.
- Martin, H.L., Smith, L., Tomlinson, D.C., 2014. Multidrug-resistant breast cancer: current perspectives. *Breast Cancer (Dove Med Press)* 6, 1-13.
- Nicholson, H., Comeau, A., Mesangeau, C., McCurdy, C.R., Bowen, W.D., 2015. Characterization of CM572, a selective irreversible partial agonist of the sigma-2 receptor with antitumor activity. *Journal of Pharmacology and Experimental Therapeutics* 354, 203-212.
- O'Driscoll, L., Clynes, M., 2006. Biomarkers and multiple drug resistance in breast cancer. *Current Cancer Drug Targets* 6, 365-384.
- Stampfer, M.R., LaBarge, M.A., Garbe, J.C., 2013. An Integrated Human Mammary Epithelial Cell Culture System for Studying Carcinogenesis and Aging, in: Schatten, H. (Ed.), *Cell and Molecular Biology of Breast Cancer*. Springer Science+Business Media, New York, pp. 323-361.
- Vilner, B.J., John, C.S., Bowen, W.D., 1995. Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Research* 55, 408-413.

- Wang X. and Bowen, W.D., 2006. Sigma-2 receptors mediate apoptosis in SK-N-SH neuroblastoma cells via caspase-10-dependent Bid cleavage and mitochondrial release of endonuclease G and apoptosis-inducing factor. Program No. 90.1. 2006 Neuroscience Meeting Planner. Atlanta, GA: Society for Neuroscience, 2006. Online.
(<http://www.abstractsonline.com/viewer/viewAbstractPrintFriendly.asp?CKey={47A7954A-EF5D-42D3-AC67-F079DDBD0568}&SKey={F4F9D514-92C8-456B-87E3-EF5F21660429}&MKey={D1974E76-28AF-4C1C-8AE8-4F73B56247A7}&AKey={3A7DC0B9-D787-44AA-BD08-FA7BB2FE9004}>)
- Wang, X. and Bowen, W.D., 2009. Sigma-2 receptor-mediated apoptosis in pancreatic cancer cells. In: *Proceedings of the American Association for Cancer Research* 50, April 18-22; Denver, CO, abstract #422, page 102.
(http://cancerres.aacrjournals.org/content/69/9_Supplement/422)
- Wheeler, K.T., Wang, L.M., Wallen, C.A., Childers, S.R., Cline, J.M., Keng, P.C., Mach, R.H., 2000. Sigma-2 receptors as a biomarker of proliferation in solid tumours. *British Journal of Cancer* 82, 1223-1232.
- Wu, Z., Bowen, W.D., 2008. Role of sigma-1 receptor C-terminal segment in inositol 1,4,5-trisphosphate receptor activation: constitutive enhancement of calcium signaling in MCF-7 tumor cells. *The Journal of Biological Chemistry* 283, 28198-28215.
- Zeng, C., Rothfuss, J., Zhang, J., Chu, W., Vangveravong, S., Tu, Z., Pan, F., Chang, K.C., Hotchkiss, R., Mach, R.H., 2012. Sigma-2 ligands induce tumour cell death by multiple signalling pathways. *British Journal of Cancer* 106, 693-701.

Zeng, C., Rothfuss, J.M., Zhang, J., Vangveravong, S., Chu, W., Li, S., Tu, Z., Xu, J., Mach, R.H., 2014. Functional assays to define agonists and antagonists of the sigma-2 receptor. *Analytical Biochemistry* 448, 68-74.

Footnotes:

This work was supported by a National Institutes of Health National Institute of General Medical Sciences T32 Predoctoral Pharmacology Training Grant [1-T32 GM077995] (HN, CZL); National Institutes of Health National Institute of General Medical Sciences R25 Initiative for Maximizing Student Development Grant [R25 GM083270] (HN); Brown University Pharmacia Pre-doctoral Fellowship (HN, CZL); and the Upjohn Professorship in Pharmacology, Brown University (WDB). We thank Dr. Robert Mach, Department of Radiology, University of Pennsylvania for providing us with SV119. We thank Dr. Christopher McCurdy, Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi for providing CM572.

Portions of this work have been previously presented in abstract form: Zongyi Liu, Hilary E. Nicholson, Wayne D. Bowen. Sigma-2 receptor-induced cell death: A novel approach to triple-negative breast cancer treatment. [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl): Abstract #5428. doi:10.1158/1538-7445.AM2015-5428 (http://cancerres.aacrjournals.org/content/75/15_Supplement/5428)

Chapter 4: Sigma-2 Receptors Play a Role in Cellular Metabolism: Stimulation of Glycolytic Hallmarks by CM764 in Human SK-N-SH Neuroblastoma

4.1 Preface

At the same time as investigations of CM572 (see Chapter 2) began, another compound with a similar core structure was first being examined. As the structure of the sigma-2 receptor binding pocket is not known, successful nucleophilic attack of the isothiocyanate group of any CM572-related compound would depend on random placement near an electrophilic residue within the binding pocket. CM769 was synthesized with the same intention as CM572—namely, that creation of an irreversible antagonist would allow for approximation of a cancer cell system lacking sigma-2 receptor input. Where the isothiocyanate moiety of CM572 is in the heterocyclic ring system of the SN79 core structure, the isothiocyanate moiety of CM769 is on the fluorophenyl ring system on the other side of the ligand (Figure I), thereby providing a distinct location within the sigma-2 receptor binding pocket for probing of an appropriate residue with which to irreversibly bind.

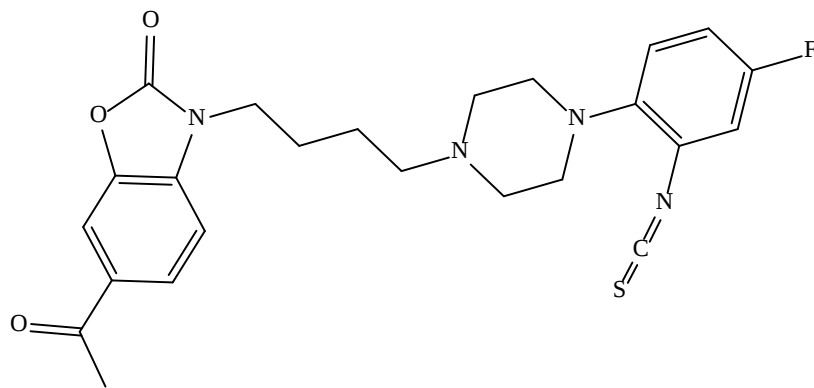
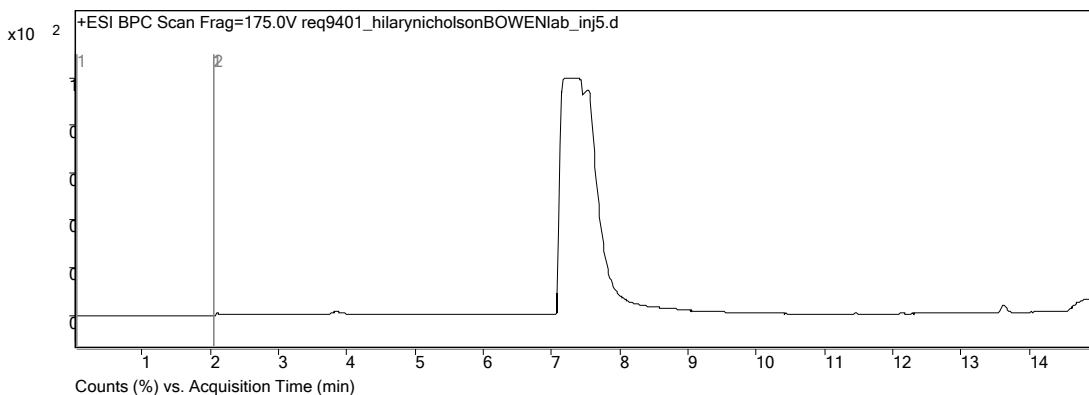


Figure I. **Structure of CM769.** CM769 is formed through the addition of an isothiocyanate moiety to the core structure of SN79, a well-characterized antagonist of the sigma-2 receptor. Synthesis described previously (McCurdy et al. 2014).

Upon initial receipt in powder form, CM769 was diluted into a 10 mM stock in pure dimethylsulfoxide and stored at -20°C. While the parent compound SN79 is very stable under these conditions (Figure II), CM769 is not (Figure III). The first use of CM769 in cells occurred after the ligand had been diluted in DMSO for several months, and therefore the ligand was degraded with nearly none of the original compound remaining. Initial investigation of the effect of this unknowingly degraded compound on cell viability demonstrated an unexpected phenotype of increased reduction of MTT in treated cells as compared to untreated cells (Figure IVa). This stimulation of MTT reduction could be attenuated by competition with the parent compound SN79, indicating a sigma-2 receptor-mediated effect (Figure IVb). After using all of the 10 mM CM769 DMSO stock that had been made initially, a new stock was made from powder and the effect of the newly diluted compound on SK-N-SH neuroblastoma cell viability was drastically altered in comparison to the unknowingly degraded previous stock (Figure V). As the initial stimulative results achieved using degraded CM769 could not be replicated with new CM769, LC/MS analysis of both fresh and aged compounds was performed and it was determined that the stimulative component of aged CM769 was likely a breakdown product.

A



B

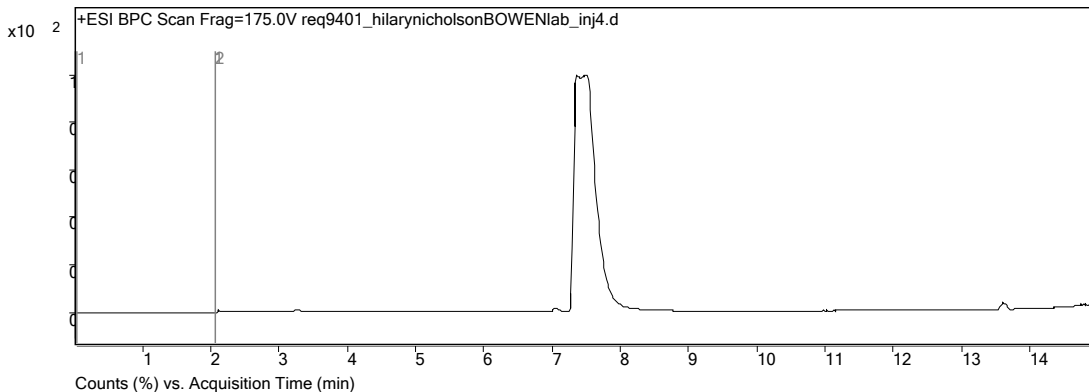
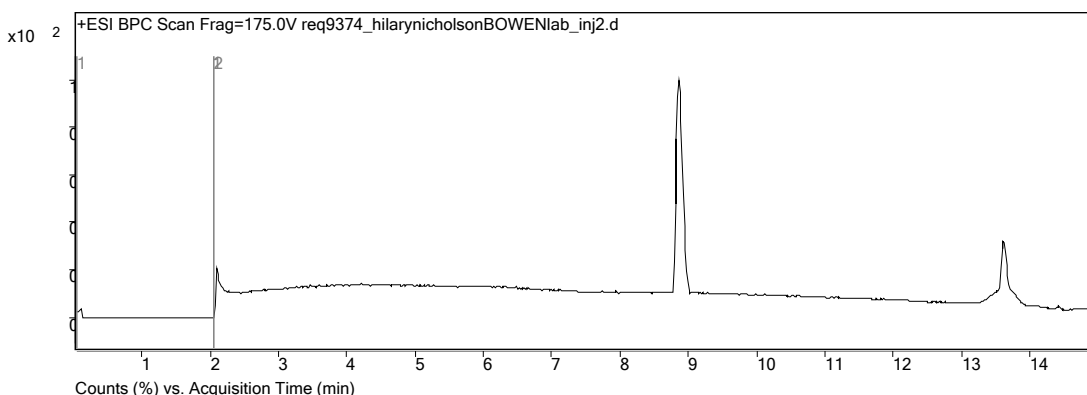


Figure II. Stability of 10 mM SN79 in DMSO stored at -20°C. Stability of SN79 (411.48 g/mol) stored in DMSO was assessed by LC/MS immediately after diluting (A) or after being stored at -20°C as a 10 mM stock in DMSO for over 2 years (B). LC traces are shown for purity assessment. No significant changes in LC traces for SN79 were observed upon storage. M+1 peaks of 412 m/z (A and B) with retention times of 7.222-7.439 min (A) and 7.399-7.549 min (B) were the only significant peaks observed in either trace, indicating no changes to the ligand over the 2-year period under these conditions. LC/MS was run by Tun-Li Shen in the Chemistry Core of Brown University.

A



B

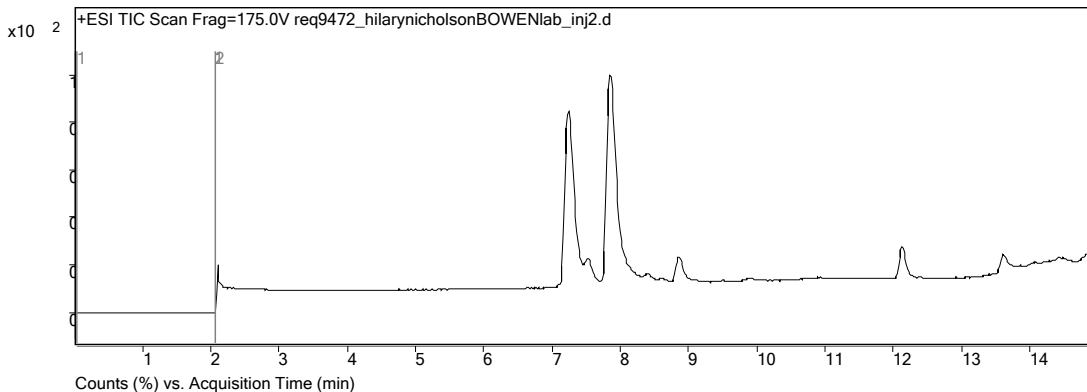
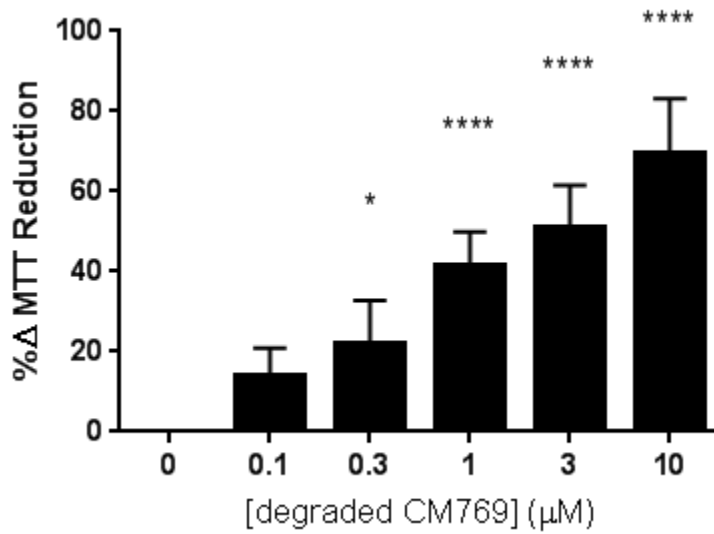


Figure III. **Stability of 10 mM CM769 in DMSO stored at -20°C.** Stability of CM769 (468.55 g/mol) stored in DMSO was assessed by LC/MS immediately after diluting (A) or after being stored at -20°C as a 10 mM stock in DMSO for 5 months (B). LC traces are shown for purity assessment. A) LC/MS analysis of CM769 immediately diluted in DMSO and then analyzed showed a single prominent M+1 peak at 469 m/z with retention time 8.811-8.928 min, consistent with the molecular weight of CM769. B) After storage at 10 mM in DMSO at -20°C for 5 months, LC/MS analysis showed significant breakdown of CM769 with two prominent peaks: 427 m/z with retention time 7.166-7.333min and 448 m/z with retention time 7.816-7.966 min. A smaller residual peak at 469 m/z with retention time 8.782-8.966 min was observed, representing

the remaining intact CM769 in the solution. CM769 was nearly entirely degraded upon storage in DMSO at -20°C for 5 months, with two major degradation products being apparent. LC/MS was run by Tun-Li Shen in the Chemistry Core of Brown University.

A



B

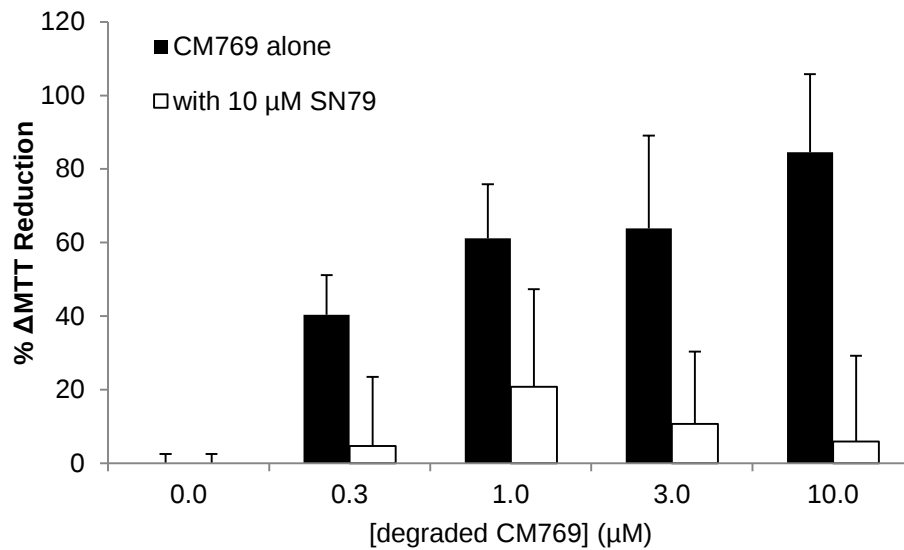


Figure IV. Effect of degraded CM769 treatment on SK-N-SH neuroblastoma cell reduction of MTT. SK-N-SH neuroblastoma cells were treated with the indicated concentrations of degraded CM769 for 24 h in the absence (A) or presence (B) of SN79 prior to MTT assay. A) Treatment with degraded CM769 increased reduction of MTT in a dose-dependent manner.

$p < 0.0001$ for one-way ANOVA ($F = 30.78$), Dunnett's post hoc test for multiple comparisons using untreated control, $*p < 0.05$, $****p < 0.0001$. Results are presented as an average of four independent experiments $\pm$ S.D. for each condition, with each experiment having five replicates per condition. B) Treatment of SK-N-SH neuroblastoma cells with degraded CM769 at the indicated concentrations with simultaneous incubation with $10 \mu\text{M}$ SN79 attenuates the increased reduction of MTT induced by treatment with degraded CM769 alone. This indicates a sigma-2 receptor-mediated effect, as SN79 selectively binds sigma-2 receptors and does not significantly bind any non-sigma receptor entities in the cell. Representative dataset is shown, with each condition presented as an average of 5 replicates $\pm$ S.D. from a single experiment.

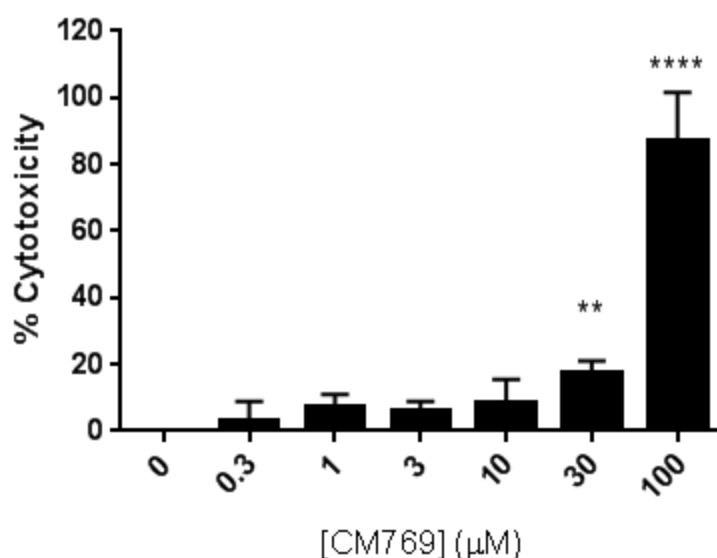


Figure V. **Effect of newly diluted CM769 treatment on SK-N-SH neuroblastoma cell**

viability. SK-N-SH neuroblastoma cells were treated with the indicated concentrations of freshly diluted CM769 for 24 h prior to MTT assay. Treatment with freshly diluted CM769 induced cytotoxicity in a dose-dependent manner. $p < 0.0001$ for one-way ANOVA ($F = 84.68$), Dunnett's post hoc test for multiple comparisons using untreated control, $**p < 0.01$, $****p < 0.0001$. Results are presented as an average of at least three independent experiments $\pm$ S.D. for each condition, with each experiment having five replicates per condition.

Working with the McCurdy lab at University of Mississippi, it was determined that the most likely cause of degradation of CM769 was hydrolysis of the isothiocyanate group into an amino group, for which the molecular weight would be 426 g/mol. This weight corresponded to a large peak in the aged CM769 LC/MS trace. This amino derivative of CM769 had been previously synthesized as CM764, and was assessed for MTT stimulative effects upon receipt. This compound was found to stimulate MTT reduction and bind sigma-2 receptors similarly to aged CM769, and a complete investigation of this effect was carried out.

While there has not previously been a non-zero, non-toxic effect associated with sigma-2 receptor activation, a long-standing question in the field surrounds the seeming contradiction of the upregulation of a receptor that induces cell death upon activation in cancer. Typically, receptors that are similarly upregulated are associated with a pro-survival benefit to the cells. Additionally, as indicated in Chapter 1, sigma-2 receptor activation can induce an immediate transient increase in cytosolic calcium that does not induce cell death. Therefore, the initial indication that the active component of degraded CM769 (CM764) could induce a stimulative, non-toxic sigma-2 receptor-mediated signal, while novel, is consistent with upregulation of the receptor in cancer and a non-toxic, potentially pro-survival function.

The following portion of this chapter has been published:

Hilary Nicholson, Christophe Mesangeau, Christopher R. McCurdy, and Wayne D. Bowen.

2015. "Sigma-2 receptors play a role in cellular metabolism: Stimulation of glycolytic hallmarks by CM764 in human SK-N-SH neuroblastoma." *Journal of Pharmacology and Experimental Therapeutics* 356(2): 232-43.

ABBREVIATIONS: BD1047, N'-[2-(3,4-dichlorophenyl)ethyl]-N,N,N'-trimethylethane-1,2-diamine; BD1063, 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine; CM572, 3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[d]oxazol-2(3H)-one; CM764, 6-acetyl-3-(4-(4-(2-amino-4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one; DCFDA, 2',7'-dichlorofluorescein diacetate; DFO, deferoxamine; DTG, 1,3-di-o-tolylguanidine; EDTA, ethylenediaminetetraacetic acid; HBSS, Hank's Balanced Salt Solution; HIF1 α , hypoxia-inducible factor 1 alpha; MEM, Minimal Essential Media; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; NE100, 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine; PBS, phosphate buffered saline; PGRMC1, progesterone receptor membrane component 1; RIPA buffer, radioimmunoprecipitation assay buffer; ROS, reactive oxygen species; RT, room temperature; SN79, 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one; TBS, Tris buffered saline; VEGF, vascular endothelial growth factor.

Authorship Contributions

Participated in research design: Nicholson, Bowen

Conducted experiments: Nicholson

Contributed new reagents or analytic tools: Mesangeau, McCurdy

Performed data analysis: Nicholson

Wrote or contributed to the writing of the manuscript: Nicholson, Bowen

4.3 Abstract

Sigma-2 receptors are attractive anti-neoplastic targets due to their ability to induce apoptosis and their upregulation in rapidly proliferating cancer cells as compared to healthy tissue. However, this role is inconsistent with overexpression in cancer, which is typically associated with upregulation of pro-survival factors. Here we report a novel metabolic regulatory function for sigma-2 receptors.. CM764 (6-acetyl-3-(4-(4-(2-amino-4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one) binds with K_i values of 86.6 ± 2.8 and 3.5 ± 0.9 nM at sigma-1 and sigma-2 receptors, respectively. CM764 increased reduction of MTT in human SK-N-SH neuroblastoma compared to untreated cells, an effect not due to proliferation. This effect was attenuated by five different sigma antagonists, including CM572, which has no significant affinity for sigma-1 receptors. This effect was also observed in MG-63 osteosarcoma and HEK293T cells, indicating that this function is not exclusive to neuroblastoma or to cancer cells. CM764 produced an immediate, robust, and transient increase in cytosolic calcium, consistent with sigma-2 receptor activation. Additionally, we observed an increase in total NAD^+/NADH level and ATP level in CM764-treated SK-N-SH cells as compared to untreated cells. After only 4 h treatment, basal levels of reactive oxygen species were reduced by 90% in cells treated with CM764 over untreated cells, and HIF1 α and VEGF levels were increased after 3-24 h treatment. These data indicate that sigma-2 receptors may play a role in induction of glycolysis, representing a possible pro-survival function for the sigma-2 receptor that is consistent with its upregulation in cancer cells as compared to healthy tissue.

4.4 Introduction

The sigma receptors comprise a pharmacologically defined family of membrane bound receptors that bind compounds from a variety of structural classes. The sigma-1 receptor is a 25 kDa protein that demonstrates stereoselectivity for (+)-benzomorphans and is known to promote cell survival (Hayashi and Su 2003; Hayashi and Su 2007; Tsai et al. 2009). The sigma-2 receptor is a 21.5 kDa protein that binds (+)-benzomorphans poorly and is significantly upregulated in rapidly proliferating tumors as compared to noncancerous tissue (Hellewell and Bowen 1990; Vilner et al. 1995; Wheeler et al. 2000). The presence of sigma-2 receptors has been validated in an extensive list of human and rodent cancer cell lines and tumors, and thus a ubiquitous role in cancer biology has been proposed (Wheeler et al. 2000; Mach et al. 2013).

Upon activation, the sigma-2 receptor induces apoptotic cell death (Crawford and Bowen 2002; Zeng et al. 2012; Zeng et al. 2014). A variety of pathways have been described in response to sigma-2 receptor activation, indicating that there exists more than one mechanism of sigma-2 receptor-induced cell death (Zeng et al. 2012). Further, discrete ligands induce independent apoptotic pathways even within a cell type, again suggesting the ability of the sigma-2 receptor to activate multiple signaling pathways (Crawford and Bowen 2002; Cassano et al. 2009; Zeng et al. 2012; Cesen et al. 2013).

The sigma-2 receptor has received attention for its potential use as a chemotherapeutic target. The significant upregulation of sigma-2 receptors in cancer as compared to healthy tissue makes it a naturally cancer-selective target, and is currently being examined clinically for diagnostic tumor imaging (Mach et al. 2013; Shoghi et al. 2013). The induction of apoptosis upon activation combined with endogenous cancer cell selectivity makes the sigma-2 receptor an attractive target for chemotherapeutic intervention. We have recently shown that the irreversible

sigma-2 receptor partial agonist CM572 selectively induces cell death in neuroblastoma, pancreatic, and breast cancer cell lines compared to effects in normal epithelial melanocytes or normal breast epithelial cells (Nicholson et al. 2015). However, the wide variety of apoptotic mechanisms induced by sigma-2 receptor activation combined with a disparity among criteria for defining functional classes of sigma-2 receptor ligands make studying the efficacy of such drugs difficult.

One intrinsic question about sigma-2 receptors that has yet to be extensively addressed surrounds their endogenous function. The endogenous ligand for sigma-2 receptors has yet to be elucidated, thus all hypotheses surrounding sigma-2 receptor function are based on observations collected through manipulation with synthetic agents. The observed apoptotic response to treatment with sigma-2 receptor ligands is widely supported; however, it is inconsistent with upregulated expression in rapidly proliferating cancer cells. This counterintuitive role is further called into question when coupled with the low but significant expression of sigma-2 receptors in many non-cancerous tissues such as liver and kidney (Hellewell et al. 1994), which indicates a role for sigma-2 receptors outside of cancer cell proliferation.

Evidence for a non-apoptotic function of sigma-2 receptors is not yet extensive, however examples of such activity do exist. For example, one criterion for classification of a sigma-2 receptor agonist is stimulated transient release of calcium from thapsigargin-sensitive pools in the endoplasmic reticulum (Vilner and Bowen 2000; Cassano et al. 2009). However, this calcium signal is not a trigger for apoptosis, as cells appear normal if ligand is removed after the transient returns to baseline (unpublished observation). In addition, there are also sigma-2 ligands that produce a calcium signal, but do not have the ability to kill cells. The sigma-2 receptor ligand SN79 (Kaushal et al. 2011; Kaushal et al. 2012) produces a small calcium signal

in SK-N-SH neuroblastoma cells, but is unable to induce significant levels of cytotoxicity even at high doses (Garcia 2012). Based on such data we have proposed that the sigma-2 receptor may signal to pathways that bifurcate to those that result in apoptotic cell death and those that result in as yet uncharacterized non-apoptotic effects (Garcia and Bowen 2010; Garcia 2012).

Recently it was proposed that progesterone receptor membrane component-1 (PGRMC1) is the putative sigma-2 receptor binding site (Xu et al. 2011). In MCF-7 cells, PGRMC1 mediates a signal that stimulates proliferation and angiogenesis-related effects (Neubauer et al. 2009), further suggesting a non-apoptotic function for ligands binding PGRMC1 or the sigma-2 receptor, whether identical or pharmacologically related. Here, we report a novel non-apoptotic, metabolically stimulative function for the sigma-2 receptor, discovered through intervention with CM764, a novel derivative of SN79. This may suggest a divergent role of relevance to sigma-2 receptor upregulation.

4.5 Materials and Methods

Radioligand binding assay

Rat liver membrane homogenates were used for receptor binding assays with slight modification to the previously reported procedure (Hellewell et al. 1994). Frozen rat livers (BioChemed, Winchester, VA) were thawed and homogenized for membrane preparation as previously described (Hellewell et al. 1994). A 150 μ g of membrane protein was incubated with 3 nM [3 H](+)-pentazocine (Perkin Elmer, Waltham, MA) (sigma-1 receptor condition) or 5 nM [3 H]DTG (Perkin Elmer, Waltham, MA) and 100 nM unlabeled (+)-pentazocine to mask sigma-1 receptors (sigma-2 receptor condition) with various concentrations of CM764 for 120 min at 25°C in 20 mM HEPES pH 7.4 with gentle shaking. Nonspecific binding was measured in the presence of 10 μ M haloperidol. Membranes were collected by filtration using a Brandel Cell Harvester (Brandel, Gaithersburg, MD) onto glass fiber filters that were presoaked in 0.5% polyethyleneimine for 30 min at room temperature. Ice cold 10 mM Tris-HCl, pH 7.4 was used to terminate reactions using 5 mL buffer followed by two 5 mL buffer washes. K_i values were determined by competition binding assay and data analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA) with K_d =17.9 nM for [3 H]DTG at sigma-2 receptors and K_d =7.5 nM for [3 H](+)-pentazocine at sigma-1 receptors in rat liver membrane homogenates.

Cell culture

Human SK-N-SH neuroblastoma cells were obtained from ATCC (Manassas, VA) and were cultured in Minimal Essential Medium (Gibco, Grand Island, NY) with 10% fetal bovine serum and 10 mg/L human insulin (Gibco, Grand Island, NY) in a humidified atmosphere at

37°C and 5% CO₂. HEK 293 T/17 human embryonic kidney cells were a generous gift from Dr. Elena Oancea, Brown University, Providence, RI, and human MG-63 osteosarcoma cells were a generous gift from Dr. Eric Darling, Brown University, Providence, RI. Both additional cell types were cultured in the same way as described for SK-N-SH neuroblastoma. Cells were passaged at 70% confluency.

MTT cell viability assay

Cell viability was measured by MTT assay (Trevigen, Gaithersburg, MD). Cells were plated at 15,000 cells/well (SK-N-SH) or 10,000 cells/well (HEK 293 T/17, MG-63) in a 96-well plate and allowed to attach overnight. Cells were then treated for 24 h prior to addition of 10 µL MTT reagent, which was allowed to be metabolized into colored formazan crystals for 3 h. A 100 µL aliquot of MTT detergent reagent was then added and formazan crystals and cell membranes were solubilized for 2 h prior to reading absorbance at 570 nm. Data was analyzed in Microsoft Excel.

CyQUANT cell proliferation assay

Cellular proliferation was measured using the CyQUANT Cell Proliferation Assay Kit (Life Technologies, Grand Island, NY). Cells were plated in 96-well plates at 15,000 cells/well for 24-48 h experiments or 10,000 cells/well for 72-96 h experiments. Cells were allowed to attach overnight prior to treatment with 10 µM CM764 or fresh media without ligand (control). Media with or without ligand was replaced every 24 h. After the indicated time, cells were

washed once with PBS and then plate was inverted and blotted dry onto a Kimwipe by gentle tapping, and frozen at -80°C for at least 24 h. Plates were then thawed to RT and stained with 1X CyQUANT GR dye, which exhibits enhanced fluorescence when bound to nucleic acids, in cell lysis buffer for 5 min protected from light prior to measurement of fluorescence at 480nm excitation/520 nm emission.

Calcium Release Assay

Fura-2 was used to measure intracellular calcium in SK-N-SH neuroblastoma. Cells were seeded at 20,000 cells/well and allowed to attach overnight prior to washing twice with HBSS and loading for 60 minutes with 2.47 μ M fura-2 acetoxymethyl ester (fura-2AM, Invitrogen, Grand Island, NY) in 0.065% pluronic acid in HBSS. Cells were then washed twice with HBSS prior to injection with indicated ligand. Stock solutions for injection were adjusted such that injection volume was 10 μ L, injected onto 90 μ L HBSS in the well for a final total volume of 100 μ L. Fura-2 ratio was measured with excitation at 340 and 380 nm and emission at 510 nm using a PerkinElmer Victor V plate reader. A baseline ratio was determined prior to injection by measuring 25 readings over 43 seconds, after which the 10 μ L injection was made and 100 readings were measured.

NAD⁺/NADH assay

NAD⁺ and NADH were measured using the NAD⁺/NADH Quantification Colorimetric Kit from BioVision (Milpitas, CA), which allows for determination of total NAD, NAD⁺, and

NADH from plated cells without the need for purification steps. Cells were plated in 35 mm petri dishes at 200,000 cells/dish and allowed to attach overnight prior to treatment with 10 μ M CM764 or fresh media without ligand (control) for 24 h. NAD⁺/NADH assay was then performed according to manufacturer's specifications. Cells were washed once with cold PBS, dissociated with 2.5 mM EDTA, pelleted by centrifugation at 223 x g for 5 min, and extracted with 400 μ L Extraction Buffer by two freeze/thaw cycles of 20 min on dry ice and 10 min at room temperature. The extract was then pelleted by centrifugation at 37,500 x g for 5 min and supernatant was collected. For NADH determination, NAD⁺ was degraded by 30 min heating at 60°C. Samples were incubated with Reaction Mix for 5 min prior to the addition of 10 μ L Developer, and then allowed to cycle for 4 h. Absorbance was measured every hour at 450 nm. NADt (NADH + NAD⁺) and NADH levels were measured directly. NAD⁺ level was inferred to be the difference between NADt and NADH. Protein concentration was measured by BCA assay (Pierce, Waltham, MA) and NADt/NADH/NAD⁺ levels were adjusted for protein levels.

ATP assay

Cellular ATP was measured using an ATP Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA). Cells were plated in 35 mm petri dishes at 600,000 cells/well and allowed to attach overnight prior to treatment with 10 μ M CM764 or fresh media without ligand (control). After 24 h, cells were washed once with PBS, lysed, and immediately deproteinized with perchloric acid and neutralized with potassium hydroxide. A 50 μ L aliquot of deproteinized supernatant was incubated with Reaction Mix for 30 min at room temperature protected from light. Fluorescence of the samples was read at 535 nm excitation/587 nm emission. Protein

concentration was measured by BCA assay (Pierce, Waltham, MA) and ATP levels were adjusted for protein levels.

Detection of cellular reactive oxygen species

Cells were plated in a black-sided 96-well plate at 25,000 cells/well and allowed to attach overnight. Cells were then washed once with HBSS and stained with 50 μ M DCFDA (Abcam, Cambridge, MA) in HBSS for 45 min in the dark at 37°C. Cells were washed once with HBSS and treated with indicated compound for 4 h in phenol red-free media with 200 μ g/mL α -tocopherol as a negative control and 500 μ M TBHP as a positive control. Fluorescence was measured at 485 nm excitation/535 nm emission.

Western blot

SK-N-SH neuroblastoma cells were plated in 35 mm petri dishes and allowed to attach overnight prior to treatment with 10 μ M CM764 for indicated time. Cells were lysed in RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA), and 200 μ M deferoxamine. Standardization was performed after protein concentration analysis by BCA assay (Pierce, Waltham, MA). Samples were run in a 10% acrylamide gel and transferred at 50V for 2.5 h at 4°C onto nitrocellulose paper prior to blocking for 1 h at RT in 10% milk/TBS/0.1% Tween-20. Blots were then washed three times with TBS/0.1% Tween-20 and probed with antibodies for HIF1 α (1:100, Santa Cruz, Dallas, TX) or VEGF (1:100, Santa Cruz, Dallas, TX) with GAPDH (1:100, Santa Cruz, Dallas, TX) or β -

tubulin (1:500, developed by Michael Klymkowsky at University of Colorado, Boulder, CO, obtained from the Developmental Studies Hybridoma Bank (NICHD of NIH), maintained at University of Iowa, Iowa City, Iowa) used as a loading control, in 10% milk/TBS/0.1% Tween-20 (or 5% BSA/TBS/0.1% Tween-20 for β -tubulin only) with overnight shaking at 4°C. Blots continued to shake at RT for 1 h and were then washed three times with TBS/0.1% Tween-20 before 1 h RT incubation with 1:3000 rabbit secondary antibody (HIF1 α probe, VEGF probe) (Santa Cruz, Dallas, TX) or 1:1000 mouse secondary antibody (GAPDH probe, β -tubulin probe) (Santa Cruz, Dallas, TX). Blots were then washed three times with TBS/0.1% Tween-20 and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Waltham, MA) per manufacturer's specifications. UltraCruz Autoradiography Film (Santa Cruz, Dallas, TX) was used to make film exposures, which were then analyzed using ImageJ software (NIH, Bethesda, MD). Protein levels were standardized using a loading control, then normalized to the level of the standardized untreated control.

Data Analysis

Data analysis was performed using Microsoft Excel and GraphPad Prism 6 (GraphPad Software, La Jolla, CA). For radioligand competition binding assays, K_i values were determined using GraphPad Prism 6 for nonlinear regression log(agonist) vs. normalized response with variable slope parameters.. For cell viability analysis, fura-2AM calcium assays, ATP assays, NADH/NAD⁺ assays, and CyQUANT Cell Proliferation assays, data was analyzed using Microsoft Excel. Two-tailed t-tests were performed where appropriate using Microsoft Excel, and one-way ANOVAs were performed using GraphPad Prism 6. For one-way ANOVAs,

Dunnet's post-hoc test was used where appropriate for comparison to control group. Power analysis was performed to 80% power using a power analysis calculator developed by the University of British Columbia Department of Statistics based on normal distributions.

Sigma receptor ligands

The ligands used in this study are shown in Table 1 along with their sigma receptor binding characteristics and reference citations. The synthesis of CM764 has been previously described (McCurdy et al., 2014).

4.6 Results

Radioligand binding competition of CM764 at sigma-1 and sigma-2 receptors

The novel SN79 derivative CM764 is derived by the addition of an amine group to the fluorophenyl ring of SN79 (synthesis previously described) (McCurdy et al. 2014). The structures of both ligands are shown in Figure 1. Competition binding was performed using [³H](+)-pentazocine to measure sigma-1 receptor binding and [³H]DTG in the presence of unlabeled (+)-pentazocine to measure sigma-2 receptor binding as described in Methods. Complete radioligand competition curves are shown in Figure 2. CM764 binds sigma-2 receptors with ~25-fold selectivity over sigma-1 receptors, with K_i values of 3.5 ± 0.9 nM at sigma-2 receptors and 86.6 ± 2.8 nM at sigma-1 receptors. This is an increase in selectivity of 5-fold over the parent compound, SN79 ($K_i=7$ nM and 27 nM at sigma-2 and sigma-1, respectively) (Kaushal et al. 2011).

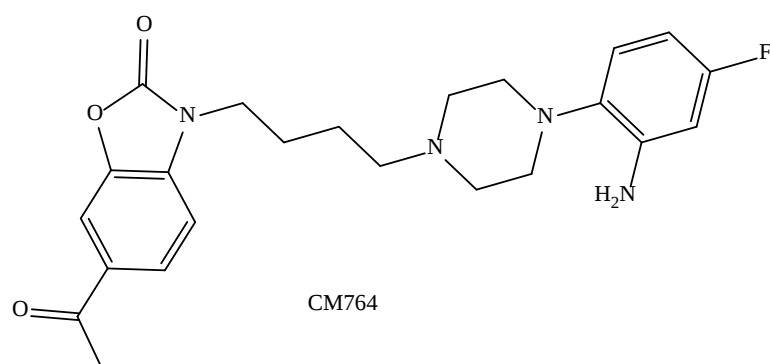
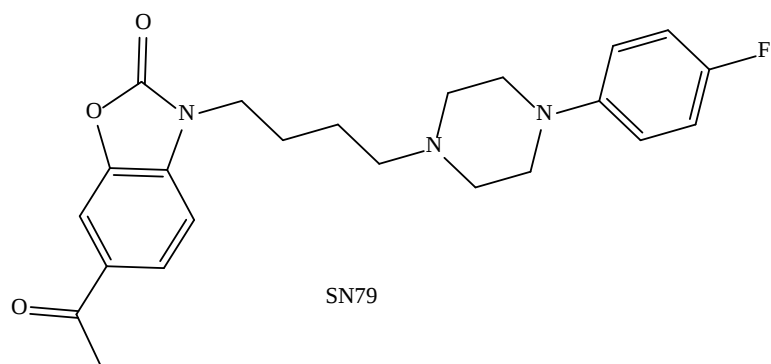


Figure 1. **Structures of SN79 and CM764.** CM764 is a novel derivative of the well-characterized sigma-2 antagonist SN79. Addition of an amine to the fluorophenyl ring of SN79 results in CM764. Synthesis of CM764 was described previously (McCurdy et al. 2014).

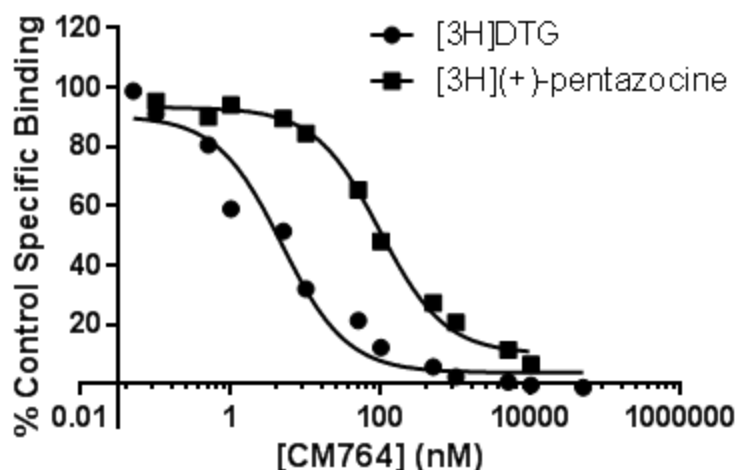


Figure 2. **CM764 binding at sigma-1 and sigma-2 receptors.** Affinity of CM764 for sigma-1 and sigma-2 receptors was determined by competition binding in rat liver membranes. Assays were carried out as described in Materials and Methods, using [^3H](+)-pentazocine to label sigma-1 receptors and [^3H]DTG in presence of unlabeled (+)-pentazocine to label sigma-2 receptors. Results are expressed as the percentage of control specific binding at each concentration of CM764. K_i values were determined by analysis of competition curves using GraphPad Prism 6 and radioligand K_d values previously determined ([^3H](+)-pentazocine K_d , 7.5 nM; [^3H]DTG K_d , 17.9 nM (Hellewell et al. 1994)). Graphpad analysis revealed K_i values of 86.6 ± 2.8 nM and 3.5 ± 0.9 nM at sigma-1 and sigma-2 receptors, respectively. Each curve represents the average of at least 3 independent experiments $\pm$ S.D., with each experiment performed in duplicate.

CM764-induced MTT reduction in SK-N-SH neuroblastoma

A well-established indicator of sigma-2 receptor activation is cell death. As CM764 was found to bind sigma-2 receptors well, we examined the effect of CM764 treatment on cell viability in order to determine the agonist or antagonist properties of the ligand. A commonly used assay to measure effects of agents on cell viability or proliferation is the MTT assay. The assay is based on the premise that reduction of the tetrazolium dye MTT to the colored formazan product, mainly in mitochondria and cytosol, is proportional to the number of viable cells. Thus, a decrease in MTT reduction usually indicates fewer cells and a cytotoxic effect, and this is the effect produced by sigma-2 receptor agonists. Human SK-N-SH neuroblastoma cells highly express sigma-2 receptors and have been demonstrated to be highly sensitive to sigma-2 receptor modulators, and were thus used to determine effects of CM764 on MTT reduction. (Vilner et al. 1995; Vilner and Bowen 2000; Hazelwood and Bowen 2006). The results are shown in Figure 3. Interestingly, treatment of SK-N-SH neuroblastoma cells with CM764 induced a dose-dependent *increase* in MTT reduction as compared to untreated control cells. This increase in MTT reduction was found to be statistically significant at 3 and 10 μ M doses.

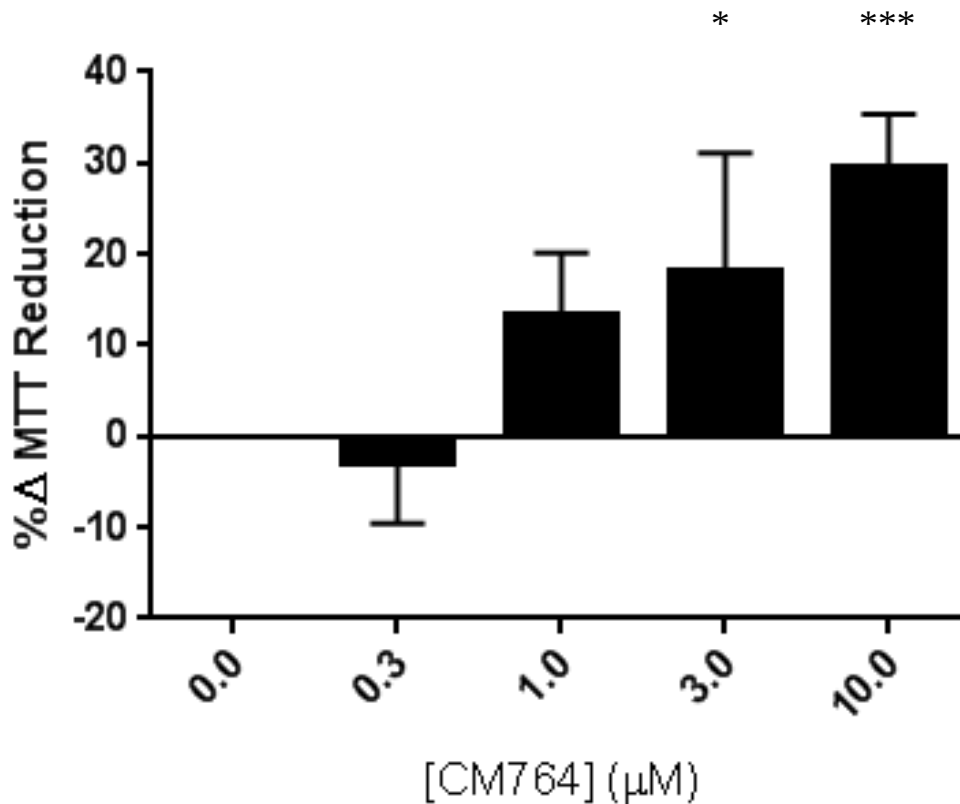


Figure 3. Effect of CM764 treatment on MTT reduction in SK-N-SH neuroblastoma. SK-N-SH neuroblastoma cells were treated with CM764 at the indicated doses for 24 h prior to MTT assay, carried out as described in Materials and Methods. CM764 treatment induced a statistically significant dose-dependent increase in MTT reduction (one-way ANOVA $F=13.94$, Dunnett's post-hoc comparison to control 3 μM $*p<0.05$, 10 μM $***p<0.001$). Results are expressed as an average percent change in MTT reduction $\pm$ S.D. in treated samples relative to an untreated control for at least three independent experiments, with each experiment having 5 replicates.

Effect of CM764 treatment on proliferation in SK-N-SH neuroblastoma

The increase in MTT reduction that resulted from treatment of SK-N-SH neuroblastoma with CM764 could occur from two potential causes: 1) an increased number of cells available to reduce MTT as compared to the untreated control condition, and/or 2) some direct effect to increase the activity of the enzymes that reduce MTT in each treated cell as compared to untreated cells. In order to determine whether the CM764-induced increase in MTT reduction was a result of increased cellular proliferation and therefore more cells being available to reduce MTT, the CyQUANT Cell Proliferation Assay was used to measure DNA replication. Cells were treated with CM764 for up to four days prior to staining with CyQUANT GR dye and quantification as described in Methods. Results are shown in Figure 4. There was no significant difference in DNA replication observed at any time point up to four days between cells treated with 10 μ M CM764 and untreated control cells. In addition, these results were confirmed by counting DAPI-stained cells, which showed no significant differences between treated and control groups (data not shown). These data indicate that treatment of SK-N-SH neuroblastoma cells with 10 μ M CM764 does not result in increased DNA replication or cellular proliferation above that of normal cell turnover and proliferation. The results further show that treatment for longer than 24 h, up to 4 days, had no toxic effect on the cells. Thus, the increased reduction of MTT observed in CM764-treated cells could be attributed to stimulated reductive metabolism without a concurrent stimulation of cell division.

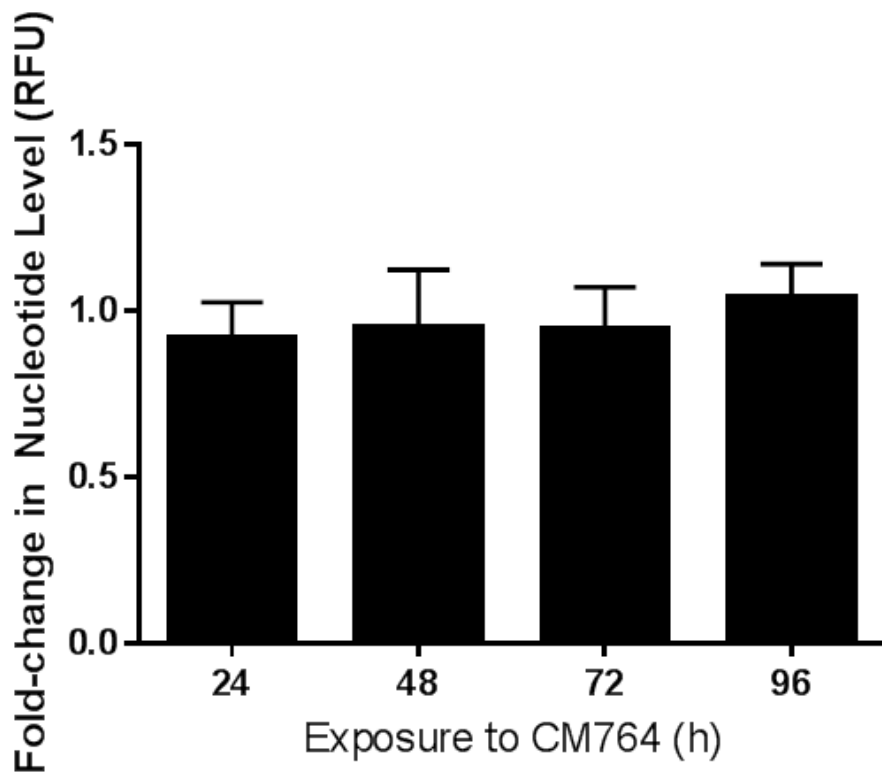


Figure 4. Effect of CM764 treatment on DNA replication in SK-N-SH neuroblastoma. DNA replication was measured to determine the effect of CM764 treatment on cellular proliferation using the CyQUANT assay as described in Materials and Methods. There was no significant change in DNA synthesis as measured by CyQUANT GR dye fluorescence in cells treated with CM764 as compared to untreated cells up to 96 h exposure. This indicates that the effect of CM764 on SK-N-SH neuroblastoma does not result in an increase in cellular proliferation. Results are presented as the fold-change in level of nucleotides as measured by ratio of RFU in treated and untreated cells at that time point. Data presented is the average of two independent experiments $\pm$ S.D., with each experiment having 5 replicates per condition. Media and ligand were changed after every 24 h period.

Pharmacological characterization of CM764-induced MTT reduction

The stimulative effect demonstrated by CM764 treatment of SK-N-SH neuroblastoma cells has yet to be reported as a result of sigma-2 receptor activation, which has previously only been associated with a decrease in cell viability (agonist activity). In order to determine whether the stimulation of MTT reduction induced by CM764 treatment is mediated by the sigma-2 receptor, several sigma-2 receptor modulators were investigated in combination with CM764 treatment to determine if the effect could be attenuated. The effects of known sigma receptor antagonists are shown in Figure 5. CM764 and each antagonist were dosed simultaneously and MTT reduction was measured after 24 h treatment. All sigma-2 receptor antagonists examined were able to significantly attenuate the stimulation of MTT reduction induced by CM764 alone, without inducing significant effect on MTT reduction when used alone. The well-characterized sigma-2 receptor antagonist SN79, the parent compound for CM764, was able to completely eliminate the stimulation induced by CM764 alone. A 24 h treatment of 30 μ M SN79 alone had no significant effect on MTT reduction in SK-N-SH neuroblastoma as compared to an untreated control. Similarly, 0.3 μ M CM572 a sigma-2 receptor partial agonist (K_i =15 nM at sigma-2 receptors and ≥ 10 μ M at sigma-1 receptors), which we have previously shown has antagonist properties at this concentration in SK-N-SH neuroblastoma (Nicholson et al., 2015), eliminated CM764-induced MTT hyper-reduction when dosed in combination with 10 μ M CM764. Sigma-1/sigma-2 antagonists BD1047 (K_i =47 nM at sigma-2 receptors and 0.93 nM at sigma-1 receptors (Matsumoto et al. 1995)) and BD1063 (K_i =449 nM at sigma-2 receptors and 9.15 nM at sigma-1 receptors (Matsumoto et al. 1995)) were both able to eliminate the increase in MTT reduction induced by CM764 treatment alone. NE100, which has selectivity for the sigma-1 receptor yet still has measurable affinity for the sigma-2 receptor (K_i =84.7 nM at sigma-2

receptors and 1.54 nM at sigma-1 receptors (Chaki et al. 1994)) was able to significantly attenuate but not completely eliminate the signal produced by CM764 when it was dosed in combination at 1 μ M. The ability of these sigma-2 receptor modulators to attenuate or entirely eliminate the CM764-induced increase in MTT reduction in SK-N-SH neuroblastoma indicate that this effect is sigma-2 mediated and represents a novel function for this receptor.

CM764 does exhibit significant affinity for the sigma-1 receptor, although it is a sigma-2-selective ligand. Some of the ligands investigated for antagonist activity against CM764 are selective for the sigma-1 receptor, though doses used in these experiments were all at least 200-fold greater than the sigma-2 receptor K_i of the ligands and therefore could be expected to be acting at the sigma-2 receptor as well as the sigma-1 receptor. In light of this, it was necessary to examine whether the stimulation of MTT reduction observed in SK-N-SH neuroblastoma in response to treatment with CM764 could be a result of sigma-1 receptor activation either exclusively or in addition to sigma-2 receptor activation. To test this, the effect of treatment with the sigma-1 agonist (+)-pentazocine (K_i =1,542 nM at sigma-2 receptors and K_d =7.5 nM at sigma-1 receptors (Hellewell et al. 1994)) was examined in SK-N-SH cells. (+)-Pentazocine was unable to induce any significant change in MTT reduction as compared to untreated control cells up to 1 μ M, which is over 130-fold above its sigma-1 receptor K_i value (experiment was repeated twice with similar results, with 5 replicates per condition in each experiment). Additionally, CM572 was able to completely attenuate the effect of CM764 on MTT reduction, although CM572 exhibits no significant binding at the sigma-1 receptor. This antagonism demonstrates that the stimulation of MTT reduction induced by CM764 treatment could be entirely eliminated by selectively blocking the sigma-2 receptor, without blocking the sigma-1

receptor at all. These data confirm that this effect is sigma-2 receptor-mediated and does not involve sigma-1 receptors.

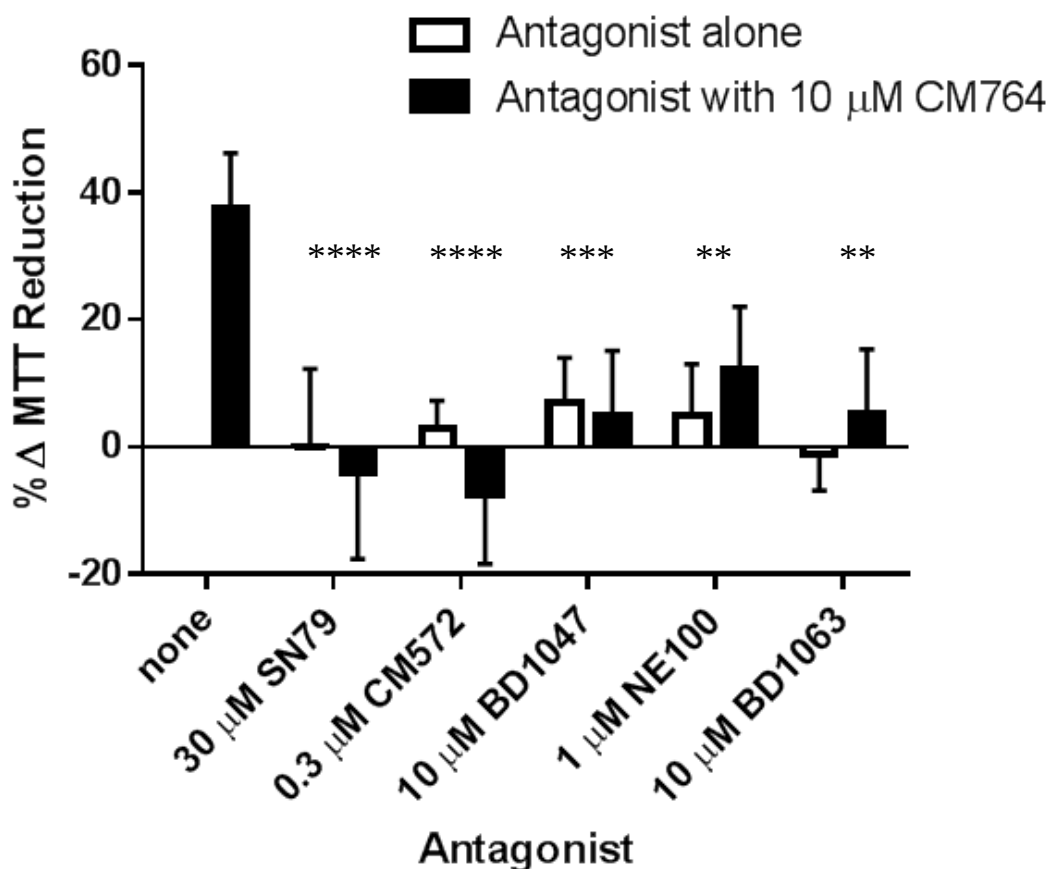


Figure 5. **Effect of sigma-2 receptor antagonists on CM764-induced MTT reduction in SK-N-SH neuroblastoma.** Cells were exposed to 10 μM CM764 alone, the indicated antagonist alone, or to the combination of 10 μM CM764 and antagonist for 24 h. MTT reduction was measured as described in Materials and Methods. No antagonist alone produced a significant effect on MTT reduction as compared to an untreated control. All antagonists were able to significantly attenuate the stimulative effect of CM764 on MTT reduction (one-way ANOVA $F=13.73$). CM572 and SN79, the most highly sigma-2 selective antagonists investigated, were both able to fully attenuate CM764-induced increase in MTT reduction (Dunnett's test for multiple comparisons as compared to an untreated control, **** $p<0.0001$). The other sigma-2 antagonists, though more selective for sigma-1 receptors, were also able to significantly attenuate

CM764-induced MTT reduction (BD1047 *** $p < 0.001$; NE100 ** $p < 0.01$; BD1063 ** $p < 0.01$).

The data support the notion that the effect is sigma-2 receptor-mediated. Results are presented as an average increase in MTT reduction as compared to an untreated control for at least 3 independent experiments, with each experiment performed with 5 replicates.

Table 1. Sigma receptor ligands and their affinities and selectivities

Common Name	Chemical Name	Sigma-1 Receptor Affinity (nM)	Sigma-2 Receptor Affinity (nM)	Sigma-2 Receptor Selectivity (fold)	References
SN79	6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one	27	7	4	Kaushal et al. 2011
CM572	3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[d]oxazol-2(3H)-one	>10,000	14.6	>685	Nicholson et al. 2015
BD1047	N'-[2-(3,4-dichlorophenyl)ethyl]-N,N,N'-trimethylethane-1,2-diamine	0.93	47	0.02	Matsumoto et al. 1995
NE100	4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine	1.54	84.7	0.02	Chaki et al. 1994
BD1063	1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine	9.15	449	0.02	Matsumoto et al. 1995
(+)-pentazocine	2-dimethylallyl-5,9-dimethyl-2'-hydroxybenzomorphan	7.5	1,542	0.005	Hellewell et al. 1994

Effect of CM764 treatment on intracellular calcium

A common characteristic of sigma-2 receptor activation is an immediate, transient increase in intracellular calcium. However, this calcium response has not been consistently coupled to apoptosis. We therefore examined whether CM764 would induce this nontoxic calcium transient, commonly resultant from sigma-2 receptor activation. Using fura-2AM ratiometric dye, we determined that injection of CM764 for a final concentration of 10 μ M and 30 μ M both induced a robust, immediate calcium transient, with calcium level decreasing towards baseline within the 5-minute recording (Figure 6). The higher dose of CM764 reached a slightly greater peak increase in calcium level, and within a faster timeframe than the lower dose. This data indicates that CM764 is able to induce the immediate calcium transient characteristic of sigma-2 receptor activation, and confirms that this signal can occur independently from the induction of apoptosis.

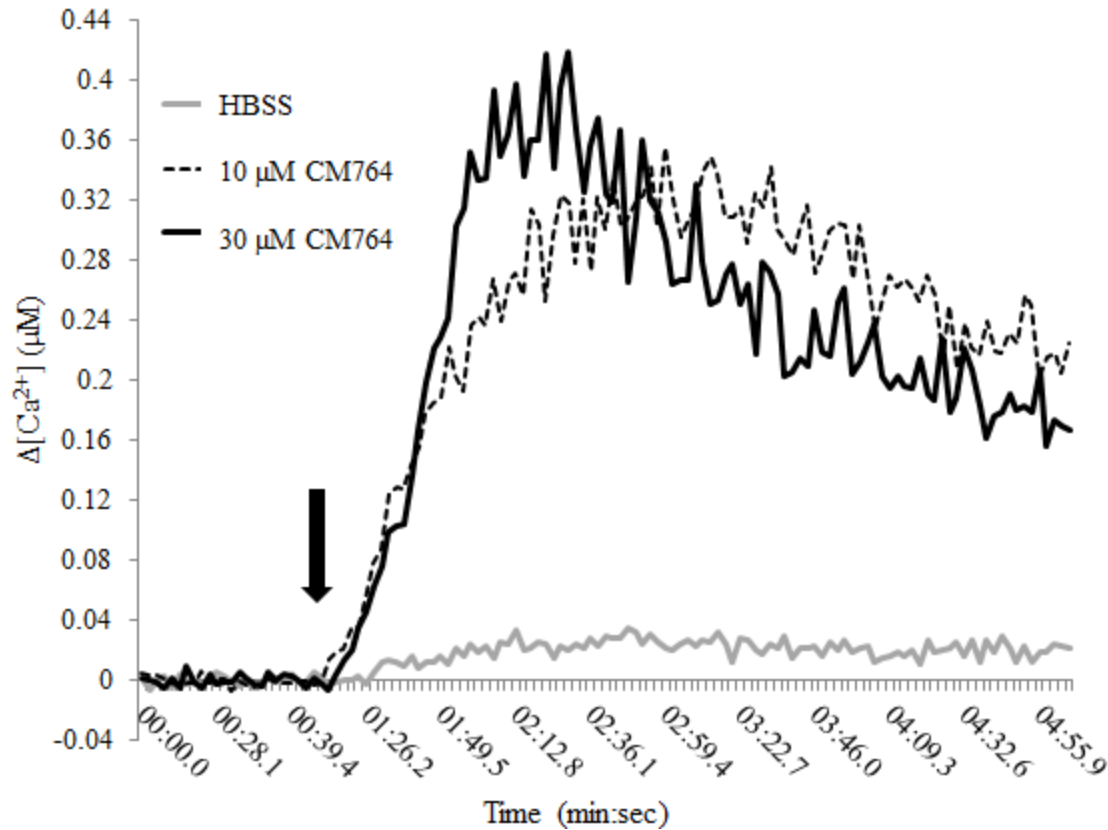


Figure 6. **Effect of CM764 treatment on intracellular calcium in SK-N-SH neuroblastoma.**

Cells were loaded with fura-2,AM for 60 min. prior to CM764 or vehicle injection (injection indicated by black arrow). CM764 injection induced an immediate increase in cytosolic calcium in SK-N-SH neuroblastoma, as has been previously demonstrated in response to sigma-2 receptor activation. A representative trace for each condition is shown. Data is presented as a change in intracellular calcium level with the baseline removed, with the baseline level of calcium determined by the average of 25 ratio measurements prior to injection. Experiment was repeated 4 times with similar results, with each experiment having 4 replicates.

Effect of CM764 on NAD⁺/NADH levels

Since MTT dye is reduced to formazan by cellular reductase enzymes that are mainly found in mitochondria and cytosol (although also present in lysosomes and endosomes) (Liu et al., 1997), these data suggest that CM764 treatment stimulates these enzymes to increase their activity. Several oxidoreductase enzymes responsible for the reduction of MTT into formazan are known to be NADH-dependent (Berridge et al., 2005). We therefore tested whether the increase in MTT reduction exhibited in SK-N-SH cells upon treatment with CM764 could be reflected in an increase in levels of this cofactor, which could facilitate enzyme activity. Using a colorimetric kit for quantification of NAD⁺/NADH levels, we were able to determine that there was a statistically significant increase in total NAD (sum of NADH and NAD⁺) in cells treated with 10 μ M CM764 for 24 h as compared with untreated cells. Results are shown in Figure 7. For both NAD⁺ individually and NADH individually neither measurement reached a statistically significant change from untreated cells. This appears to be due to variability between which reduction state increased more than the other, as NAD⁺ levels were determined indirectly as the difference between NAD total and NADH, which were both measured directly. Therefore, the variability in NAD⁺ levels was dependent on the variability in NADH measurements, which did not allow for significance in NAD⁺ unless significance was reached for NADH. However, the overall increase in total NAD in CM764-treated cells as compared to untreated cells demonstrate that increased levels of the cofactor required for enzymatic reduction of MTT was indeed induced by CM764. This is likely a contributing factor to the observed increase in MTT reduction in CM764-treated cells, perhaps in addition to direct stimulation of oxidoreductase enzymes.

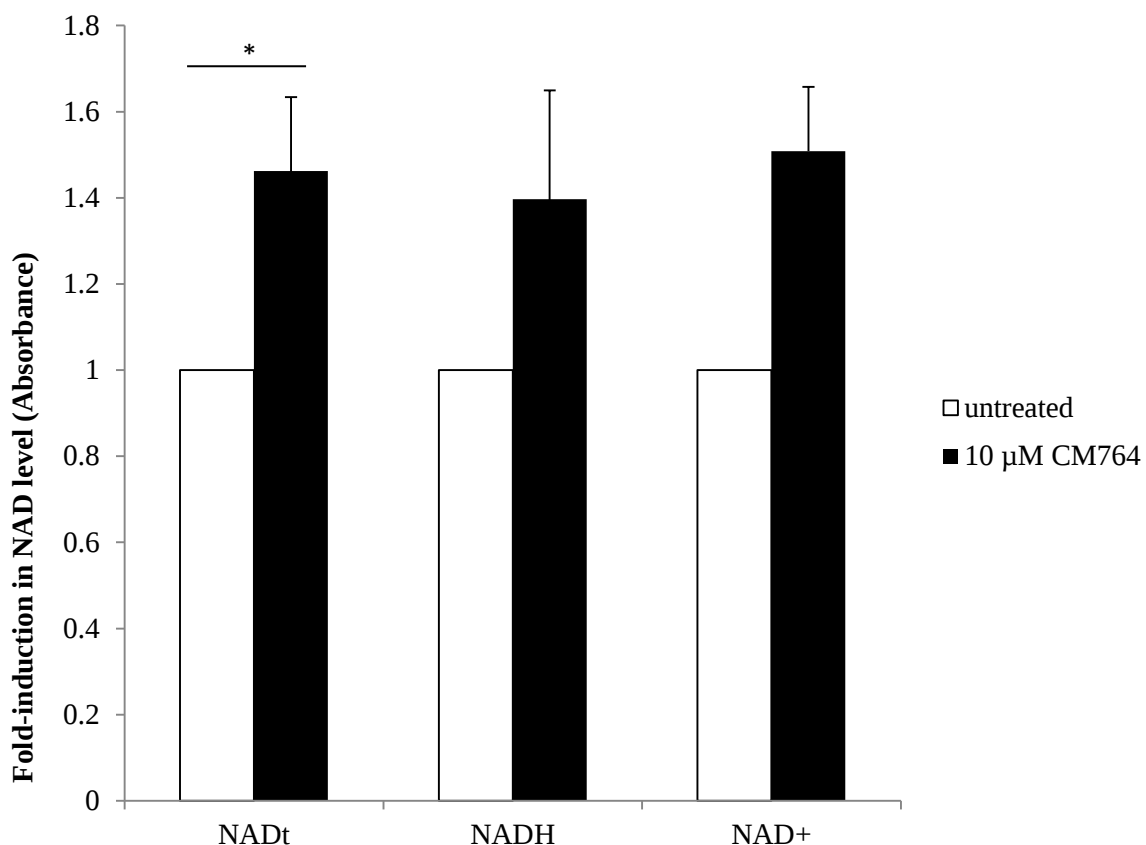


Figure 7. **Changes in NADH/NAD⁺ in response to CM764 exposure.** Cells were exposed to 10 μ M CM764 for 24 h prior to NADH/NAD⁺ assay, carried out as described in Methods.

Treatment with CM764 induced a statistically significant, yet not robust ~1.4-fold increase in total NAD (NADH + NAD⁺) as compared to an untreated control (Student's t-test, $p < 0.05$ ($p = 0.043$)). NADH and NAD⁺ levels each showed a similar trend to total NAD, but did not reach statistical significance. Results are presented as an average fold increase in absorbance in treated wells as compared to an untreated control for 3 independent experiments $\pm$ S.D., with each experiment being performed in triplicate.

Effect of CM764 treatment on ATP level

The increase in NADt and reduction of MTT that were induced by CM764 treatment of SK-N-SH neuroblastoma cells indicate an overall increase in metabolic function. In order to test this hypothesis, we measured ATP levels in CM764-treated cells as compared to untreated cells using a fluorescent kit as described in Methods. Results are shown in Figure 8. Cells treated for 24 h with 10 μ M CM764 showed a modest yet statistically significant increase in ATP with average ATP levels being 13% higher in treated cells as compared to untreated cells (range of 8% to 23%). These data suggest that CM764 treatment does indeed stimulate metabolism, perhaps through induction of glycolysis rather than TCA cycle. TCA cycle stimulation would be expected to create a more significant increase in ATP as compared to the addition of glycolysis, which would produce only a modest increase in ATP production.

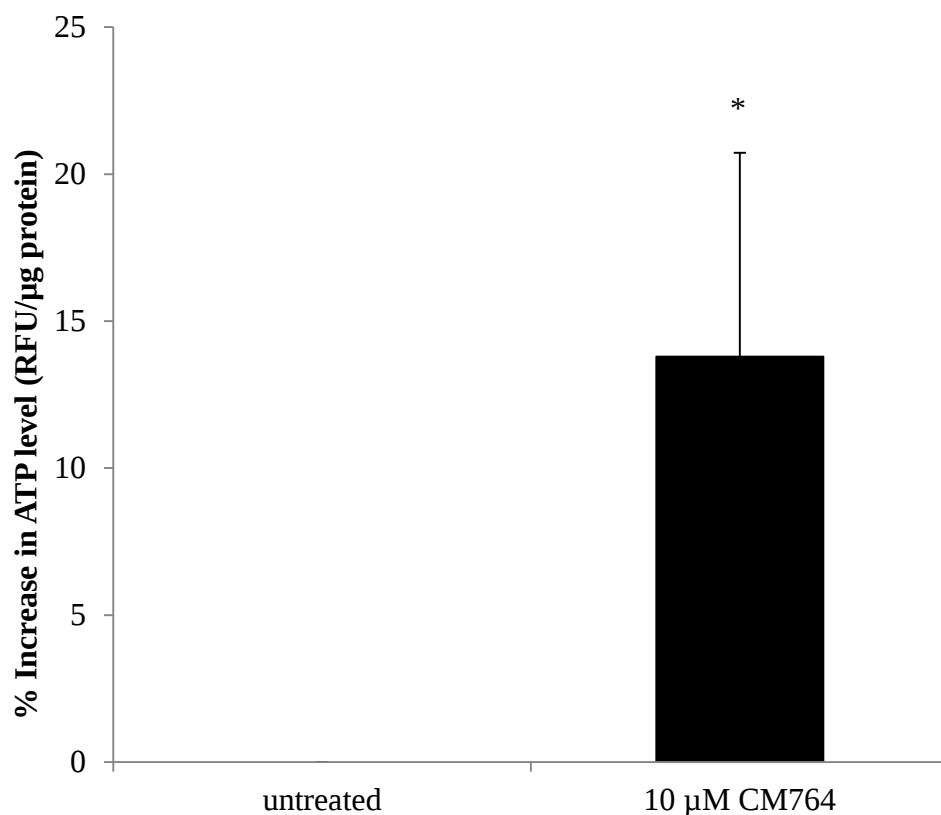


Figure 8. **Changes in ATP levels in response to CM764 exposure.** Cells were exposed to 10 μM CM764 for 24 h prior to ATP assay as described in Methods. Treatment with CM764 induced a ~13% increase in ATP level as compared to an untreated control (Student's t-test, * $p < 0.05$ ($p = 0.032$)). Results are presented as an average % change in ATP levels as determined by an increase in relative fluorescence normalized to protein concentration in treated cells compared to an untreated control for 3 independent experiments and controlled for cell number in 1 experiment for a total of 4 independent experiments $\pm$ S.D., with each experiment being performed in duplicate.

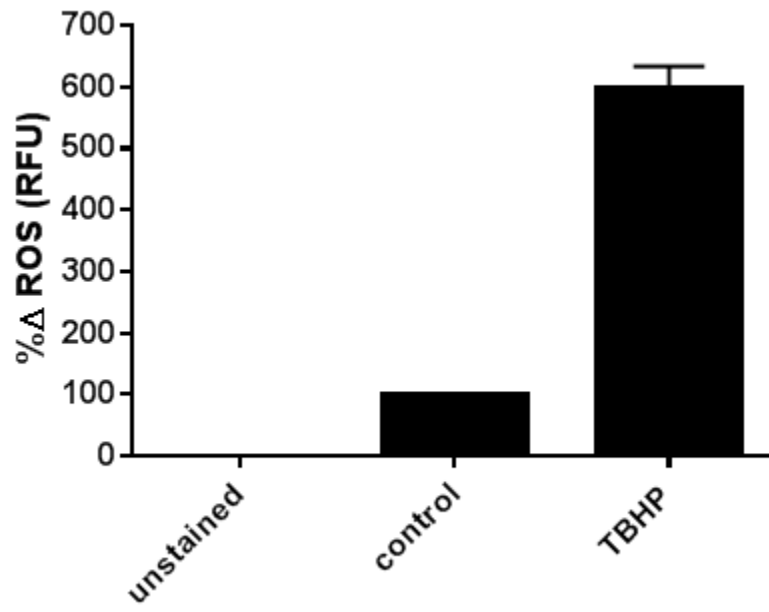
CM764-induced production of reactive oxygen species

Under glycolysis cells produce pyruvate, which can act as an antioxidant. Further, if some ATP production that results from oxidative phosphorylation in untreated cells were indeed resulting from glycolysis in CM764-treated cells as hypothesized above, then we would expect to see a decrease in reactive oxygen species generated from oxygen consumption in oxidative phosphorylation. In order to determine whether this effect could result from CM764 treatment, we measured ROS accumulation using the ROS-sensitive DCFDA dye. Cells were stained with DCFDA and then treated with 10 μ M CM764 for 4 h prior to measurement of fluorescence as described in Methods. Results are shown in Figure 9. To ensure effectiveness of the assay, *tert*-butyl hydrogen peroxide (TBHP) was used as a positive control (Panel A) as compared to untreated DCFDA-stained cells, which were normalized to 100% RFU. Basal fluorescence of unstained cells was subtracted from each measurement. As a negative control, treatment of SK-N-SH neuroblastoma with the antioxidant α -tocopherol (Vitamin E) showed a decrease in ROS of 76% as compared to an untreated control. Treatment with 10 μ M CM764 for just 4 h showed a decrease in ROS of 95% as compared to an untreated control. These data indicate a very significant decrease in ROS in response to treatment with CM764, indicating a strong antioxidant effect resultant from activation of the sigma-2 receptor through this mechanism. This may be due to induction of glycolysis, which could explain the antioxidant effect of CM764 treatment.

In order to determine whether the reduction in ROS induced by treatment with CM764 was a result of direct radical scavenging, as with α -tocopherol, the experiment was repeated using 10 μ M CM572. CM572 is a close structural analog of CM764, lacking the amine group on the fluorophenyl ring and instead having an isothiocyanate moiety on the heterocyclic ring system. A 10 μ M dose of CM572 did not induce an increase in MTT reduction as compared to

control cells, and also did not result in any significant change in level of ROS as compared to untreated cells even after 6 h of treatment (experiment was performed using 4 replicates per condition; data not shown). Thus the effect of CM764 on ROS is due to a signaling mechanism and not to direct radical scavenging.

A



B

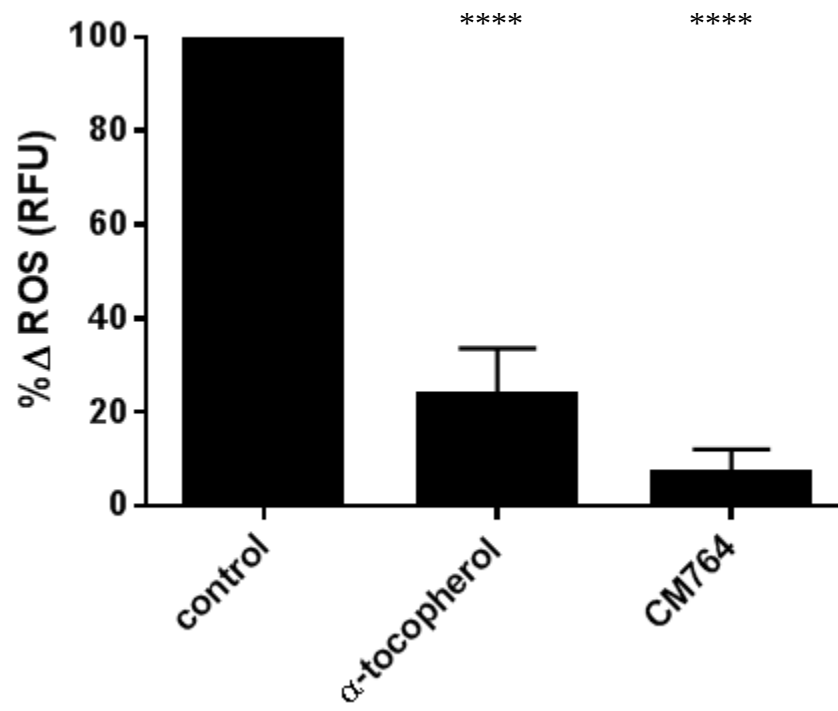
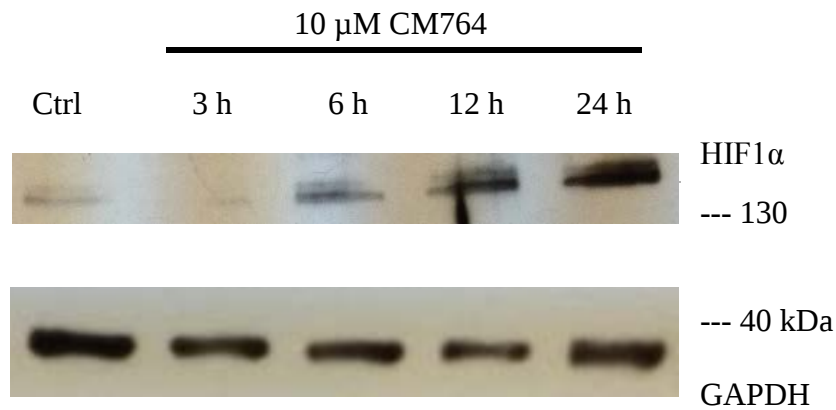


Figure 9. Effect of CM764 on reactive oxygen species levels in SK-N-SH neuroblastoma cells. Cells were stained with DCFDA prior to treatment with 10 μ M CM764 for 4 h, followed by fluorescence measurement as described in Methods. Panel A demonstrates the effectiveness of the assay, showing the effect of 500 μ M tert-butyl hydrogen peroxide (TBHP) as a positive control for the production of reactive oxygen species. Panel B shows the results of treatment with the antioxidant α -tocopherol (Vitamin E, 200 μ g/ml) and treatment with 10 μ M CM764. Treatment of SK-N-SH neuroblastoma cells with CM764 resulted in a marked decrease in reactive oxygen species that was more effective than that of α -tocopherol (95% and 76%, respectively). Results were highly significant (one-way ANOVA $F=238.4$, Dunnett's test for multiple comparisons as compared to the untreated control **** $p<0.0001$ for α -tocopherol, **** $p<0.0001$ for CM764). Results are presented as an average of the percent change in ROS levels achieved $\pm$ S.D. as compared to an untreated control normalized to 100% for three independent experiments, each experiment having four replicates per condition.

Effect of CM764 on HIF1 α protein level

A hallmark of cells undergoing glycolysis under normoxic conditions (aerobic glycolysis) is the expression of HIF1 α . A link between treatment of tumor cells with the sigma modulator rimcazole and increased expression of HIF1 α has been established previously (Achison et al. 2007). We therefore decided to investigate whether this transiently expressed protein might be stabilized or increasingly translated upon treatment with CM764. Cell lysates of SK-N-SH neuroblastoma were made after treatment with 10 μ M CM764 for the indicated length of time and were used to Western blot for presence of HIF1 α as described in Methods. A representative blot of HIF1 α and loading control GADPH are shown (Panel A) and quantified (Panel B) in Figure 10. Our results indicate a strong induction of HIF1 α , either by stabilization of the translated protein or by increased translation of mRNA, in response to treatment with CM764. Increased expression was most significant after 24 h, showing >14-fold induction, however expression was already >4-fold increased after only 6 h treatment. The observed induction of HIF1 α protein level is consistent with previous data that shows increased expression of HIF1 α in cells that preferentially employ glycolysis under conditions of normoxia (Dery et al., 2005; Kuschel et al., 2012). These data suggest that CM764 treatment does induce aerobic glycolysis in SK-N-SH neuroblastoma cells, consistent with our results from ROS and ATP studies described above.

A



B

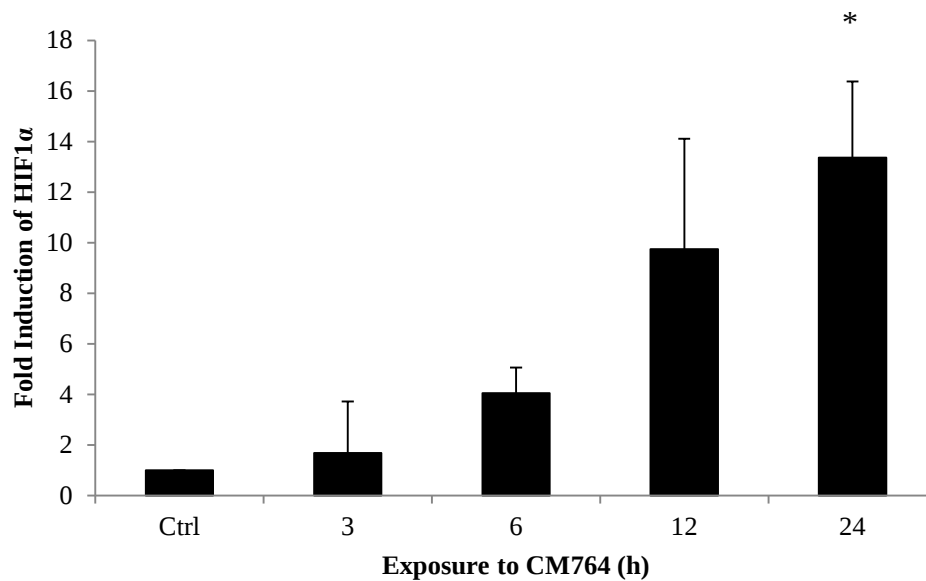


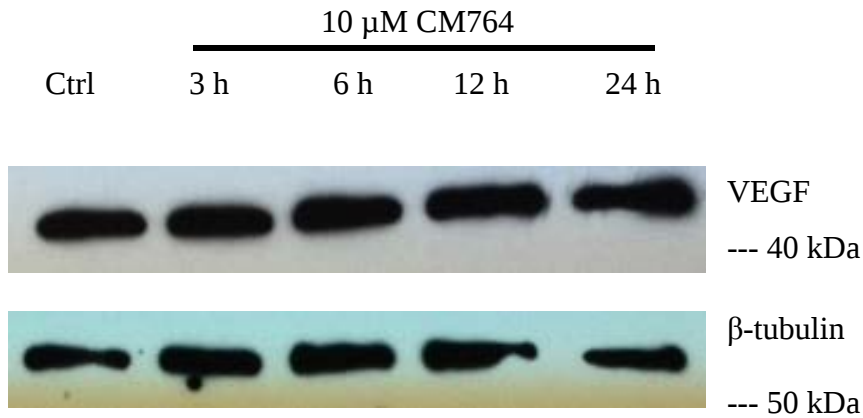
Figure 10. Effect of CM764 treatment on levels of HIF1 α in SK-N-SH neuroblastoma. Cell lysates of SK-N-SH neuroblastoma cells were made after treatment with 10 μ M CM764 for the indicated amount of time and were used for Western blotting for HIF1 α as described in Materials and Methods. Panel A: HIF1 α Western blot and GAPDH loading control. Panel B: Independent blots were quantified and ratio of HIF1 α /GAPDH determined and averaged for all experiments.

Data is expressed as fold increase in ratio relative to control treated without CM764. Treatment with CM764 resulted in an increase in HIF1 α level as early as 3 h after exposure (one-way ANOVA F=8.716, Dunnett's test for multiple comparisons as compared to untreated control *p<0.05). Results are an average of two independent experiments. A representative blot is shown.

Effect of CM764 on VEGF protein level

To confirm the activity of increased HIF1 α protein levels in SK-N-SH neuroblastoma cells in response to treatment with 10 μ M CM764, a transcriptional target of HIF1 α was measured in response to the same treatment. Vascular endothelial growth factor (VEGF) has been extensively described as a target of HIF1 α transcriptional regulation, and anti-VEGF therapy has been shown to impair ATP level (Curtarello et al., 2015). We therefore examined the effect of CM764 treatment on VEGF expression in SK-N-SH neuroblastoma. Results are shown (Panel A) and quantified (Panel B) in Figure 11. Our results confirm that protein level of VEGF is increased by over 4-fold in cells treated with 10 μ M CM764 as compared to untreated cells. This increased expression was most significant after 24 h treatment, lagging behind the observed increased expression of HIF1 α , which presumably regulated the VEGF transcription.

A



B

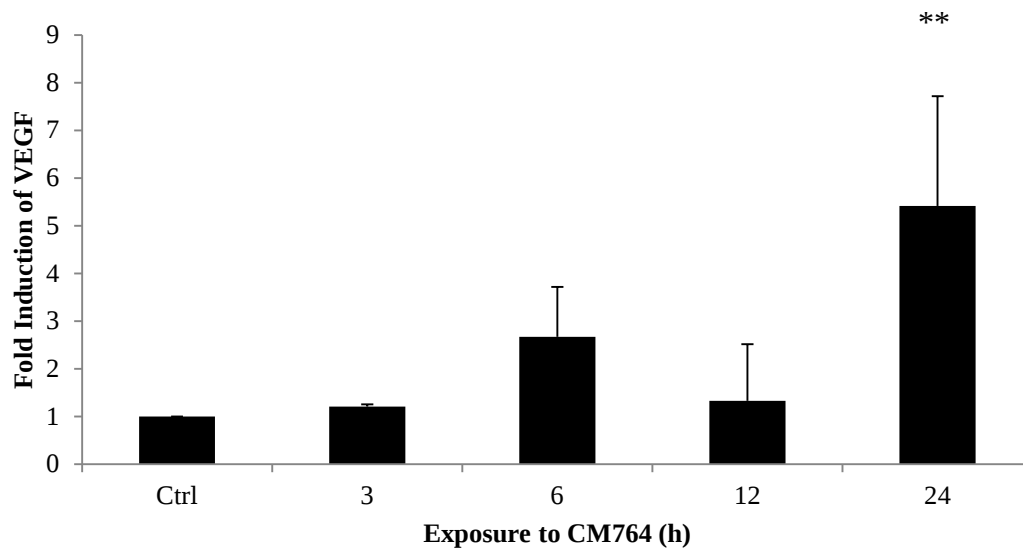


Figure 11. **Effect of CM764 treatment on expression of VEGF in SK-N-SH neuroblastoma.**

Cell lysates of SK-N-SH neuroblastoma cells were made after treatment with 10 μ M CM764 for indicated amount of time and were used for Western blotting for VEGF as described in Materials and Methods. Panel A: VEGF Western blot and β -tubulin loading control. Panel B:

Independent blots were quantified and ratio of VEGF/loading control (β -tubulin or GAPDH)

determined and averaged for all experiments. Data is expressed as fold increase in ratio relative to control treated without CM764. Treatment with CM764 induced protein expression of VEGF after 24 h exposure, following an earlier increase in HIF1 α expression as shown in Figure 10 (one-way ANOVA F=6.558, Dunnett's test for multiple comparisons as compared to untreated control **p<0.01). Results shown are an average of three independent experiments. A representative blot is shown.

Effect of CM764 across multiple cell types

In order to determine whether the increase in MTT reduction induced by CM764 treatment is exclusive to SK-N-SH neuroblastoma, the effect of this treatment was investigated in multiple cell lines. Human embryonic kidney (HEK293 T/17) cells and human osteosarcoma (MG-63) cells were treated with CM764 at the indicated doses for 24 h prior to MTT assay. Results are shown in Figure 12. Maximal stimulation achieved with 10 μ M CM764 in SK-N-SH neuroblastoma after 24 h treatment was matched using the same treatment in HEK293 T/17 cells and MG-63 cells. These data demonstrate that the increased reduction of MTT induced by CM764 activity at sigma-2 receptors is not exclusive to one cell type but rather may be present across a variety of cell types. Additionally, this effect is not exclusive to cancerous cell lines, as it is also effective in non-cancerous HEK cells, though there may be a slight trend toward less activity in the HEK cells compared to SK-N-SH and MG-63 cells at the lower concentrations. Together, these data suggest a role for sigma-2 receptors outside of cytotoxicity in cancer cells.

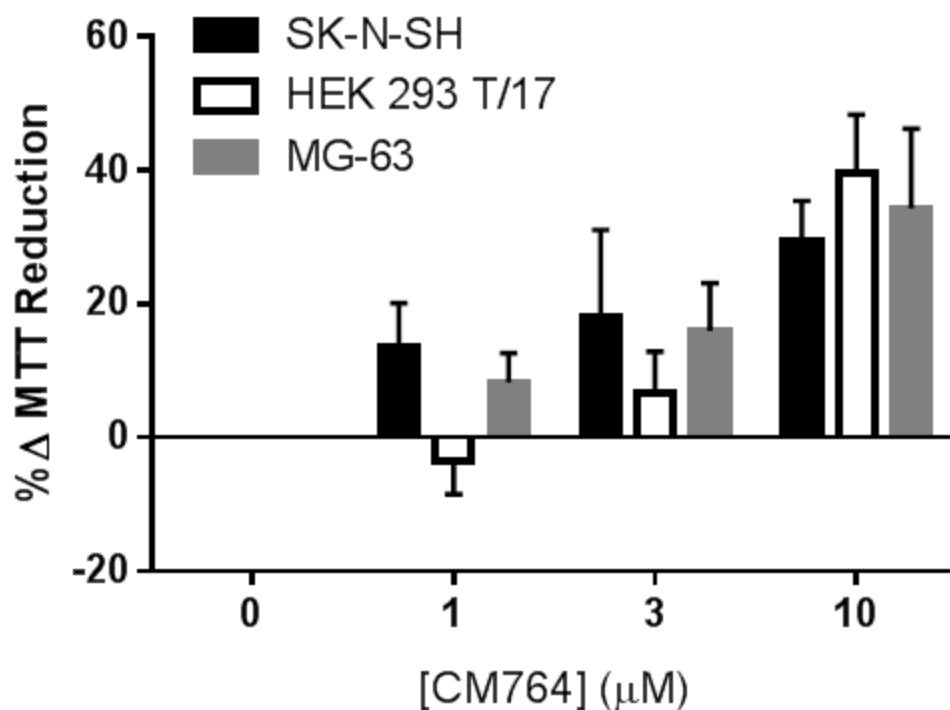


Figure 12. **Effect of CM764 treatment across cell types of different tissues.** Human SK-N-SH neuroblastoma, HEK293T human embryonic kidney cells, and MG-63 human osteosarcoma cells were treated with the indicated dose of CM764 for 24 h. MTT assay was then carried out as described in Materials and Methods. CM764 treatment induced an increase in reduction of MTT reagent in all three cell types examined. These data indicate that the effect of CM764 observed in the neuroblastoma is not a cell line-specific effect but is consistent across multiple cell types, both cancerous and non-cancerous. Results are shown as an average percent change in MTT reduction $\pm$ S.D. as compared to an untreated control in each cell line for at least three independent experiments, with each experiment performed with 5 replicates.

4.7 Discussion

Here we demonstrate a previously unreported metabolic regulatory role of sigma-2 receptors. CM764 binds sigma-2 receptors with high affinity ($K_i=3.5$ nM) and 25-fold selectivity over sigma-1 receptors (Figure 2), a significant increase in selectivity over the parent compound SN79. Since sigma-2 receptor activation has traditionally been associated with a reduction in cell viability, we used the MTT assay to measure potential loss of cell viability in response to CM764 exposure. Interestingly, treatment of SK-N-SH neuroblastoma cells with CM764 resulted in a significant increase in MTT reduction compared to untreated cells (Figure 3). This could result from cellular proliferation, stimulation of oxidoreductase activity, or both. Results from CyQUANT cell proliferation assays indicated no change in proliferation or DNA replication in response to treatment with CM764, suggesting a strictly metabolic effect (Figure 4).

This stimulative effect on MTT reduction could be attenuated by sigma-2 receptor antagonists with and without measurable affinity at sigma-1 receptors, but could not be replicated by sigma-1 receptor activation by sigma-1-selective agonist (+)-pentazocine. All sigma-2 receptor modulators investigated attenuated CM764-induced stimulation of MTT reduction while no effect on MTT reduction was observed for each antagonist independently (Figure 5). Specifically of note is the attenuation of the CM764-induced increase in MTT reduction by antagonism with CM572, which has high affinity for the sigma-2 receptor ($K_i=15$ nM) but no significant affinity for the sigma-1 receptor ($K_i \geq 10,000$ nM). These data indicate that this is a novel sigma-2-mediated effect that is independent of sigma-1 receptor activation.

CM764 injection onto fura-2-loaded SK-N-SH neuroblastoma cells induced an immediate, robust, transient calcium response, consistent with previous reports of sigma-2

receptor activation (Figure 6). This observation lends further support to the action of CM764 at sigma-2 receptors, as well as confirms that this calcium response can be uncoupled from induction of apoptosis. As changes in calcium level are also known to be early steps in several important metabolic pathways, this data is also consistent with a metabolic effect resultant from sigma-2 receptor activation by CM764.

As reduction of MTT is known to be NADH-dependent, NADH/NAD⁺ levels were investigated in response to CM764 treatment. CM764 treatment induced an increase in total NAD (NADH + NAD⁺) compared to untreated cells, indicating that levels of this cofactor are increased as a result of sigma-2 receptor activation by CM764 (Figure 7). However, the increase was modest, and while likely not limiting, the increase in NADH induced by CM764 activation of sigma-2 receptors is unlikely to be the driving factor for the observed increase in MTT reduction. The increase in NADt observed in response to CM764 treatment indicated moderate stimulation of a metabolic pathway, perhaps less efficient than an overall stimulation of oxidative phosphorylation.

CM764 also induced an increase in ATP levels of treated cells compared to untreated cells, further indicating a metabolic stimulation (Figure 8). However, this increase was modest (~13% increase in ATP level as compared to untreated control). CM764 also induced extensive reduction of ROS in SK-N-SH neuroblastoma as compared to untreated cells (Figure 9B). This effect was rapid, with near-complete elimination of basal levels of ROS occurring within 4 h of treatment. Interestingly, Ostensfeld et al. showed that the sigma-2 agonist siramesine caused an *increase* in ROS in WEHI-S fibrosarcoma and MCF-7 breast tumor cells that contributed to cell death (Ostensfeld et al., 2005). In addition, methamphetamine may induce ROS production and cell death in differentiated NG108-15 neuroblastoma-glioma hybrid cells via activation of sigma-

2 receptors that is antagonized by SN79 (Kaushal et al., 2014). These opposing effects on ROS by sigma-2 ligands are consistent with the notion of bifurcating toxic and non-toxic pathways mediated by sigma-2 receptors.

The modest increase in ATP levels and NADH could possibly be explained by the hypothesis that CM764 induces stimulation of glycolysis, rather than oxidative phosphorylation. Stimulation of glycolysis would add only a small amount of both of these factors in comparison to the highly efficient production of ATP and increased reduction of NAD^+ occurring by oxidative phosphorylation. The reduction in ROS observed in response to treatment with CM764 is also consistent with a hypothesis that CM764 induces stimulation of glycolysis, as the glycolytic pathway can reduce ROS in two ways. First, a product of glycolysis is pyruvate, which has antioxidant properties. Second, preferential use of glycolysis for ATP production can reduce oxidative phosphorylation, which decreases the amount of oxygen channeled into superoxide formation. Thus induction of glycolysis by CM764 is consistent with the observed ATP, NADH/NAD⁺, and ROS data.

CM764 induced an increase in HIF1 α protein level, known to be stabilized under conditions of aerobic glycolysis (Figure 10). Induction of HIF1 α by CM764 treatment is consistent with activation of glycolysis, and has been shown to be induced without hypoxia in such cases. Further, CM764 treatment also induced expression of VEGF, a transcriptional target of HIF1 α (Figure 11). This suggests that the HIF1 α protein level increase is active protein, as the increased expression in VEGF followed ~18 h after the first significant observed increase in HIF1 α , which occurred after 6 h of treatment. Taken together, these data suggest that CM764 induces a sigma-2-modulated stimulation of cellular glycolysis under normoxia. This is a novel function of the sigma-2 receptor that is more consistent with observations of sigma-2 receptor

expression in rapidly proliferating non-cancerous cells such as HEK (Johannessen et al. 2009; Xu et al. 2011) and COS (Monassier et al. 2007; Johannessen et al. 2009) cells, as well as in tumor cells, as it may offer protection against damage from reactive oxygen species. Consistent with this hypothesis, CM764 induced an increase in MTT reduction that matched that induced in SK-N-SH neuroblastoma cells when dosed in human embryonic kidney cells and human osteosarcoma cells indicating that this effect is present across a variety of tumor and noncancerous cell types.

Overexpression of HIF1 α is a common observation in many cancers (Zhong et al. 1999; Talks et al. 2000). HIF1 α allows cells to overcome hypoxic conditions, as are common in the core of tumors and for metastases that may not have an established blood supply. Further, through its transcriptional activation of VEGF, HIF1 α promotes angiogenesis and therefore oxygen supply for metastatic sites (Lin et al. 2004; Liang et al. 2008; Kim et al. 2014). The novel function of the sigma-2 receptor that our study has revealed is consistent with this role of HIF1 α in rapidly proliferating cancer cells and allows for congruous explanation of the high expression of sigma-2 receptors in the same. Interestingly, sigma-1 receptors have also been linked to HIF1 α . Blockade of sigma-1 receptors by the putative antagonist rimcazole in normoxic colorectal and mammary carcinoma cells increases HIF1 α , but with a cytotoxic effect in this case (Achison et al., 2007). Thus, regulation of HIF1 α by sigma-1 and sigma-2 receptor subtypes may be complex, with different viability outcomes depending on cell type and sigma ligand.

It has been proposed that the sigma-2 receptor binding site resides within the PGRMC1 protein complex (Xu et al. 2011). However, whether the sigma-2 receptor and PGRMC1 are one in the same molecule remains controversial (Abate et al., 2015; Chu et al., 2015). Furthermore, the physiological effects of PGRMC1 activation have largely been shown to promote cell

survival and inhibit apoptosis, which is in direct contrast with classical pro-apoptotic models of sigma-2 receptor activation (Losel et al. 2008; Neubauer et al. 2009; Ahmed et al. 2010; Peluso et al. 2010). Interestingly, activation of PGRMC1 by cell-impermeable progesterone was shown to significantly stimulate VEGF gene expression in MCF-7 cells (Neubauer et al., 2009). The data shown in Figure 5 and the lack of (+)-pentazocine activity show that the pharmacological profile of CM764-induced stimulation of MTT reduction is consistent with mediation by sigma-2 receptors. Yet stimulation of VEGF expression by CM764 resembles an effect of PGMRC1 activation (Neubauer et al., 2009). In view of the controversy over the identity of the sigma-2 receptor, there are three possible explanations for the data reported here: 1) the sigma-2 receptor is the binding site of PGRMC1, with bifurcating apoptotic and non-apoptotic pathways being initiated from a single sigma-2/PGRMC1 receptor entity, depending on the specific ligand involved, 2) PGRMC1 and sigma-2 receptors are distinct molecules, but have overlapping pharmacological profiles allowing some sigma-2 ligands to have effects at PGRMC1, or 3) PGRMC1 and sigma-2 receptors are distinct entities with distinct pharmacological profiles, but binding of some compounds to sigma-2 receptors results in complexation with PGRMC1 and activation of non-toxic PGRMC1 signaling events. Distinguishing these possibilities will require further investigation. It should be mentioned here that several other SN79 analogs with high sigma-2 affinity are also able to stimulate MTT reduction (in preparation). In deference to the observed pharmacological profile, we have referred to the effects described herein as being sigma-2 receptor-mediated.

In conclusion, this study unveils a novel metabolically stimulative, non-toxic sigma-2 receptor function. It is consistent with an evolutionary benefit to upregulation of sigma-2 receptors in cancer cells and rapidly proliferating noncancerous cells, suggesting potential for

protection against oxidative damage, hypoxic conditions, and stimulation of angiogenesis via VEGF production.

4.8 Supplementary Figures

Supplemental Figure 1:

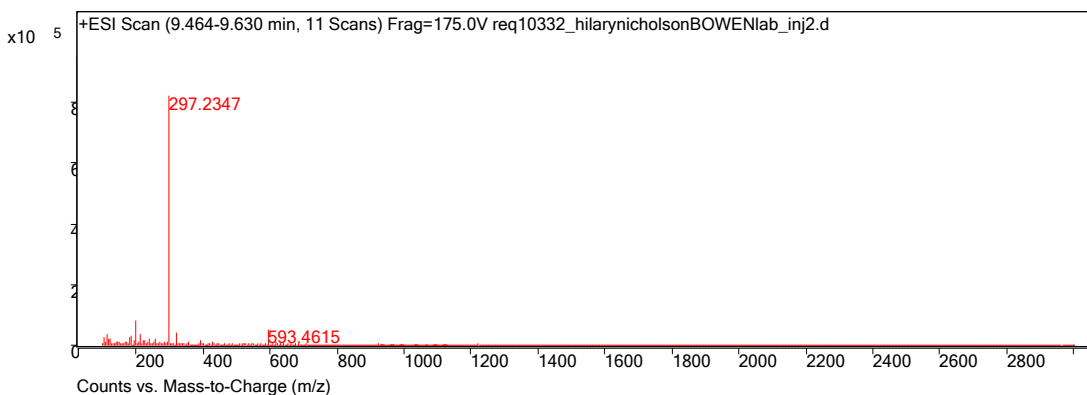
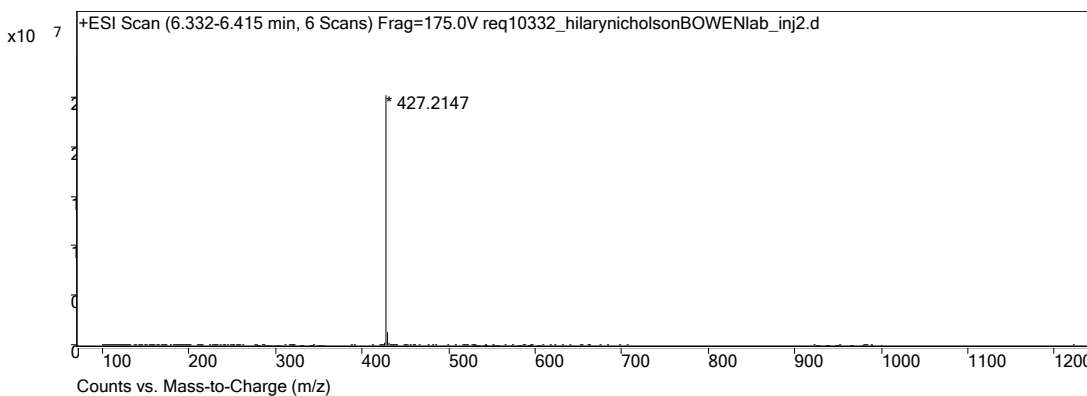
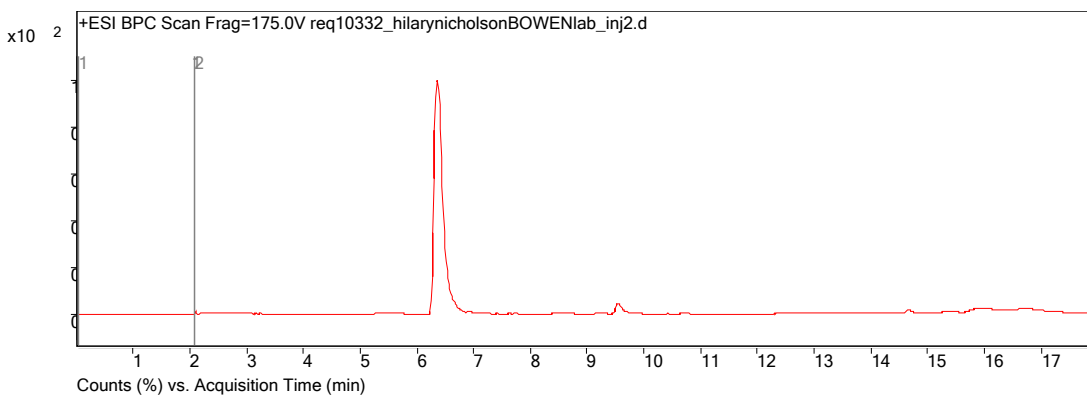
Methods for extraction experiment:

SK-N-SH cells were plated at 60,000 cells per well in 24 well plates. Other wells were left without cells. Wells (with or without cells) were incubated in 1 ml of complete cell culture medium (with 10% FBS) containing 30 μ M CM764 for 0 h (control) or for 24 h in a humidified atmosphere at 37°C and 5% CO₂. For the 0 h control, CM572 was added to the well, mixed, and media immediately removed for extraction. The culture medium (1 ml) was removed from the wells and placed in a glass extraction tube on ice. The pH was brought from pH 7.4 to pH 8.5 using NaOH. One ml of incubation mixture was extracted with 1 ml of ethyl acetate by vigorous vortexing. After phases separated, 0.7 ml of organic phase was removed and evaporated under nitrogen stream. The residue was reconstituted into 0.2 ml of methanol for HPLC/MS analysis. Media without cells that contained no CM764 was extracted to assess components in the ethyl acetate extract that are due to just the media. The analyses were carried out in the Brown University Chemistry Department analytical core facility by Dr. Tun-Li Shen.

Summary of Results:

There was some concern about oxidative breakdown of CM764 over the 24 h timeline of the experiments due to the potentially sensitive 1,2-diaminophenyl ring moiety that could be oxidized to compounds analogous to an o-quinone. We incubated 30 μ M CM764 in normal culture media under the conditions of cell incubation for up to 24 h, with and without the presence of SK-N-SH cells. We followed this by extraction of the media and analysis by LC/MS. The results of these experiments are shown in Supplemental Figures 2-4. There

appears to be no significant degradation of the compound in 24 h compared to either compound incubated for “zero” time and extracted out of media or to pure (authentic) compound from stock stored in DMSO. The major peak under the various conditions had the correct retention time and mass of the pure compound. There were no additional peaks present that were not already present in media without CM764. The only potentially questionable peak was a very small peak of RT=6.16-6.27, m/z=429.23 that was present at 24 h, not seen at 0 h, and not seen without cells (see Supplemental Figure 4). This could either be a component from the cells or a product related to CM764. The latter is unlikely since the increased mass by 2 units could only indicate reduction, yet the compound is slightly more polar than CM764. The difference in mass by only 2 units higher shows that this is not an oxidation product of CM764, and most likely a compound coming from the cells. These results show that CM764 is apparently stable under the conditions of the experiments described.

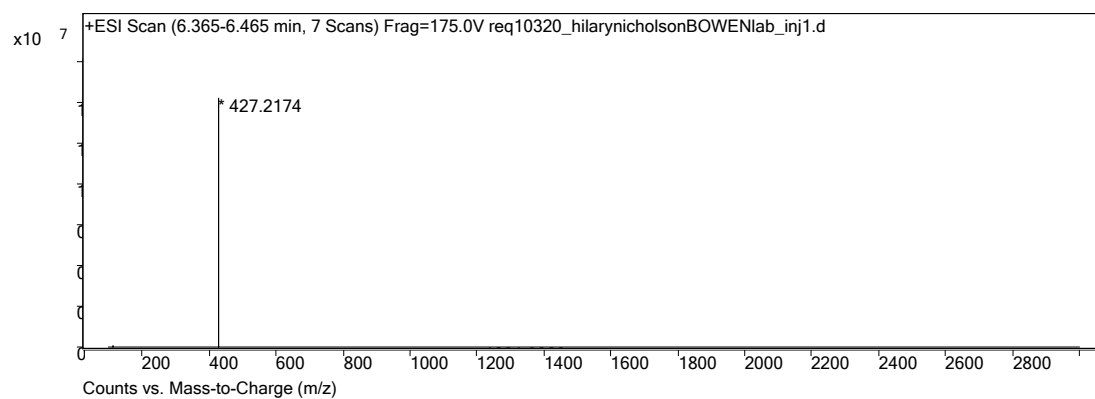
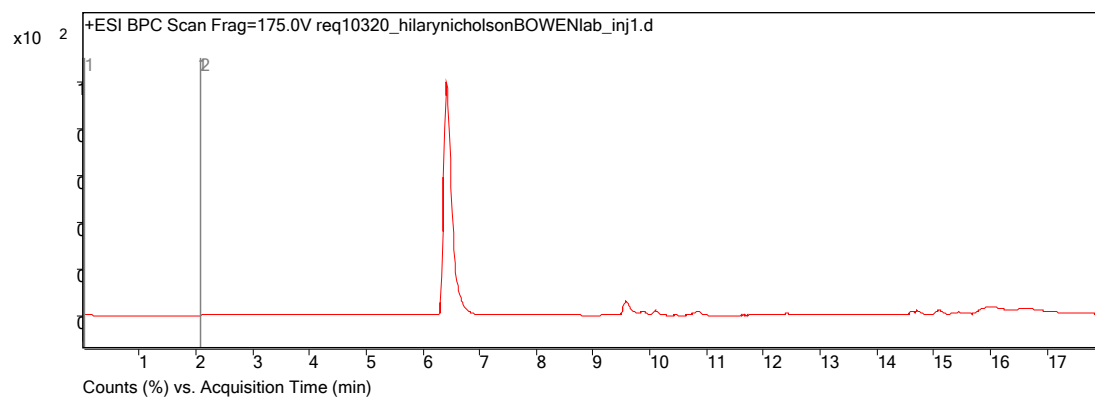


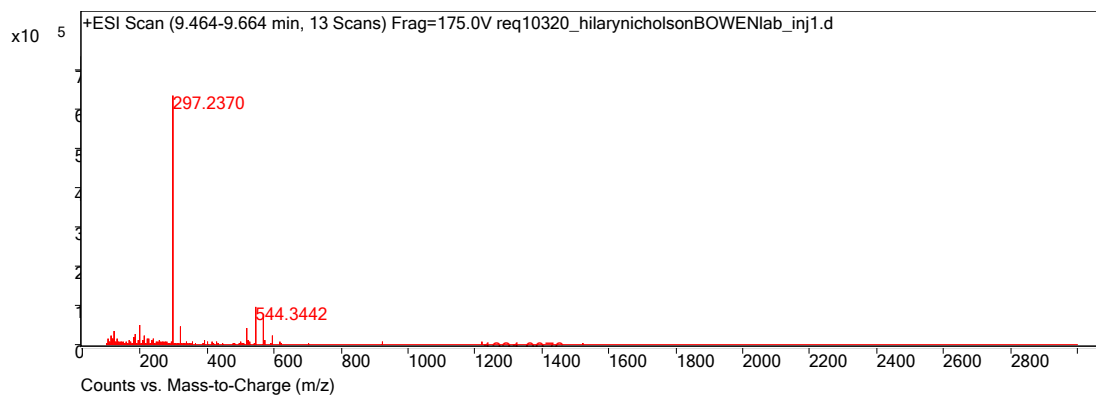
Supplemental Figure 2: Pure CM764 from DMSO stock

CM764 m/z = 427.21, RT = 6.33-6.41 min

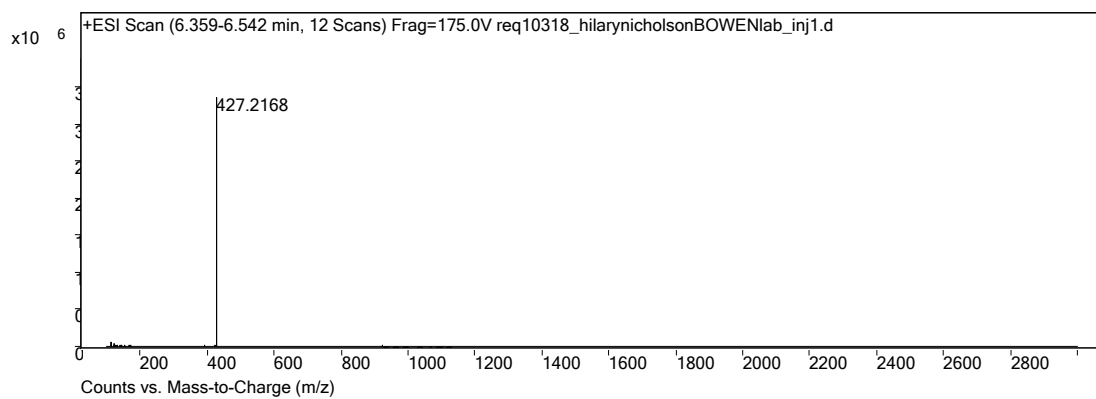
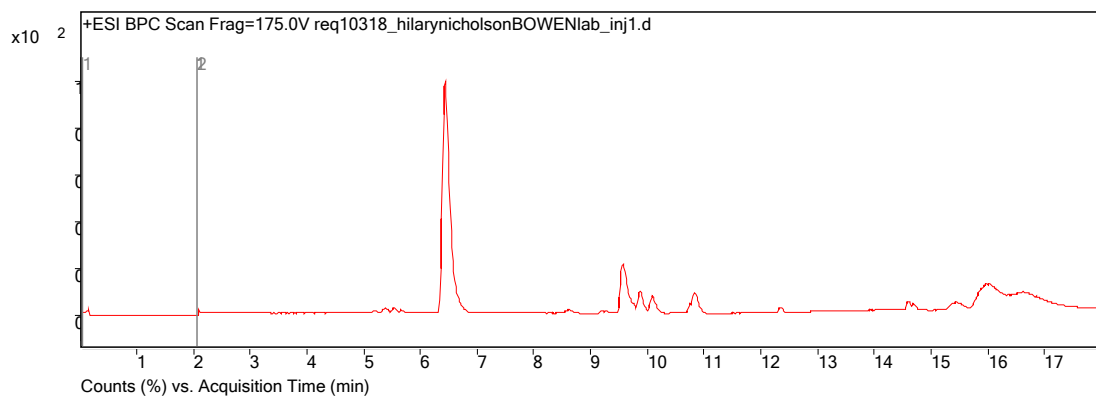
Small peak at RT=9.46-9.63, m/z = 297.23 is not an impurity in CM764, as it has appeared in all chromatographs, even those without CM764 present.

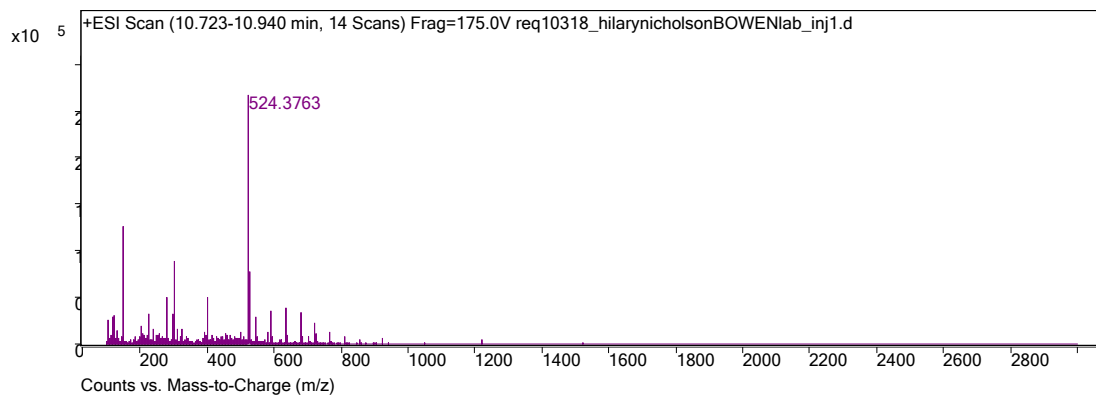
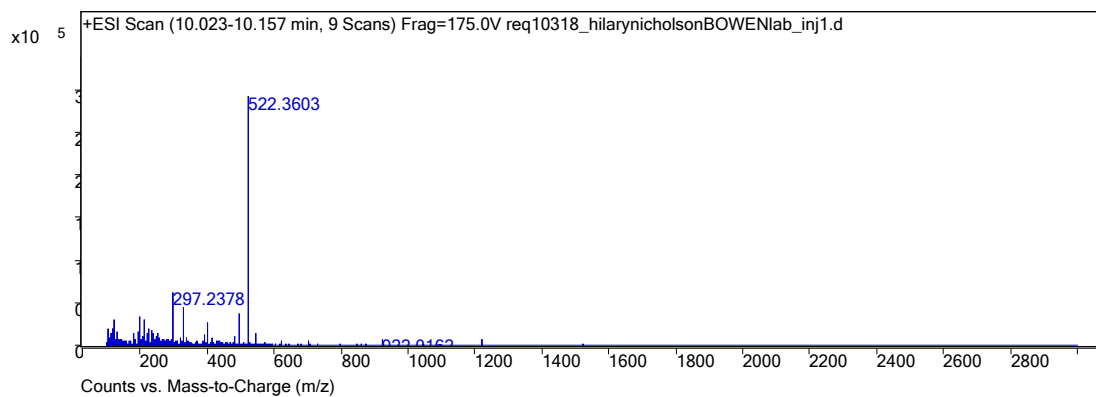
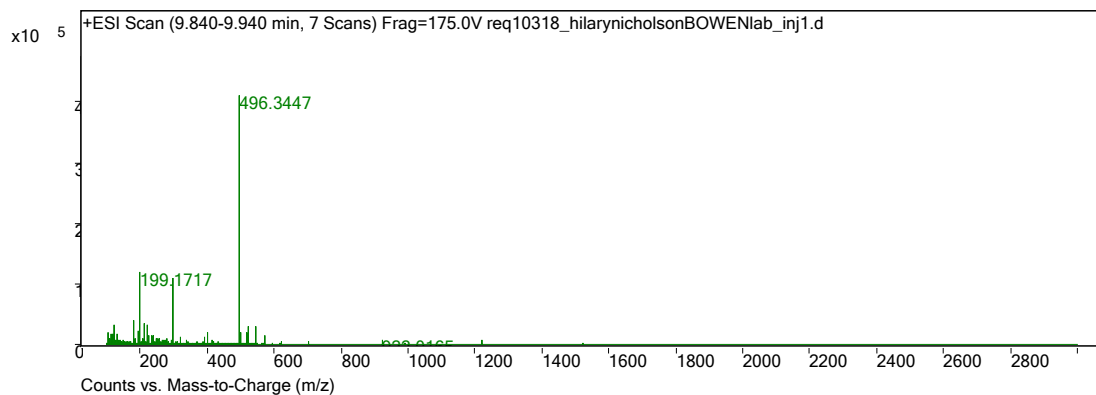
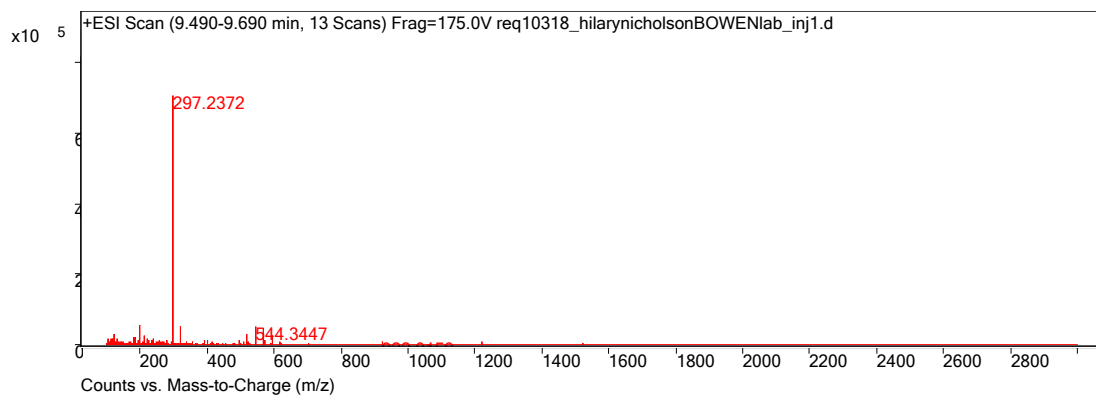
A





B





Supplemental Figure 3: CM764 (30 μ M) incubated in culture media without cells for 0 h or 24 h

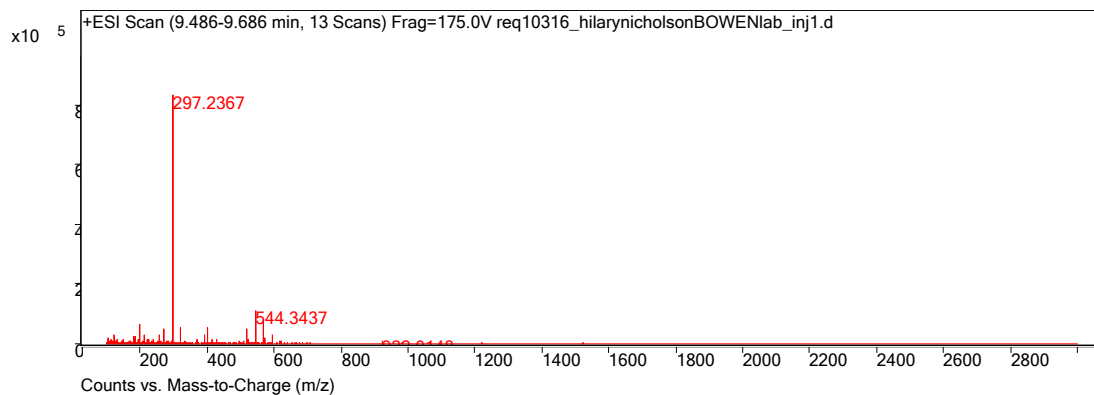
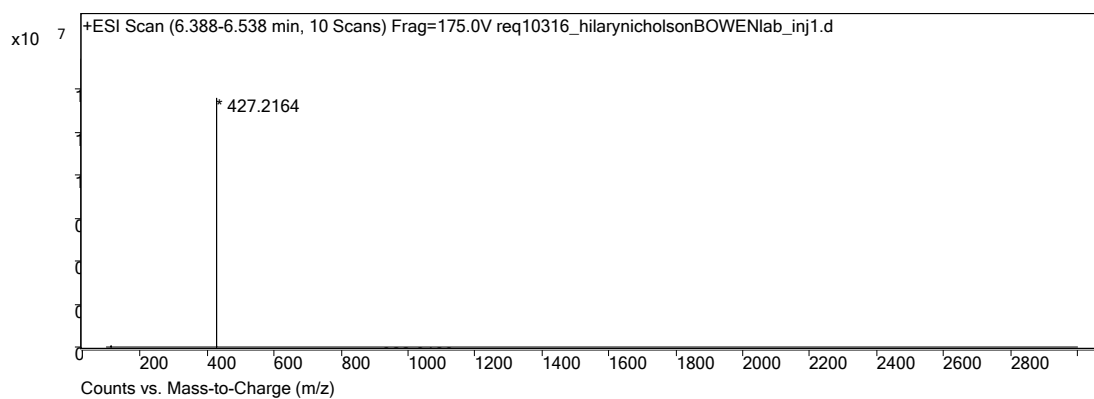
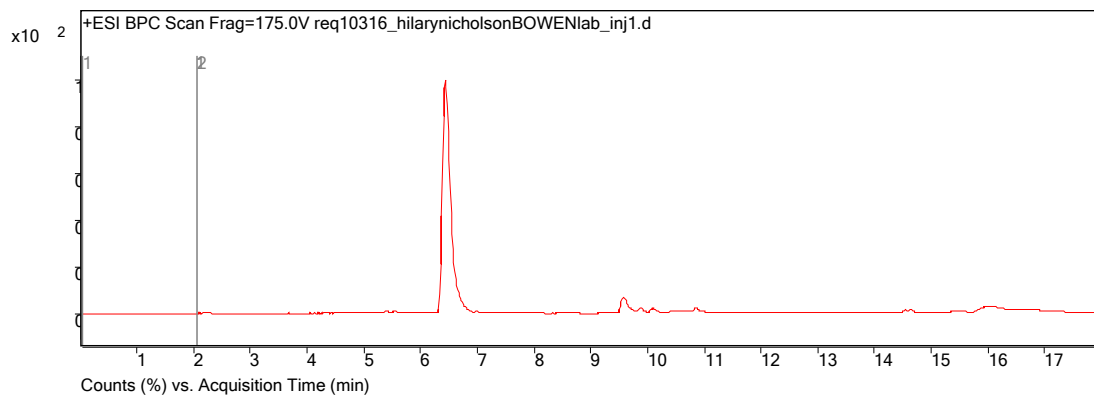
A) Incubation for 0 h (compound added to media and immediately extracted), without cells

B) Incubation for 24 h in media at 37°C in 5% CO₂ humidified incubator, with no cells present

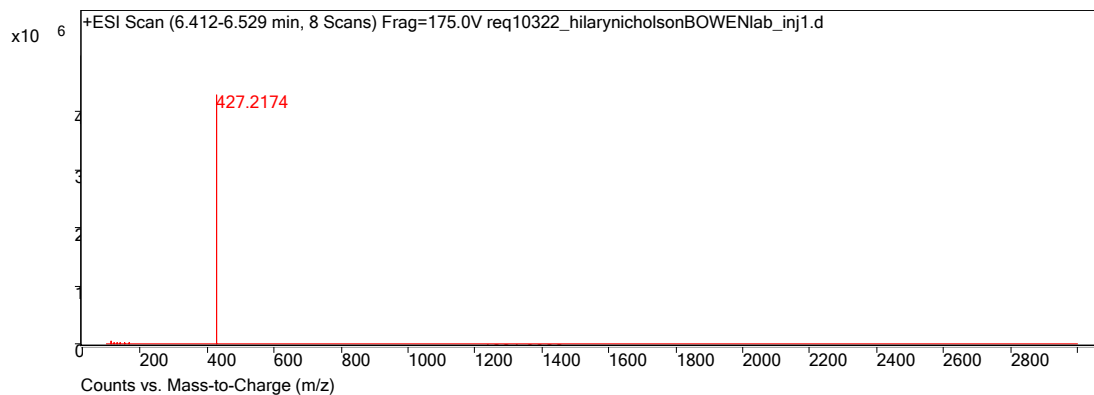
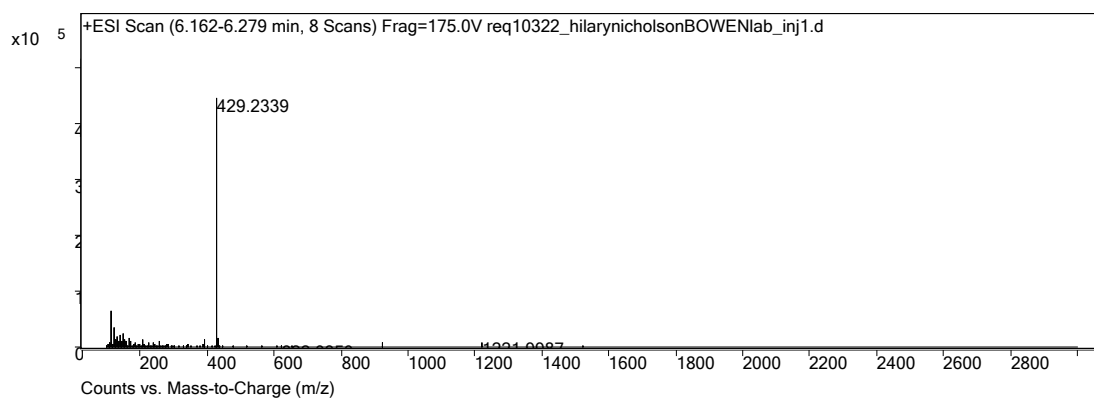
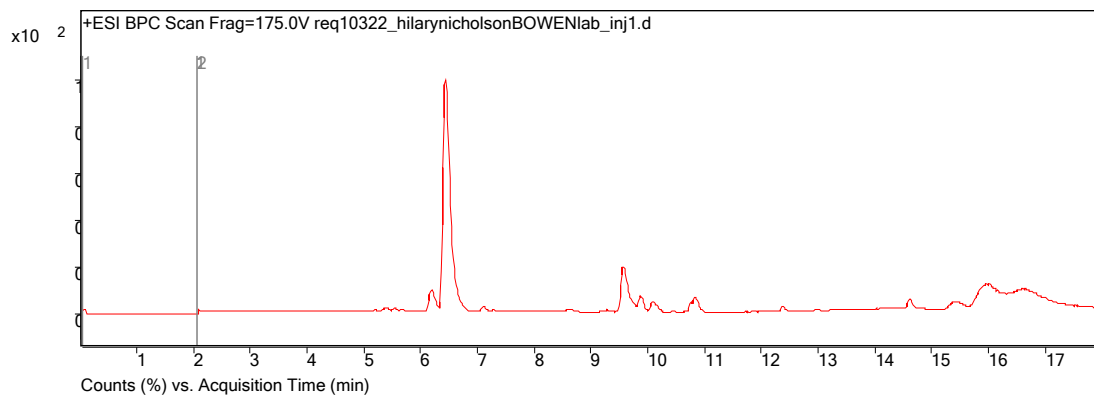
CM764 m/z = 427.21, RT = 6.36-6.46 min

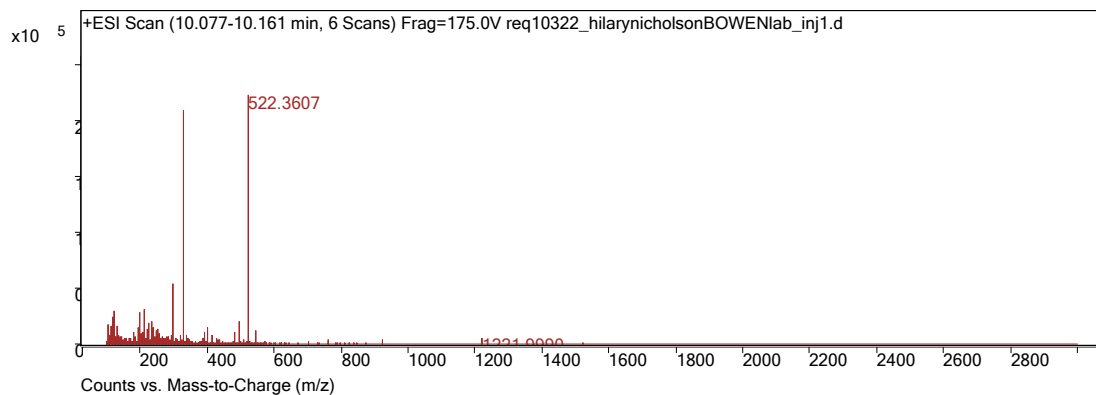
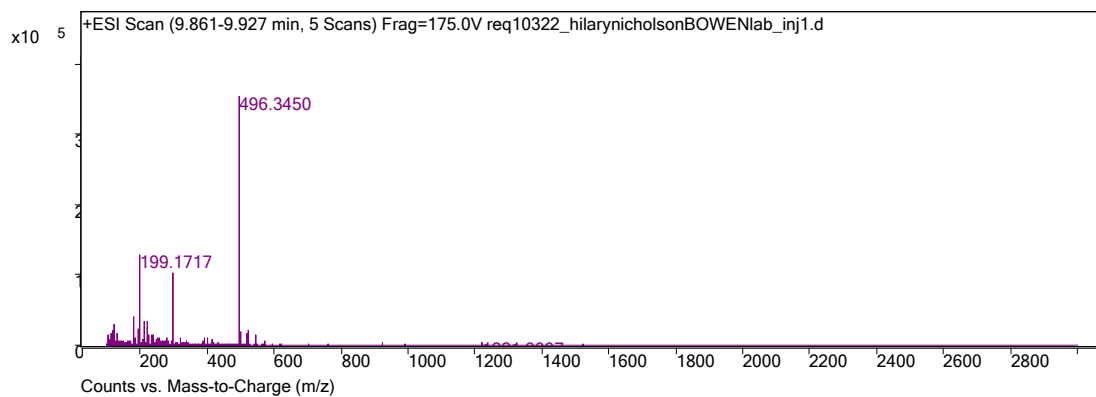
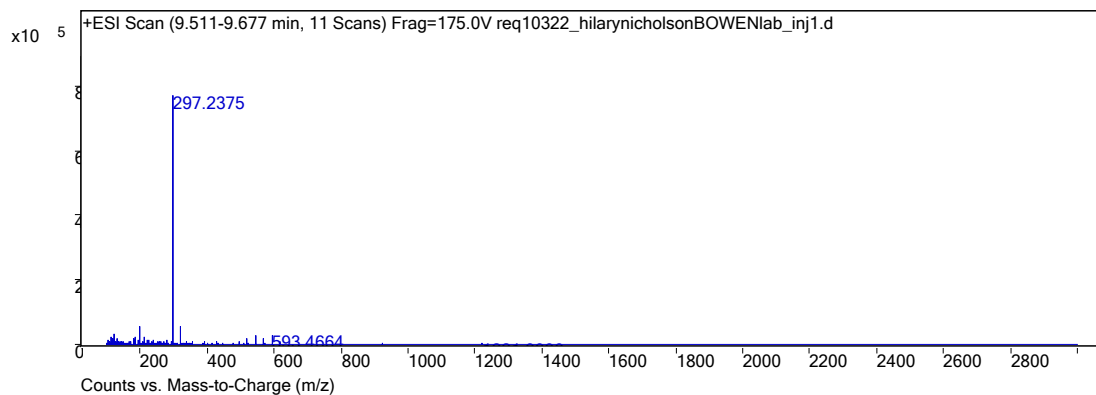
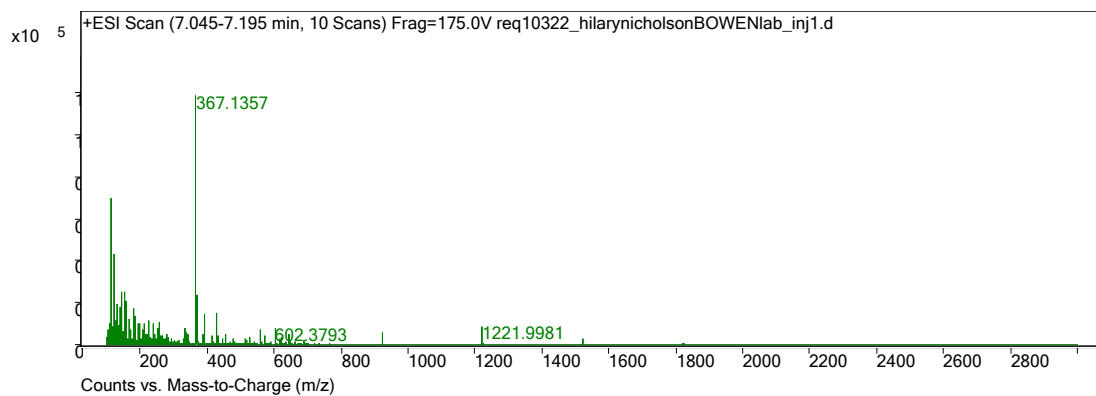
The small peaks in the RT range 9.49 – 10.94 are not related to CM764 as they appear in extracted media that has not been exposed to CM764.

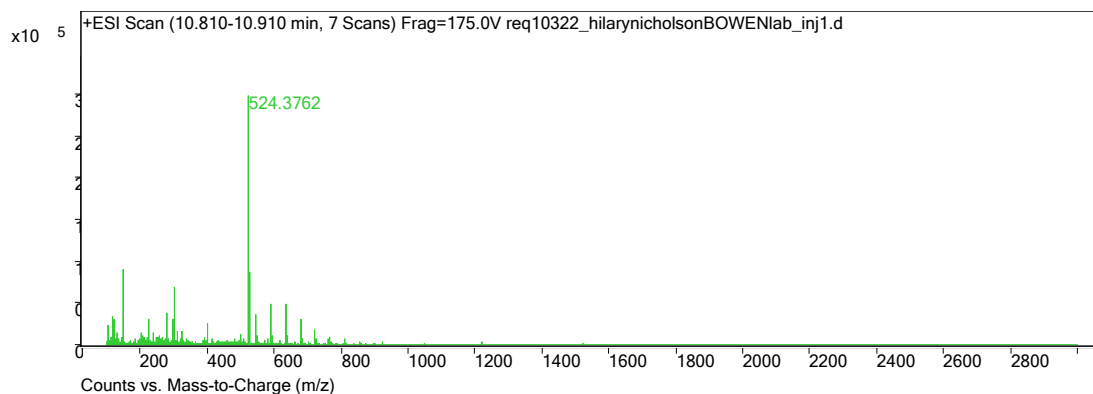
A



B







Supplemental Figure 4: CM764 (30 μ M) incubated in media with cells for 0 h or 24 h

- A) Incubation for 0 h with cells present (compound added, mixed, and immediately extracted)
- B) Incubation for 24 h in media at 37°C in 5% CO₂ humidified incubator, with cells present

CM764 m/z = 427.21, RT = 6.41-6.53 min

The small peaks in the RT range 9.51 – 10.91 are not related to CM764 as they appear in extracted media that has not been exposed to CM764.

The small peak at RT=6.16-6.27, m/z =429.23 was not seen without cells (Supplemental Figure 2) and could be either be a component from the cells or a product related to CM764. The latter is unlikely since the increased mass by 2 units could only indicate reduction, yet the compound is slightly more polar than CM764.

4.9 References

- Abate C, Niso M, Infantino V, Menga A, and Berardi F (2015) Elements in support of the 'non-identity' of the PGRMC1 protein with the sigma(2) receptor. *Eur J Pharmacol* **758**: 16-23.
- Achison M, Boylan MT, Hupp TR, and Spruce BA (2007) HIF-1alpha contributes to tumour-selective killing by the sigma receptor antagonist rimcazole. *Oncogene* **26**(8): 1137-1146.
- Ahmed IS, Rohe HJ, Twist KE, Mattingly MN, and Craven RJ (2010) Progesterone Receptor Membrane Component 1 (Pgrmc1): A heme-1 domain protein that promotes tumorigenesis and is Inhibited by a small molecule. *J Pharmacol Exp Ther* **333**(2): 564-573.
- Berridge MV, Herst PM, and Tan AS (2005) Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnol Annual Rev* **11**: 127-152.
- Cassano G, Gasparre G, Niso M, Contino M, Scalera V, and Colabufo NA (2009). F281, synthetic agonist of the sigma-2 receptor, induces Ca^{2+} efflux from the endoplasmic reticulum and mitochondria in SK-N-SH cells. *Cell Calcium* **45**(4): 340-345.
- Cesen MH, Repnik U, Turk V, and Turk B (2013). Siramesine triggers cell death through destabilisation of mitochondria, but not lysosomes. *Cell Death & Disease* **4**.
- Chaki S, Tanaka M, Muramatsu M, and Otomo S (1994). NE-100, a novel potent sigma-ligand, preferentially binds to sigma(1) binding-sites in guinea-pig brain. *Eur J Pharmacol* **251**(1): R1-R2.
- Chu U, Mavlyutov T, Chu ML, Yang H, Mesangeau C, McCurdy C, Guo LW, and Ruoho A (2015) The 18 kDa sigma-2 receptor and PGRMC1 are derived from separate genes. *FASEB J* **29**(1).

- Crawford K W and Bowen WD (2002) Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines. *Cancer Res* **62**(1): 313-322.
- Curtarello M, Zulato E, Nardo G, Valtorta S, Guzzo G, Rossi E, Esposito G, Msaki A, Pasto A, Rasola A, Persano L, Ciccarese F, Bertorelle R, Todde S, Plebani M, Schroer H, Walenta S, Mueller-Klieser W, Amadori A, Moresco RM, and Indraccolo S (2015) VEGF-targeted therapy stably modulates the glycolytic phenotype of tumor cells. *Cancer Res* **75**(1):120-133.
- Dery MC, Michaud MD, and Richard DE (2005) Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. *IJBCB* **37**: 535-540.
- Garcia D and Bowen WD (2010) Sigma-2 receptor-mediated apoptosis and calcium signaling: Are they bifurcating pathways? Society for Neuroscience, San Diego, CA, Meeting Planner. Program No. 470.17.
- Garcia DR (2012) Sigma-2 receptor-mediated cytotoxicity and calcium signaling: Evidence for bifurcating pathways. PhD, Brown University.
- Hayashi T and Su T P (2003) Intracellular dynamics of sigma-1 receptors (sigma(1) binding sites) in NG108-15 cells. *J Pharmacol Exp Ther* **306**(2): 726-733.
- Hayashi T and Su T P (2007) Sigma-1 receptor chaperones at the ER-Mitochondrion interface regulate Ca²⁺ signaling and cell survival. *Cell* **131**(3): 596-610.
- Hazelwood S and Bowen W D (2006) Sigma-2 receptor-mediated apoptosis in human SK-N-SH neuroblastoma cells: Role of lipid rafts, caspases, and mitochondrial depolarization. American Association for Cancer Research Annual Meeting, Washington, D.C.

- Hellewell S B and Bowen W D (1990) A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Res* **527**(2): 244-253.
- Hellewell S B, Bruce A, Feinstein G, Orringer J, Williams W, and Bowen W D (1994) Rat liver and kidney contain high densities of sigma 1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur J Pharmacol* **268**(1): 9-18.
- Johannessen M, Ramachandran S, Riemer L, Ramos-Serrano A, Ruoho A E, and Jackson M B (2009) Voltage-gated sodium channel modulation by sigma-receptors in cardiac myocytes and heterologous systems. *Am J Physiol Cell Physiol* **296**(5): C1049-1057.
- Kaushal N, Robson M J, Rosen A, McCurdy C R, and Matsumoto R R (2014) Neuroprotective targets through which 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79), a sigma receptor ligand, mitigates the effects of methamphetamine in vitro. *Eur J Pharmacol* **724**: 193-203.
- Kaushal N, Robson M J, Vinnakota H, Narayanan S, Avery B A, McCurdy C R, and Matsumoto R R (2011) Synthesis and pharmacological evaluation of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79), a cocaine antagonist, in rodents. *AAPS J* **13**(3): 336-346.
- Kaushal N, Seminerio M J, Robson M J, McCurdy C R, and Matsumoto R R (2012) Pharmacological evaluation of SN79, a sigma (sigma) receptor ligand, against methamphetamine-induced neurotoxicity in vivo. *Eur Neuropsychopharmacol*.

- Kim A, Im M, Yim N H, and Ma J Y (2014) Reduction of metastatic and angiogenic potency of malignant cancer by *Eupatorium fortunei* via suppression of MMP-9 activity and VEGF production. *Sci Rep* **4**: 6994.
- Kuschel A, Simon P, and Tug S (2012) Functional regulation of HIF-1 α under normoxia--is there more than post-translational regulation? *J Cell Physiol* **227**(2): 514-524.
- Liang X, Yang D, Hu J, Hao X, Gao J, and Mao Z (2008) Hypoxia inducible factor-alpha expression correlates with vascular endothelial growth factor-C expression and lymphangiogenesis/angiogenesis in oral squamous cell carcinoma. *Anticancer Res* **28**(3A): 1659-1666.
- Lin C, McGough R, Aswad B, Block J A, and Terek R (2004) Hypoxia induces HIF-1alpha and VEGF expression in chondrosarcoma cells and chondrocytes. *J Orthop Res* **22**(6): 1175-1181.
- Liu Y, Peterson DA, Kimura H, Schubert D (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurosci* **69**(2):581-93.
- Losel R M, Besong D, Peluso J J, and Wehling M (2008) Progesterone receptor membrane component 1--many tasks for a versatile protein. *Steroids* **73**(9-10): 929-934.
- Mach R H, Zeng C, and Hawkins W G (2013) The sigma2 receptor: a novel protein for the imaging and treatment of cancer. *J Med Chem* **56**(18): 7137-7160.
- Matsumoto R R, Bowen W D, Tom M A, Vo V N, Truong D D, and De Costa B R (1995) Characterization of two novel sigma receptor ligands: antidystonic effects in rats suggest sigma receptor antagonism. *Eur J Pharmacol* **280**(3): 301-310.

- McCurdy C R, Mesangeau C, Matsumoto R R, Poupaert J H, Avery B A, and Abdelazeem A H A (2014) Highly selective sigma receptor ligands. United States of America. **Patent # US8686008 B2.**
- Monassier L, Manoury B, Bellocq C, Weissenburger J, Greney H, Zimmermann D, Ehrhardt J D, Jaillon P, Baro I, and Bousquet P (2007) Sigma(2)-receptor ligand-mediated inhibition of inwardly rectifying K(+) channels in the heart. *J Pharmacol Exp Ther* **322**(1): 341-350.
- Neubauer H, Adam G, Seeger H, Mueck A O, Solomayer E, Wallwiener D, Cahill M A, and Fehm T (2009) Membrane-initiated effects of progesterone on proliferation and activation of VEGF in breast cancer cells. *Climacteric* **12**(3): 230-239.
- Nicholson H, Comeau A, Mesangeau C, McCurdy C R, and Bowen W D (2015) Characterization of CM572, a Selective Irreversible Partial Agonist of the Sigma-2 Receptor with Antitumor Activity. *Journal of Pharmacology and Experimental Therapeutics*.
- Ostenfeld M S, Fehrenbacher N, Hoyer-Hansen M, Thomsen C, Farkas T, and Jaattela M (2005) Effective tumor cell death by sigma-2 receptor ligand siramesine involves lysosomal leakage and oxidative stress. *Cancer Res* **65**(19): 8975-8983.
- Peluso J J, Liu X, Gawkowska A, Lodde V, and Wu C A (2010) Progesterone inhibits apoptosis in part by PGRMC1-regulated gene expression. *Mol Cell Endocrinol* **320**(1-2): 153-161.
- Shoghi K I, Xu J B, Su Y, He J, Rowland D, Yan Y, Garbow J R, Tu Z D, Jones L A, Higashikubo R, Wheeler K T, Lubet R A, Mach R H, and You M (2013) Quantitative Receptor-Based Imaging of Tumor Proliferation with the Sigma-2 Ligand [F-18]ISO-1. *Plos One* **8**(9).
- Talks K L, Turley H, Gatter K C, Maxwell P H, Pugh C W, Ratcliffe P J, and Harris A L (2000) The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-

- 2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* **157**(2): 411-421.
- Tsai S Y, Hayashi T, Mori T, and Su T P (2009) Sigma-1 receptor chaperones and diseases. *Cent Nerv Syst Agents Med Chem* **9**(3): 184-189.
- Vilner B J and Bowen W D (2000) Modulation of cellular calcium by sigma-2 receptors: release from intracellular stores in human SK-N-SH neuroblastoma cells. *J Pharmacol Exp Ther* **292**(3): 900-911.
- Vilner B J, John C S and Bowen W D (1995) Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Res* **55**(2): 408-413.
- Wheeler K T, Wang L M, Wallen C A, Childers S R, Cline J M, Keng P C, and Mach R H (2000) Sigma-2 receptors as a biomarker of proliferation in solid tumours. *British Journal of Cancer* **82**(6): 1223-1232.
- Xu J B, Zeng C B, Chu W H, Pan F H, Rothfuss J M, Zhang F J, Tu Z D, Zhou D, Zeng D X, Vangveravong S, Johnston F, Spitzer D, Chang K C, Hotchkiss R S, Hawkins W G, Wheeler K T, and Mach R H (2011) Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site. *Nature Communications* **2**.
- Zeng C, Rothfuss J, Zhang J, Chu W, Vangveravong S, Tu Z, Pan F, Chang K C, Hotchkiss R, and Mach R H (2012) Sigma-2 ligands induce tumour cell death by multiple signalling pathways. *Br J Cancer* **106**(4): 693-701.
- Zeng C, Rothfuss J M, Zhang J, Vangveravong S, Chu W, Li S, Tu Z, Xu J, and Mach R H (2014) Functional assays to define agonists and antagonists of the sigma-2 receptor. *Anal Biochem* **448**: 68-74.

Zhong H, De Marzo A M, Laughner E, Lim M, Hilton D A, Zagzag D, Buechler P, Isaacs W B, Semenza G L, and Simons J W (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* **59**(22): 5830-5835.

Footnotes:

*This work was supported by the National Institutes of Health National Institute of General Medical Sciences T32 Predoctoral Pharmacology Training Grant [1-T32 GM077995-01A2] (HN); National Institutes of Health National Institute of General Medical Sciences Initiative for Maximizing Student Development Grant [R25 GM083270] (HN); Brown University Pharmacia Pre-doctoral Fellowship in Pharmacology (HN); National Institutes of Health National Institute on Drug Abuse Grant [R01 DA023205] (CM, CRM); National Institutes of Health National Institute of General Medical Sciences Grant [P20 GM104932] (CM, CRM); and the Upjohn Professorship in Pharmacology, Brown University (WDB)

This work has been previously presented in part at the Society for Neuroscience Annual Meeting 2014 (299.17).

Chapter 5: Structure-activity relationship of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79) derivatives at sigma receptors

5.1 Preface

Taken together, the results of the investigations of CM572 and CM764 demonstrate that small changes to the SN79 core structure, such as substitution with an isothiocyanate or addition of an amino group, can have a large impact on sigma-2 receptor function. Further, while both ligands are structurally very similar to SN79 and retain the ability to bind sigma-2 receptors with high affinity and selectivity, neither compound retained the antagonist activity of the parent. From this, it is apparent that it is not currently possible to predict the activity of a ligand based solely on its parent compound without a profound understanding of what trends exist correlating structure, binding, and function.

SN79 is one of a very small number of sigma-2 receptor ligands that does not bind off-target proteins with any significant affinity. Commonly overlapping binding sites for other high affinity sigma-2 receptor binding compounds include dopamine receptors and transporters, alpha-adrenergic receptors, and opioid receptors (R. R. Matsumoto 2007). SN79 was therefore chosen as a starting compound for the establishment of a clear structure-activity relationship among a series of single-element changes to its core structure. A prospective outcome of this study would be a more well-informed approach to function-based ligand design, which may help

avoid the “guess and check” model that has been demonstrated to be unsuccessful by the antagonist-based hypotheses that drove investigations of CM572 and CM764. Another benefit of such a study might be the prediction of characteristics of the sigma-2 receptor binding pocket, based on substituents that promote or inhibit binding or subtype selectivity. Areas of steric strain or electronegativity clash may be possible to extrapolate, depending on binding affinity trends.

With two distinct functions resultant from sigma-2 receptor activation now apparent, one cytotoxic and one stimulative, it is also important to have a variety of ligands that are able to induce each phenotype for confirmation and optimization studies. As both ligands are based on the same core structure, it becomes even more important to understand the relationship between ligand structure and sigma-2 receptor activity, as differential induction of each function may have diverse therapeutic indications. This relationship may also be able to be exploited for synergistic approaches to therapy. Therefore, a series of compounds with single-element changes to the core structure of SN79 were synthesized for evaluation of a complete structure-activity relationship at sigma-2 receptors.

5.2 Abstract

Sigma-2 receptors are pharmacologically-defined protein receptors that have been implicated in a growing number of human diseases and disorders in recent years. There exists a paucity of highly selective ligands for the sigma-2 receptor and a lack of understanding of the relationship between structure and function for these ligands, which this study sought to elucidate for a variety of single-element changes to the core structure of the sigma-2-selective antagonist SN79. Substitutions on the heterocyclic ring of the core structure of SN79 resulted in high-affinity sigma-2 receptor ligands, with replacement of the heterocyclic oxygen with –NMe decreasing sigma-1 receptor affinity and a sulfur substitution at this position imparting high affinity at both subtypes and thus low subtype selectivity. Substitution of the methyl ketone moiety on SN79 with an isothiocyanate group resulted in ligands that irreversibly bound the sigma-2 receptor and induced cytotoxicity in SK-N-SH neuroblastoma cells, an effect that was not conserved with –NO₂, –NH₂, or –F substitutions. The results of this study will help guide directed synthesis for the development of selective sigma-2 receptor-targeting ligands with predictable function for use in basic and clinical studies involving the sigma-2 receptor.

5.3 Introduction

The sigma-2 receptor is a 21.5 kDa membrane receptor that is constantly becoming of increased interest to a wide variety of health-directed initiatives. Due to its upregulation in tumors as compared to healthy tissue, the sigma-2 receptor has been implicated in cancer cell proliferation as well as being developed into a tool for imaging tumors (Vilner et al. 1995; Mach et al. 1997; Wheeler et al. 2000; Mach and Wheeler 2009; Zeng et al. 2011; Shoghi et al. 2013). This trajectory of pathological functionality for sigma-2 receptors has been extended to include virus-associated tumors and implications in viral diseases such as HIV have been proposed (Bowen et al. 2004; Roperto et al. 2010). Additionally, a connection between sigma-2 receptors and Alzheimer's disease recently has been described (Izzo et al. 2014; Izzo et al. 2014; Sahlholm et al. 2015). It is inevitable that further implications for the use of the sigma-2 receptor to treat disease states will surface as investigations progress, uncovering novel functions and interactions for this unique target.

One aspect of sigma-2 receptor biology that remains enigmatic is that this receptor has not yet been cloned. The progesterone receptor membrane component 1 (PGRMC1) has been associated with the sigma-2 receptor binding site through pharmacological manipulation and knockdown of this known protein, although stark controversy over the exact relationship between these two entities remains (Xu et al. 2011; Abate et al. 2015; Chu et al. 2015). Thus, it is imperative that the pharmacology of the sigma-2 receptor be effectively developed and described in order to understand the pathways, mechanisms, and functions associated with it. The endogenous ligand for sigma-2 receptor is not yet known, but a range of synthetic ligands have been synthesized and characterized to aid in the investigation of this receptor (Decosta et al.

1994; Bowen et al. 1995; Prezzavento et al. 2007; Cassano et al. 2009; Hornick et al. 2010; Kaushal et al. 2011; Zeng et al. 2012).

Directed synthesis of ligands for a specified function has not yet had consistent success, highlighted by recent findings that a single-moiety change to the structure of sigma-2 antagonist SN79 that was expected to result in a ligand with a similar function, but instead resulted in formation of a potent, selective partial agonist, CM572 (Nicholson et al. 2015). Generally, an agonist of the sigma-2 receptor is able to induce cell death; however functional classification of sigma-2 receptors is not standardized, with different groups preferring different criteria for agonist determination. Cleavage of BH3 interacting-domain death agonist (Bid), activation of caspase-3, induction of an immediate transient rise in cytosolic calcium, and positive TUNEL staining have all been proposed as markers of agonist function through sigma-2 receptors, among several others (Bowen et al. 1995; Crawford and Bowen 2002; Hazelwood and Bowen 2006; Wang and Bowen 2006; Zeng et al. 2014; Nicholson et al. 2015a). Final result of a reduction in cell viability through sigma-2 receptor binding is accepted in the field as an endpoint of receptor activation by an agonist.

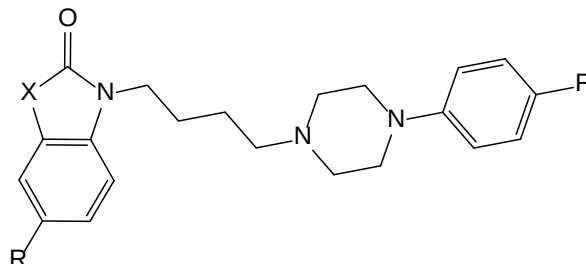
A significant challenge for pharmacological development of the sigma-2 receptor is the paucity of sigma-2 selective ligands. The sigma-1 receptor is a pharmacologically related protein receptor with distinct size (25 kDa), function, and subcellular localization from the sigma-2 receptor, and it has been successfully cloned. There exist several sigma-1-selective ligands for analysis of this receptor, but ligands with opposing selectivity for the sigma-2 receptor are sparse. SN79 is a moderately selective sigma-2 receptor antagonist ($K_i = 7$ nM and 27 nM at sigma-2 and sigma-1, respectively) (Kaushal et al. 2011). Here, we describe the synthesis and characterization of a novel series of SN79-derived ligands for their affinity and function at

sigma-2 receptors with the aim of developing an understanding of the relationships between ligand structure, binding, and function through single-element variations. This study sought to form a foundation from which to design targeted sigma-2 receptor ligands that are highly selective and result in inclusion into a predicted functional class.

5.4 Results

All ligands were examined for their ability to bind sigma-1 and sigma-2 receptors using [3H](+)-pentazocine to measure sigma-1 receptors and [3H]DTG to measure sigma-2 receptors in the presence of unlabeled (+)-pentazocine to mask sigma-1 receptors as described in Experimental Section. Competition assays were performed for each compound against each receptor subtype independently and K_i values were determined using Graphpad Prism. Selectivity was determined as the ratio of the sigma-1 receptor K_i to the sigma-2 receptor K_i . Results are summarized in Table 1.

Table 1:

Binding affinities of SN79 derivatives at sigma-1 and sigma-2 receptors.

Ligand	X	R	Sigma binding affinities ($K_i \pm SD$) (nM)		
			$[^3H](+)$ -pentazocine	$[^3H]$ DTG	s1/s2
			sigma-1	sigma-2	
SN79	O	COCH ₃	28.03 $\pm$ 3.39*	6.89 $\pm$ 0.09*	4.07
CM572	O	NCS	$\geq 10,000^\dagger$	14.6 $\pm$ 7.0 [†]	>650
CM458	O	NO ₂	22.2 $\pm$ 5.3	0.56 $\pm$ 0.38	39.6
CM571	O	NH ₂	15.5 $\pm$ 2.4	21.7 $\pm$ 5.3	0.7
NF7	NMe	COCH ₃	182.9 $\pm$ 13.9	20.6 $\pm$ 2.4	8.9
WA404	NMe	NCS	448.5 $\pm$ 44.4	36.3 $\pm$ 7.8	12.4
WA402	NMe	NO ₂	20.3 $\pm$ 6.5	7.4 $\pm$ 3.0	2.7
WA403	NMe	NH ₂	2987.7 $\pm$ 506.9	17.9 $\pm$ 8.0	166.9
WA504	S	COCH ₃	8.1 $\pm$ 2.3	2.5 $\pm$ 2.7	3.2
WA435	S	NCS	56.9 $\pm$ 10.2	2.0 $\pm$ 1.5	5.6
WA413	S	NO ₂	6.1 $\pm$ 1.0	3.2 $\pm$ 0.3	1.9
WA416	S	NH ₂	15.6 $\pm$ 2.8	3.9 $\pm$ 0.5	4.0
WA379	NMe	F	100.7 $\pm$ 10.3	6.1 $\pm$ 1.7	16.5

Radioligand competition binding assays were performed using [3H](+)-pentazocine to measure sigma-1 receptor and [3H]DTG in the presence of unlabeled (+)-pentazocine to measure sigma-2 receptors as described in Experimental Section. Single-position changes to the core structure of SN79 resulted in drastic changes in affinity and selectivity at sigma-1 and sigma-2 receptors.

Generally, changes in the X-position determined selectivity, with –S- substitutions resulting in reduced selectivity and –O- substitutions resulting in increased selectivity for the sigma-2 receptor. R-group substitutions did not demonstrate a discernably consistent pattern across the ligand series. An accurate K_i value could not be determined for CM572 (X=O, R=NCS) at sigma-1 receptors due to insolubility above 1 mM, however ~50% of total sigma-1 receptors were bound by 10,000 nM CM572 at compared to total available sigma-1 receptor binding capacity as measured by [^3H](+)-pentazocine alone. Results are presented as an average K_i values from at least three independent experiments for each ligand (except for CM572 at sigma-1 receptors), $\pm$ S.D. All experiments were performed in duplicate. *(Kaushal et al. 2011)
†(Nicholson et al. 2015)

When ligands with the same R-group were compared, there existed a trend towards a loss of high-affinity binding at sigma-1 receptors for compounds with –NMe- X-group substitutions. This was most clearly exemplified with WA403 (R=NH₂) (and to a lesser extent with NF7 (R=COCH₃)), where there is a striking rightward shift in the sigma-1 binding competition curves for the –NMe- substituted ligand when compared to the other ligands with R=NH₂ but X=O or X=S, as demonstrated in Figure 1. When X=NMe sigma-1 affinity was decreased by at least 185-fold as compared to X=O and X=S for ligands with the same R-group, and when X=NMe, the sigma-1 affinity was decreased by at least 6.5-fold for R=COCH₃ in the same comparisons. When R=NO₂, the X=NMe substitution still demonstrated the lowest affinity binding as compared to other X-group substitutions, however the shift was less drastic. For WA404 (R=NCS) the –NMe- substituted ligand bound sigma-1 receptors with ~8 times lower affinity than the corresponding X=S substitution (WA435). For CM572 (R=NCS, X=O), a valid K_i value could not be determined at sigma-1 receptors due to solubility limits of the ligand. However, it is clear that CM572 (X=O, R=NCS) is an outlier in this series due to its lack of significant sigma-1 receptor binding and therefore its extreme selectivity.

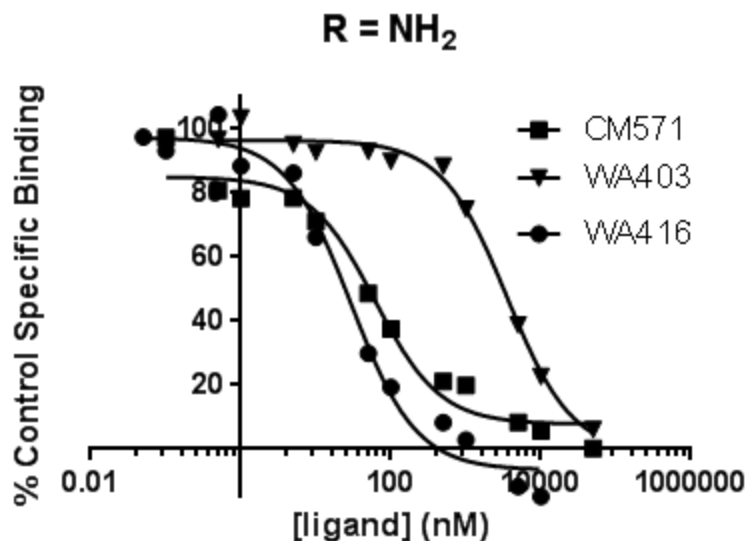


Figure 1. **Effect of X-group substitutions on sigma-1 receptor binding affinity.** Radioligand competition assays were performed in rat liver membrane preparations using [³H](+)-pentazocine to measure sigma-1 receptor occupancy. K_i values were determined using the previously determined $K_d=7.5$ nM for [³H](+)-pentazocine in rat liver and GraphPad Prism 6 software for analysis. K_i values for sigma-1 receptor binding of R=NH₂ substituted ligands were determined to be 15.5 ± 2.4 nM, 2987.7 ± 506.9 nM, and 15.6 ± 2.8 nM for CM572 (X=O, R=NH₂), WA403 (X=NMe, R=NH₂), and WA416 (X=S, R=NH₂) substitutions, respectively. This series dramatically demonstrates the loss of sigma-1 receptor affinity for X=NMe substituted ligands, which showed a 185-fold decrease in affinity at the sigma-1 receptor as compared to other X-group substitutions. Competition curves shown are an average of 3 independent experiments for CM571, 5 independent experiments for WA403, and 3 independent experiments for WA416. All experiments were performed in duplicate. K_i values reported are an average of the individual K_i value from each independent experiment, $\pm$ S.D.

Comparing ligands with the same X-group substitution allowed for a comparison of the effects of R-group substitutions on sigma-1 receptor binding affinity. Ligands with R=NCS generally had a significantly decreased affinity for sigma-1 receptors as compared to any other R-group substitution. This is best demonstrated by CM572 (R=NCS, X=O), where the isothiocyanate substituted ligand has a decrease in sigma-1 receptor binding affinity of over 500-fold when compared to any other R-group substitution, as shown in Figure 2. For X=S substitutions, there was at least a 3-fold loss of sigma-1 affinity for the isothiocyanate (WA435) substitution as compared to the other R-group substitutions. As mentioned above, sigma-1 receptor binding affinity was not high for any ligand when X=NMe (Figure 1), and WA404 (R=NCS, X=NMe) had a low affinity K_i value of 449 nM at the sigma-1 receptor, although this was not the lowest affinity of the X=NMe substituted ligands.

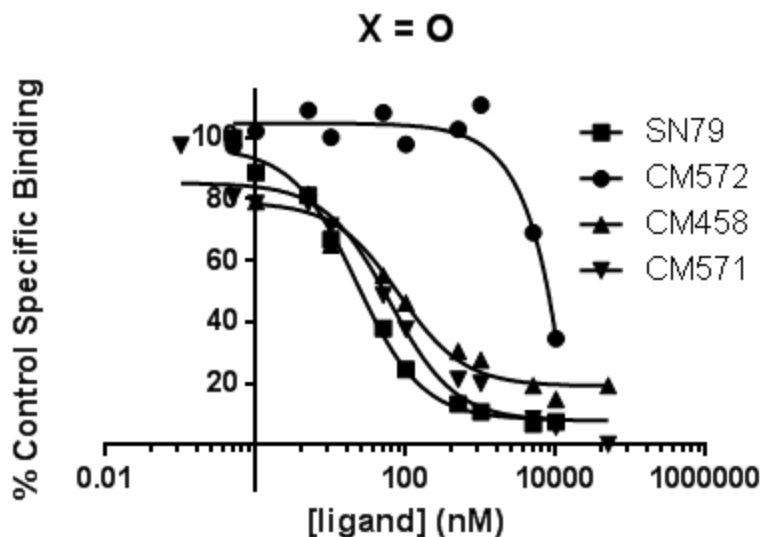


Figure 2. Effect of R-group substitutions on sigma-1 receptor binding affinity. Sigma-1 receptor binding affinity was determined by competition with [^3H](+)-pentazocine and analyzed using GraphPad Prism 6 software, using the predetermined $K_d=7.5$ nM for [^3H](+)-pentazocine at sigma-1 receptors in rat liver membrane homogenates. Sigma-1 receptor binding affinity was significantly decrease when $R=\text{NCS}$ for all X-group substitutions. This pattern is most clearly illustrated for $X=\text{O}$ substituted ligands, with CM572 ($X=\text{O}$, $R=\text{NCS}$) showing at least a 350-fold loss in sigma-1 receptor binding affinity as compared to other R-group substitutions. K_i values for $X=\text{O}$ substituted ligands at sigma-1 receptors were determined to be $28.03 \pm 3.39^* \text{ nM}$, $>10,000 \text{ nM}$, $22.2 \pm 5.3 \text{ nM}$, and $15.5 \pm 2.4 \text{ nM}$ for SN79 ($X=\text{O}$, $R=\text{COCH}_3$), CM572 ($X=\text{O}$, $R=\text{NCS}$), CM458 ($X=\text{O}$, $R=\text{NO}_2$), and CM571 ($X=\text{O}$, $R=\text{NH}_2$), respectively. An accurate K_i value for CM572 ($X=\text{O}$, $R=\text{NCS}$) at sigma-1 receptors could not be determined due to insolubility above 1 mM, however $\sim 50\%$ of [^3H](+)-pentazocine binding sites remained with 10,000 nM CM572 present. Competition curves shown are an average of 4 independent experiments for SN79, 2 independent experiments for CM572, 3 independent experiments for CM458, and 3 independent experiments for CM571. All experiments were performed in

duplicate. K_i values reported are an average of the individual K_i value from each independent experiment, $\pm$ S.D.

The effect of X-group substitutions on receptor affinity was not limited to the sigma-1 subtype. When sigma-2 affinities were compared, X=S substituted ligands had generally high sigma-2 affinity as compared to X=O and X=NMe substituted ligands (Figure 3). WA435 (R=NCS, the X=S) showed at least a 7-fold increase in sigma-2 receptor affinity as compared to other X-group substitutions. Similarly, for R=COCH₃ and R=NH₂ the sulfur X-group substitution increased sigma-2 receptor binding affinity by at least ~3-fold and ~4-fold, respectively, as compared to other X-group substitutions. R=NO₂ substitutions resulted in generally very high affinity ligands at the sigma-2 receptor, with all ligands in this group having binding affinities of better than 8 nM. In this group, WA413 (R=NO₂, X=S) did not demonstrate the highest sigma-2 receptor binding affinity, but was still a very high affinity ligand with a K_i value of 3.2 nM. All X=S substitutions resulted in sigma-2 receptor affinities that were between 2-4 nM, indicating that this substitution in the X-position may play an important role in the binding pocket of the sigma-2 receptor.

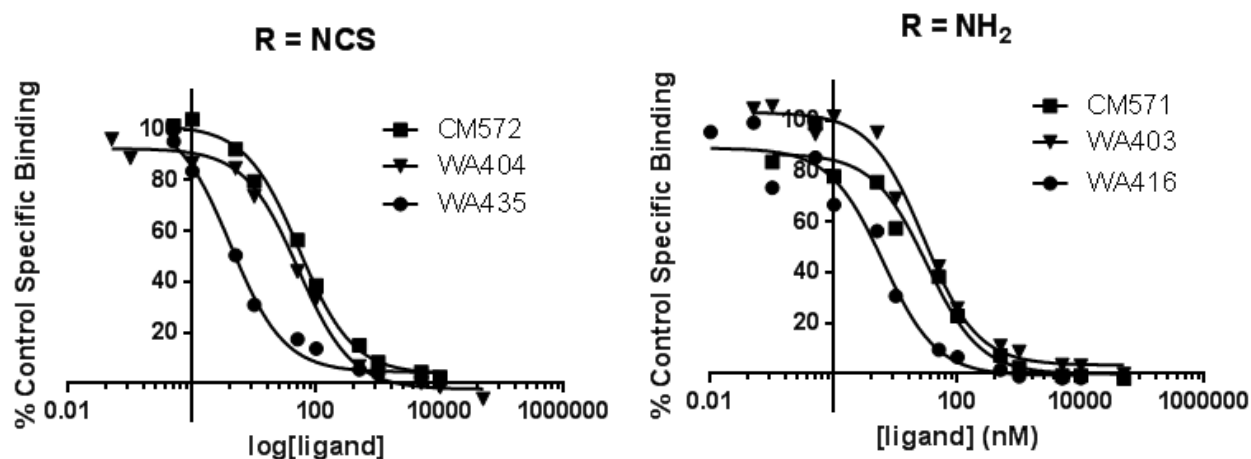
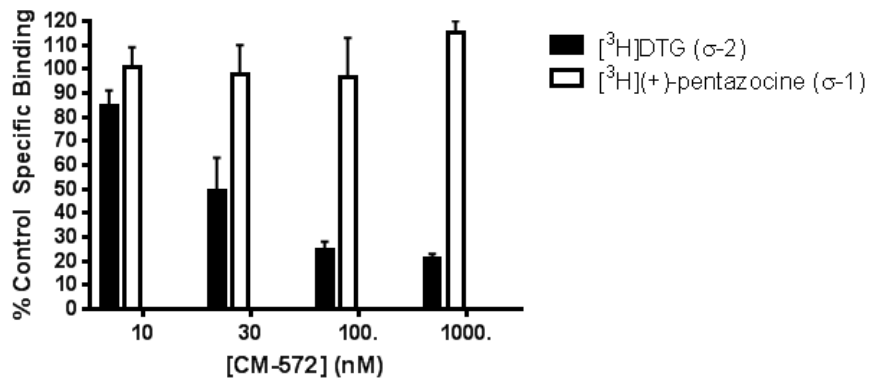


Figure 3. **Effect of X-group substitutions on sigma-2 receptor binding affinity.** Sigma-2 receptor binding affinity was determined by competition of each ligand with [3 H]DTG and K_i values obtained using GraphPad Prism 6 software and the predetermined K_d value of 17.9 nM for [3 H]DTG in rat liver homogenates. Generally, X-group substitutions with sulfur resulted in ligands with very high sigma-2 receptor binding affinity, ranging between 2-4 nM K_i values. This trend generally held true across R-group changes, and was most drastically demonstrated by WA435 (X=S, R=NCS) and WA403 (X=S, R= NH_2) comparisons depicted here. For R=NCS, WA435 (X=S, R=NCS) resulted in an increase in sigma-2 receptor binding affinity of at least 7-fold as compared to other X-group substituted ligands. Similarly, for R= NH_2 WA416 (X=S, R= NH_2) substitution increased sigma-2 receptor binding affinity by at least 4.5-fold over other X-group substituted ligands. Competition curves shown are an average of 4 independent experiments for CM572, 6 independent experiments for WA404, 3 independent experiments for WA435, 3 independent experiments for CM571, 5 independent experiments for WA403, and 3 independent experiments for WA416. All experiments were performed in duplicate.

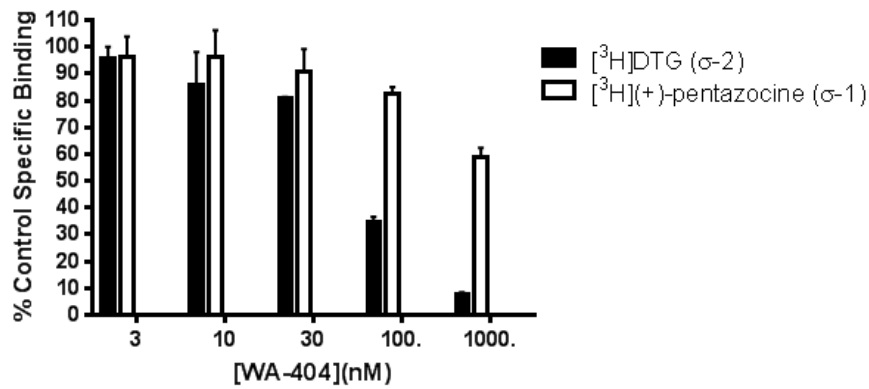
The changes in the X-position that resulted in drastic effects on binding affinity at both sigma-1 and, to a lesser extent, sigma-2 receptor binding affinity naturally also demonstrated an effect on subtype selectivity of the ligands. It should be noted that all ligands demonstrated at least slightly selective binding for the sigma-2 receptor over the sigma-1 receptor except for CM571 (X=O, R=NH₂). Generally, having an oxygen in the X-position led to more highly sigma-2 selective ligands, while having a sulfur in this position generally decreased selectivity (Table 1). However, this trend was less closely adhered to than trends in binding at each individual receptor. Several ligands in this study were found to have extreme selectivity for the sigma-2 receptor over the sigma-1 subtype, with over 50-fold differences in affinity between the two.

We have previously shown that CM572 (X=O, R=NCS) is able to irreversibly bind the sigma-2 receptor (Nicholson et al. 2015). In this study, we examined two additional R=NCS substituted ligand for their ability to irreversibly bind sigma-1 and sigma-2 receptors. Both the WA404 (X=NMe, R=NCS) and WA435 (X=S, R=NCS) were able to irreversibly bind sigma-2 receptors, with the WA435 (X=S, R=NCS) having a higher potency for irreversible binding (Figure 4). This trend correlates with sigma-2 receptor binding affinity differences, as the X=S substituted ligands have a higher affinity for sigma-2 receptors than the X=NMe substituted ligands. Neither isothiocyanate potently irreversibly bound sigma-1 receptors, although WA404 (X=NMe, R=NCS) did show some ability to irreversibly block sigma-1 receptor binding at high doses.

A



B



C

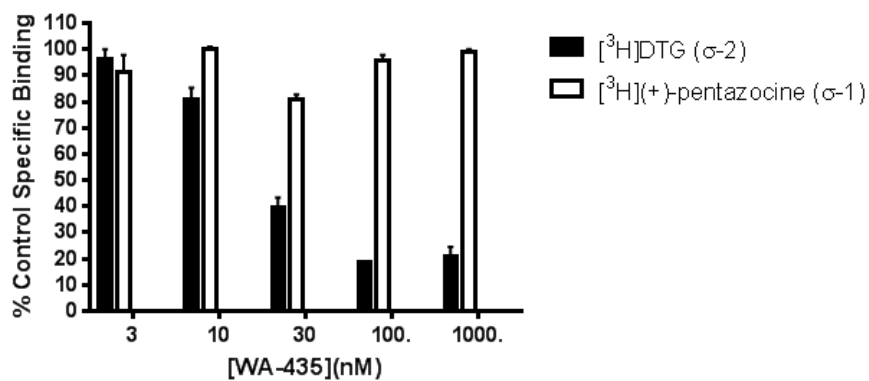


Figure 4. Irreversible binding of isothiocyanate-substituted ligands at sigma-1 and sigma-2 receptors. Rat liver membrane homogenates were treated with isothiocyanate-substituted ligands

for 60 minutes prior to washing to remove any reversibly bound ligand. Recovery of sigma-1 and sigma-2 binding sites was then determined using [^3H]DTG to measure sigma-2 receptors and [^3H](+)-pentazocine to measure sigma-1 receptors. Data is presented as the percentage of total sigma receptors of each subtype recovered. A) CM572 (X=O, R=NCS) was previously shown to irreversibly bind sigma-2 receptors but not sigma-1 receptors (Nicholson et al. 2015). WA404 (X=NMe, R=NCS) (B) and WA435 (X=S, R=NCS) (C) maintained the ability to selectively irreversibly bind sigma-2 receptors. WA404 (X=NMe, R=NCS) demonstrated some slight ability to irreversibly bind sigma-1 receptors at high doses. Data shown is the average of at least two independent experiments for each condition, with each experiment performed in duplicate.

One globally accepted criterion for classification of a sigma-2 receptor ligand as an agonist is the ability to induce cell death. In order to classify the function of the ligands in this study, MTT cell viability assays were used to determine the efficacy and potency of the ability of each ligand to induce cell death in SK-N-SH neuroblastoma. The R-group substitution had the greatest effect on the ability of each ligand to induce cell death. The only compounds in the series with the ability to potently decrease cell viability were those with R=NCS, regardless of X-group substitution. Data for the active compounds is depicted in Figure 5. The isothiocyanate substituted ligands all were able to induce a reduction in cell viability in the SK-N-SH neuroblastoma with an EC_{50} below 35 μ M, with the CM572 (X=O, R=NCS) being the most potent with an EC_{50} of 7.6 μ M, as previously reported (Nicholson et al. 2015). Other X-group substitutions (X=NMe and X=S) demonstrated less potent EC_{50} values (WA404= 30.4 ± 1.1 μ M and WA435= 32.8 ± 1.1 μ M). The only other ligand in this study that was able to induce a significant level of cell death was the WA413 (X=S, R=NO₂), which reached ~36% cytotoxicity at a dose of 50 μ M after 24 h treatment. An EC_{50} value for this ligand could not confidently be determined due to insolubility at high doses, although the highly concentrated dose that was required in order to induce even moderate cell death suggests the possibility of a non-specific mechanism.

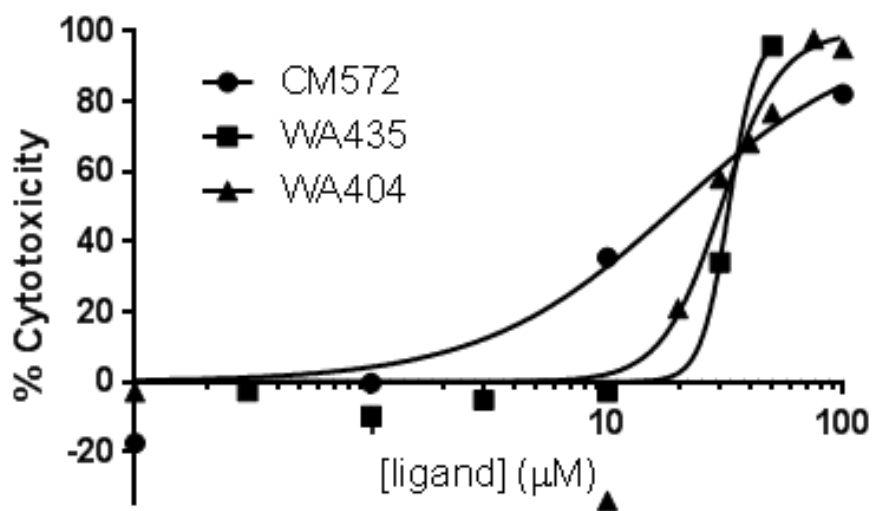


Figure 5. **Effect of isothiocyanate derivatives on cell viability of SK-N-SH neuroblastoma.**

SK-N-SH neuroblastoma cells were plated at 15,000 cells/well and allowed to attach overnight prior to dosing with indicated dose of each ligand. Cells were incubated with ligand for 24 hours prior to determination of viable cells remaining with MTT assay. Results are presented as a percentage of cells still viable after 24 hour drug treatment as compared to untreated control cells. All three isothiocyanate substituted ligands were able to induce significant levels of cytotoxicity after 24 hour exposure. No other ligands examined were able to induce cytotoxicity with EC_{50} values that could be attributed to specific action at sigma-2 receptors. All three isothiocyanate derivatives of SN79 showed moderate to high potency for the ability to induce cell death in SK-N-SH neuroblastoma cells, with EC_{50} values determined to be $7.6 \pm 1.7 \mu M^*$, $32.76 \pm 1.05 \mu M$, and $30.35 \pm 1.13 \mu M$ for CM572 (X=O, R=NCS), WA435 (X=S, R=NCS), and WA404 (X=NMe, R=NCS), respectively. Dose-response curves shown are an average of 3 independent experiments each ligand, with each experiment performed using 5 replicates per condition. EC_{50} values were determined from average cytotoxicity values for each dose using

GraphPad Prism 6 software for analysis and are presented as average $\pm$ S.D. *(Nicholson et al. 2015)

The isothiocyanate moiety was added with the idea of imparting irreversible binding capability to the ligands through potential nucleophilic attack from an amine or thiol group appropriately positioned in the binding pocket of the sigma-2 receptor. Our irreversible binding study demonstrated that this substitution did impart the ability of the ligands to irreversibly bind sigma-2 receptors for all ligands examined, however sigma-1 receptor irreversible binding was not widely observed (Figure 4). The effect of this irreversible binding on cell viability was further tested by treating SK-N-SH neuroblastoma with an acute 60 minute pretreatment with the isothiocyanate ligands, followed by extensive washing and a 24 hour incubation in fresh ligand-free media. Cell viability was assessed after this period, and any induced cell death was attributed to ligand irreversibly bound and therefore continuously activating sigma-2 receptors throughout the 24 hour incubation without free ligand. All isothiocyanate derivatives were found to continue to induce cell death after the acute 60 minute pretreatment, washing, and 24 hour incubation in media without free ligand. Results are shown in Figure 6. For both CM572 (X=O, R=NCS) and WA404 (X=NMe, R=NCS), the acute treatment reached comparable levels of cell death as the 24 hour incubation with ligand, with no statistically significant differences between the two exposure times ($p=0.13$ and $p=0.056$ for comparison of 24 hour incubation with ligand and 60 minute acute treatment followed by washing and 24 hour incubation in media without free ligand, for CM572 (X=O, R=NCS) and WA404 (X=NMe, R=NCS), respectively, Student's t-test). Interestingly, for WA435 (X=S, R=NCS) the efficacy of the ligand increased when treated acutely as compared to the 24 hour continuous exposure, although both conditions induced significant cell death and the difference between treatments did not reach statistical significance ($p=0.052$, Student's t-test).

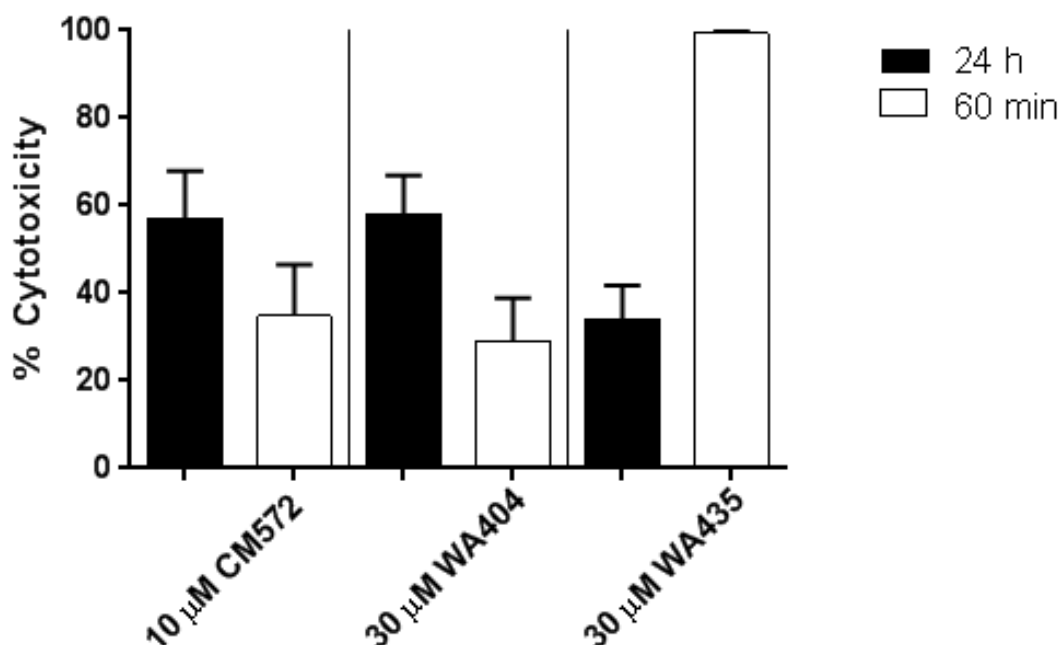


Figure 6. **Comparison of acute and continuous exposure to isothiocyanate substituted ligands on cell viability of SK-N-SH neuroblastoma.** Cells were plated at 15,000 cells/well and allowed to attach overnight prior to dosing with indicated concentration of each ligand. For 24 hour experiments, cells were incubated with ligand continuously for 24 hours. For acute treatments, cells were treated with ligand for 60 minutes followed by extensive washing with fresh media and 24 hour incubation in ligand-free media. At the end of the 24 hour period, viable cells were determined by MTT assay. All three isothiocyanate derivatives resulted in comparable levels of cell death in the acute exposure experiment as in the 24 hour continuous exposure ($p=0.13$, 0.056 , and 0.052 for CM572, WA404, and WA435 comparisons of the two exposure conditions, Student's t -test). These data indicate that the isothiocyanate substituted ligands continue to induce cell death as a result of their capacity to irreversibly bind sigma-2 receptors even after free ligand is removed. Results are presented as the percentage of cells killed in response to each treatment compared to untreated control cells. Data is presented as an average

of 3 independent experiments for each condition for each ligand, $\pm$ S.D. All experiments were performed with 5 replicates per condition.

The data clearly shows that in order for the compounds in this series to be cytotoxic, the isothiocyanate group must be present. This would indicate that irreversible binding to sigma-2 receptors is a requirement for cytotoxicity. However another explanation for the efficacy of the isothiocyanate ligands is that the strong electron-withdrawing character of this moiety influences the receptor interaction such that the efficacy is altered. In order to discriminate between the effect of irreversible binding and the effect of the strong electron withdrawing character of the isothiocyanate moiety on cell viability, an R-group substitution with fluorine was investigated to represent the strong electron withdrawing character of the isothiocyanate but without the capability to irreversibly bind to the sigma-2 receptor. This ligand, WA379 (X=NMe, R=F), bound with high affinity and selectivity to sigma-2 receptors ($K_i=100.7 \pm 10.3$ nM at sigma-1 receptors and 6.1 ± 1.7 nM at sigma-2 receptors, average $\pm$ S.D. of at least three independent experiments with each experiment performed in duplicate). When the effect of treatment with the fluorine substituted ligand on cell viability was examined, no significant cytotoxicity was induced (data not shown). These results demonstrate that the strong electron withdrawing character of the isothiocyanate group alone is not sufficient to induce significant levels of cell death, and that the irreversible effect is necessary for agonist activity at the sigma-2 receptor in this class of ligands.

We have recently described a novel, non-toxic, non-zero function for sigma-2 receptors using the SN79 derivative CM764 (Nicholson et al. 2016). Four of the ligands investigated in this series replicated this effect, inducing an increase in reduction of MTT upon 24 h treatment as compared to untreated cells. Results are shown in Figure 7. The four ligands (CM458, CM571, NF7, and WA504) that promoted significantly increased MTT reduction in SK-N-SH neuroblastoma have R-group substitutions representing all possibilities examined except

R=NCS, which was shown to be toxic in all cases. Additionally, this subset of four ligands also encompasses all three possible X-group substitutions examined. These data indicate that neither X-group nor R-group alone determine the ability to induce increased reduction of MTT. All four ligands exhibited high sigma-2 receptor binding affinity, and receptor subtype selectivity varied greatly.

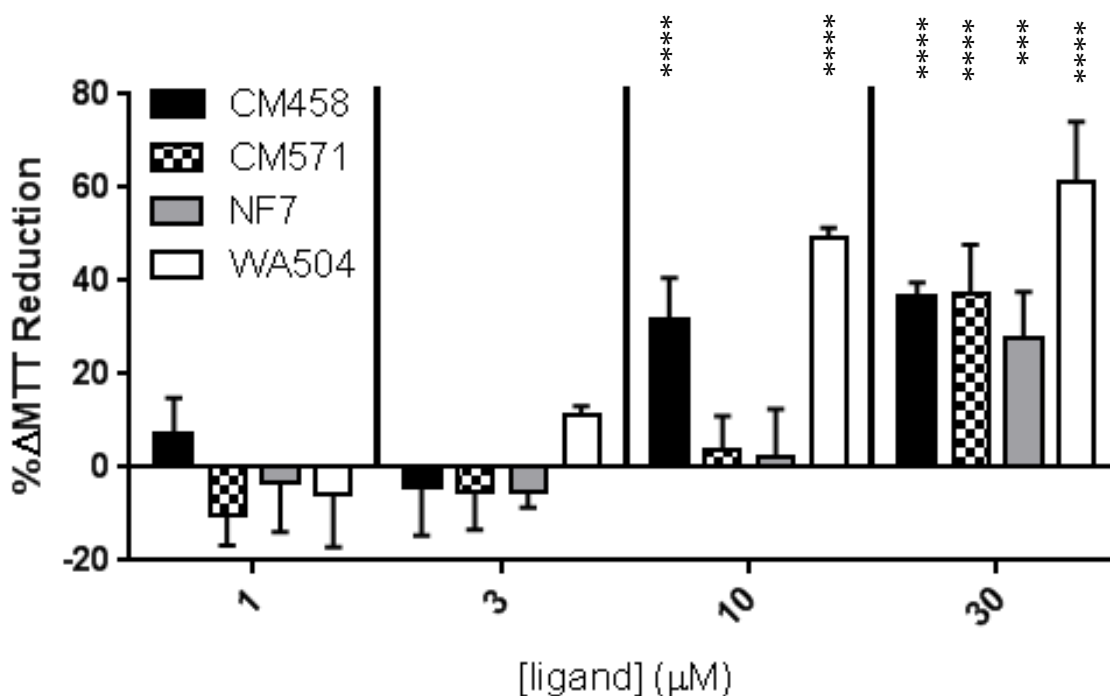


Figure 7. **Ability of SN79 derivatives to increase reduction of MTT in SK-N-SH**

neuroblastoma. Cells were plated at 15,000 cells/well and allowed to attach overnight prior to treatment with indicated ligand and concentration for 24 h, followed by MTT assay. Four out of twelve SN79 derivatives in this series demonstrated the ability to induce a significant increase in the amount of MTT reduced as compared to untreated cells (two-way ANOVA overall $p < 0.0001$. Dunnett's multiple comparisons post-hoc test *** $p < 0.001$, **** $p < 0.0001$ compared to untreated cells). All R-group substitutions were represented except R=NCS, which is consistent with the ability of R=NCS ligands to induce cell death. All X-group substitutions were also represented in this subset, and all ligands demonstrated high sigma-2 receptor binding affinity. Results are presented as the percent change in MTT reduction as compared to an untreated control, which was set to zero. Data are presented as an average $\pm$ S.D. of at least three independent experiments for each condition, which each experiment performed using five replicates per condition.

5.5 Discussion

The goal of this study was to establish a relationship between ligand structure, binding affinity and selectivity, and functional efficacy of a series of novel SN79 derivatives at sigma-1 and sigma-2 receptors. The parent compound SN79 previously has been shown to display ~4-fold selectivity for the sigma-2 receptor (Kaushal et al. 2011), and has been screened for off-target binding without any significant hits. SN79 is a sigma-2 receptor antagonist, and does not induce cell death even at high doses (Garcia 2012). As one of the few sigma-2 selective ligands that has been characterized, this was a natural starting point for the development of additional sigma-2 selective ligands with which to study the relationships between ligand structure, binding affinity, receptor subtype selectivity, and *in vitro* function. We synthesized a series of ligands with single-moiety changes using the core structure of SN79 to examine these relationships, with an aim towards future targeted synthesis with intended binding capacity and function.

Using [^3H](+)-pentazocine to measure sigma-1 receptors and [^3H]DTG in the presence of unlabeled (+)-pentazocine to measure sigma-2 receptors with sigma-1 receptors masked, binding affinity of each ligand was measured at both receptor subtypes using radioactive competition binding assays (Table 1). Affinity at sigma-1 receptors was significantly decreased for ligands with X=NMe substitutions, suggesting potential steric strain at this position in the binding pocket of the sigma-1 receptor (Figure 1). Sterically smaller X-group substitutions (X=O and X=S) did not result in a general decrease in sigma-1 receptor binding affinity, lending strength to this hypothesis. Another possibility is that the sigma-1 receptor binding pocket may have an ideally situated hydrogen bond donor that the X=O and, to a weaker extent, the X=S substitutions could participate in to promote higher affinity binding. However, this is a less likely cause as X=S substitutions had generally higher affinity binding yet sulfur is a much weaker hydrogen bond

acceptor than oxygen. Another factor that demonstrated a decrease in sigma-1 receptor binding affinity is the isothiocyanate R-group substitution. Derivatives with R=NCS had generally lowered affinity for the sigma-1 receptor, which resulted in a trend of increased selectivity for the sigma-2 receptor. All ligands in this series except CM571 (X=O, R=NH₂) demonstrated at least slight selectivity for the sigma-2 receptor, which was maintained from the patent compound SN79.

Sigma-2 receptor binding affinity was increased for ligands with R=NO₂ substitutions (Figure 2) and independently for ligands with X=S substitutions (Figure 3). The X=S substitution also generally resulted in an increase in affinity at sigma-1 receptors (although to a lesser extent), which resulted in reduced sigma-2 receptor selectivity. Similar to the driving determinants of sigma-1 receptor binding affinity, sigma-2 receptor binding affinity appears to be dependent on both X- and R-group constituents. These results are consistent with the ligand core structure, in which the X-position and R-position are conjugated. Therefore, resonance allows for changes in the X-group to affect electron density at the R-position, and vice versa.

Substitution of the R-group with an isothiocyanate allowed for the possibility of irreversible binding if there were an ideally positioned amine or thiol group in the binding pocket of the receptor. This irreversible binding capability was confirmed by measuring recovered radioligand binding after pretreatment with isothiocyanate-substituted ligands followed by washout of any non-irreversibly bound ligand. All isothiocyanate substituted ligands were able to irreversibly bind sigma-2 receptors, while only WA404 (X=NMe, R=NCS) showed any ability to irreversibly bind sigma-1 receptors and not with high potency. This indicates that the isothiocyanate in the R-position may be ideally placed for covalent bond formation in the sigma-2 receptor binding pocket, but not in the sigma-1 receptor binding pocket. Interestingly, only

R=NCS substituted ligands were able to induce significantly potent levels of cytotoxicity in SK-N-SH neuroblastoma, indicating agonist activity (Figure 5). This ability to induce cell death was determined to be a result of the capacity to irreversibly bind sigma-2 receptors, rather than a result of the highly electron withdrawing group in the R-group position, as neither R=NO₂ substituted ligands nor an R=F substituted ligand were able to induce cytotoxicity under replicated conditions.

When SK-N-SH cells were acutely treated with isothiocyanate substituted ligands for 60 minutes followed by extensive washing and 24 hour incubation in media without free ligand, the levels of cytotoxicity achieved were comparable to those after 24 hour incubation with ligand for both CM572 (X=O, R=NCS) and WA404 (X=NMe, R=NCS) (Figure 6). This indicates that the ability to irreversibly bind sigma-2 receptors also imparts the ability to continue to activate these receptors and induce cytotoxicity without free ligand present in the media. WA435 (X=S, R=NCS) was found to induce cell death more potently when acutely treated and then incubated in media without free ligand for 24 hours as compared to a 24 hour exposure to ligand. WA435 (X=S, R=NCS) is the only R=NCS substitution that retains significant affinity for the sigma-1 receptor, although it does not irreversibly bind the sigma-1 subtype. This may explain the increased potency in the acute treatment, as the 24 hour continuous exposure to ligand would allow for binding to the sigma-1 receptor, which is known to promote cell survival (Hayashi and Su 2007; Wu and Bowen 2008), and would therefore compete with the cytotoxic effect of sigma-2 receptor activation. In the acute treatment, all ligand would be washed out of sigma-1 receptors and this pro-survival pathway would no longer be activated.

We have recently shown the ability of a novel SN79 derivative, CM764, to induce a non-toxic, non-zero effect through the sigma-2 receptor (Nicholson et al. 2016). This effect

comprised of an increase in MTT reduction, an increased level of NAD⁺/NADH, a marked decrease in basal ROS level, and an increase in HIF1 α which may be connected to an observed increase in VEGF expression. In the series with which the current study was concerned, four of the twelve SN79 derivative shared this CM764-like phenotype with respect to MTT reduction. Although it was not possible to determine a clear relationship between structure and ability to increase MTT reduction in SK-N-SH neuroblastoma cells, our data demonstrate that high sigma-2 receptor affinity was a common factor for all ligands with this function. As neither R-group alone nor X-group alone could explain the relationship between ligand structure and the ability to induce increased MTT reduction, it seems probable that this phenotype derives from a combinatorial effect. The X-group and R-group are conjugated in the series investigated in this study, and therefore both positions could contribute to the development of this non-toxic function. In addition, with CM764, X= O, R=COCH₃ and there is an amine moiety in fluorophenyl ring, indicating that modifications of the other ring system also affect this stimulatory efficacy. Further studies on the combinatorial effects and electron density changes with additional X-group and R-group substitutions may help elucidate this relationship.

Overall, the results of this study demonstrate several trends that can be employed for targeted synthesis of sigma receptor ligands in this series. Highest affinity ligands generally resulted from X=S substituted groups, however these ligands were not highly selective. Increased sigma-2 receptor selectivity resulted from X=NMe substitutions, for which the selectivity was largely derived from a loss of sigma-1 receptor affinity. Substitution of the R-group for strongly electron withdrawing nitro and fluorine moieties was not sufficient to impart agonist activity, but irreversible binding capability resulted in ligands with cytotoxic capabilities. The results of this study will aid in the design of sigma receptor ligands with desired selectivity and functionality

from structure design through *in vitro* testing, and may have implications for targeted therapeutic development for the many implications of sigma-2 receptor involvement in health and disease.

5.6 Materials and Methods

Compound Syntheses

General synthesis of compounds is previously reported (McCurdy et al. 2014).

Radioligand Binding Assays

Radioligand competition binding assays were performed as previously described (Nicholson et al. 2015). Briefly, 150 μ g rat liver homogenate was incubated for 120 minutes at 25°C in 20 mM HEPES with varying concentrations of each novel ligand and 3 nM [3 H](+)-pentazocine (PerkinElmer, Waltham, MA) for measuring sigma-1 receptors or 5 nM [3 H]DTG (PerkinElmer, Waltham, MA) to measure sigma-2 receptors in the presence of 100 nM (+)-pentazocine to mask sigma-1 receptors in a final volume of 0.5 mL. Haloperidol (10 μ M) was used to determine nonspecific binding. After incubation, assays were terminated by filtration with 5 mL ice cold 10 mM Tris pH 7.4 through 0.5% polyethyleneimine-soaked fiberglass filters using a Brandel Cell Harvester (Brandel, Gaithersburg, MD) and two additional 5 mL washes in the same buffer. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) was used to determine K_i values with [3 H](+)-pentazocine K_d =7.5 nM at sigma-1 receptors in rat liver homogenates and [3 H]DTG K_d =17.9 nM at sigma-2 receptors in rat liver homogenates.

For irreversible binding studies, 0.3 mg/mL membrane homogenate was treated with the indicated concentration of each isothiocyanate-substituted ligand individually for 60 minutes in 20 mM HEPES pH 7.4 at 25°C. This preparation was then diluted to 0.018 mg/mL using ice-cold 20 mM HEPES and centrifuged for 10 min at 37,000 x g. The pellet was resuspended to the original volume with ice-cold buffer and centrifuged again, and then resuspended again to the

original volume. Non-covalently bound ligand was then allowed to dissociate during a 60 minute incubation at 25°C prior to centrifugation of the preparation at 37,000 x g for 10 minutes and resuspension of the pellet in 50 mM Tris, pH 8.0 to a concentration of 0.6 mg/mL. This homogenate was used directly in the competition binding studies as described above for a final concentration of 0.3 mg/mL in 0.5 mL total volume. Control membranes were treated in the same manner without exposure to ligand. This membrane preparation procedure was able to dissociate 500 nM SN79 from both sigma-1 and sigma-2 receptors, indicating successful removal of non-irreversibly bound ligand.

Cell Culture

Human SK-N-SH neuroblastoma cells (ATCC, Manassas, VA) were cultured in MEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 10 mg/L human insulin, and 1X Pen-Strep (Gibco, Grand Island, NY) in a humidified atmosphere at 37 °C and 5% CO₂. Cells were passaged at 70% confluency.

Cell Viability Assays

Cell viability was measured using MTT assays (Trevigen, Gaithersburg, MD). Cells were plated in 96-well plates at 15,000 cells/well and allowed to attach overnight. Cells were then treated with indicated doses of ligand in a final volume of 100 µL for 24 hours. After this period, 10 µL of MTT Reagent was added to each well and allowed to be metabolized for 3 hours at 37 °C, then 100 µL MTT Detergent Reagent was added and allowed to solubilized formazan

crystals and cell membranes for 2 additional hours. Absorbance was read at 570 nm and cytotoxicity was calculated as the percent loss in formazan formation in treated cells as compared to untreated cells. EC₅₀ values were determined using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

For acute treatments, cells were treated with ligand for 60 minutes and then washed twice with fresh ligand-free media to remove any non-irreversibly bound ligand. Fresh ligand-free media (100 µL) was then added to each well and allowed to incubate for 24 hours at 37 °C prior to MTT assay as described above.

5.7 References

- Abate, C., Niso, M., Infantino, V., Menga, A. and Berardi, F. (2015). "Elements in support of the 'non-identity' of the PGRMC1 protein with the sigma2 receptor." European Journal of Pharmacology **758**: 16-23.
- Bowen, W. D., Bertha, C. M., Vilner, B. J. and Rice, K. C. (1995). "CB-64D and CB-184 - Ligands with High Sigma(2) Receptor Affinity and Subtype Selectivity." European Journal of Pharmacology **278**(3): 257-260.
- Bowen, W. D., Crawford, K. W. and Hildreth, J. E. (2004). Sigma-2 receptor agonists and their use in the treatment of hiv infection. United States of America. **WO2004064775 A3**.
- Cassano, G., Gasparre, G., Niso, M., Contino, M., Scalera, V. and Colabufo, N. A. (2009). "F281, synthetic agonist of the sigma-2 receptor, induces Ca²⁺ efflux from the endoplasmic reticulum and mitochondria in SK-N-SH cells." Cell Calcium **45**(4): 340-345.
- Chu, U., Mavlyutov, T., Chu, M.-L., Yang, H., Mesangeau, C., McCurdy, C., Guo, L.-W. and Ruoho, A. (2015). "The 18 kDa Sigma-2 Receptor and PGRMC1 are Derived From Separate Genes." The FASEB Journal **29**(1 Supplement).
- Crawford, K. W. and Bowen, W. D. (2002). "Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines." Cancer Research **62**(1): 313-322.
- Decosta, B. R., He, X. S., Dominguez, C., Cutts, J., Williams, W. and Bowen, W. D. (1994). "A New Approach to the Design of Sigma-2-Selective Ligands - Synthesis and Evaluation of N-[2-(3,4-Dichlorophenyl)Ethyl]-N-Methyl-2(1-Pyrrolidinyl)Ethylamine-Related Polyamines at Sigma-1 and Sigma-2 Receptor Subtypes." Journal of Medicinal Chemistry **37**(2): 314-321.

Garcia, D. R. (2012). Sigma-2 receptor-mediated cytotoxicity and calcium signaling: Evidence for bifurcating pathways. PhD, Brown University.

Hayashi, T. and Su, T. P. (2007). "Sigma-1 receptor chaperones at the ER-Mitochondrion interface regulate Ca²⁺ signaling and cell survival." Cell **131**(3): 596-610.

Hazelwood, S. and Bowen, W. D. (2006). Sigma-2 receptor-mediated apoptosis in human SK-N-SH neuroblastoma cells: Role of lipid rafts, caspases, and mitochondrial depolarization. #4932. American Association for Cancer Research Annual Meeting, Washington, D.C.

Hornick, J. R., Xu, J. B., Vangveravong, S., Tu, Z. D., Mitchem, J. B., Spitzer, D., Goedegebuure, P., Mach, R. H. and Hawkins, W. G. (2010). "The novel sigma-2 receptor ligand SW43 stabilizes pancreas cancer progression in combination with gemcitabine." Molecular Cancer **9**(298).

Izzo, N. J., Staniszewski, A., To, L., Fa, M., Teich, A. F., Saeed, F., Wostein, H., Walko, T., 3rd, Vaswani, A., Wardius, M., Syed, Z., Ravenscroft, J., Mozzoni, K., Silky, C., Rehak, C., Yurko, R., Finn, P., Look, G., Rishton, G., Safferstein, H., Miller, M., Johanson, C., Stopa, E., Windisch, M., Hutter-Paier, B., Shamloo, M., Arancio, O., LeVine, H., 3rd and Catalano, S. M. (2014). "Alzheimer's therapeutics targeting amyloid beta 1-42 oligomers I: Abeta 42 oligomer binding to specific neuronal receptors is displaced by drug candidates that improve cognitive deficits." PLoS One **9**(11): e111898.

Izzo, N. J., Xu, J., Zeng, C., Kirk, M. J., Mozzoni, K., Silky, C., Rehak, C., Yurko, R., Look, G., Rishton, G., Safferstein, H., Cruchaga, C., Goate, A., Cahill, M. A., Arancio, O., Mach, R. H., Craven, R., Head, E., LeVine, H., 3rd, Spires-Jones, T. L. and Catalano, S. M. (2014). "Alzheimer's therapeutics targeting amyloid beta 1-42 oligomers II: Sigma-2/PGRMC1 receptors mediate Abeta 42 oligomer binding and synaptotoxicity." PLoS One **9**(11): e111899.

Kaushal, N., Robson, M. J., Vinnakota, H., Narayanan, S., Avery, B. A., McCurdy, C. R. and Matsumoto, R. R. (2011). "Synthesis and pharmacological evaluation of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79), a cocaine antagonist, in rodents." The AAPS Journal **13**(3): 336-346.

Mach, R. H., Smith, C. R., al-Nabulsi, I., Whirrett, B. R., Childers, S. R. and Wheeler, K. T. (1997). "Sigma 2 receptors as potential biomarkers of proliferation in breast cancer." Cancer Research **57**(1): 156-161.

Mach, R. H. and Wheeler, K. T. (2009). "Development of molecular probes for imaging sigma-2 receptors in vitro and in vivo." Central Nervous System Agents in Medicinal Chemistry **9**(3): 230-245.

McCurdy, C. R., Mesangeau, C., Matsumoto, R. R., Poupaert, J. H., Avery, B. A. and Abdelazeem, A. H. A. (2014). Highly selective sigma receptor ligands. United States of America. **US8686008 B2**.

Nicholson, H., Comeau, A., Mesangeau, C., McCurdy, C. R. and Bowen, W. D. (2015). "Characterization of CM572, a Selective Irreversible Partial Agonist of the Sigma-2 Receptor with Antitumor Activity." Journal of Pharmacology and Experimental Therapeutics **354**(2): 203-212.

Nicholson, H., Mesangeau, C., McCurdy, C. R., and Bowen, W. D. (2016). "Sigma-2 Receptors Play a Role in Cellular Metabolism: Stimulation of Glycolytic Hallmarks by Cm764 in Human SK-N-SH Neuroblastoma." Journal of Pharmacology and Experimental Therapeutics **356**(2): 232-243.

Prezzavento, O., Campisi, A., Ronsisvalle, S., Volti, G. L., Marrazzo, A., Bramanti, V., Cannavo, G., Vanella, L., Cagnotto, A., Mennini, T., Ientile, R. and Ronsisvalle, G. (2007).

"Novel sigma receptor ligands: Synthesis and biological profile." Journal of Medicinal Chemistry **50**(5): 951-961.

Roperto, S., Colabufo, N. A., Inglese, C., Urraro, C., Brun, R., Mezza, E., Staibano, S., Raso, C., Maiolino, P., Russo, V., Palma, E. and Roperto, F. (2010). "Sigma-2 Receptor Expression in Bovine Papillomavirus-Associated Urinary Bladder Tumours." Journal of Comparative Pathology **142**(1): 19-26.

Sahlholm, K., Liao, F., Holtzman, D. M., Xu, J. B. and Mach, R. H. (2015). "Sigma-2 receptor binding is decreased in female, but not male, APP/PS1 mice." Biochemical and Biophysical Research Communications **460**(2): 439-445.

Shoghi, K. I., Xu, J. B., Su, Y., He, J., Rowland, D., Yan, Y., Garbow, J. R., Tu, Z. D., Jones, L. A., Higashikubo, R., Wheeler, K. T., Lubet, R. A., Mach, R. H. and You, M. (2013).

"Quantitative Receptor-Based Imaging of Tumor Proliferation with the Sigma-2 Ligand [F-18]ISO-1." PLoS One **8**(9).

Vilner, B. J., John, C. S. and Bowen, W. D. (1995). "Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines." Cancer Research **55**(2): 408-413.

Wang, X. and Bowen, W. D. (2006). Sigma-2 receptors mediate apoptosis in SK-N-SH neuroblastoma cells via caspase-10-dependent Bid cleavage and mitochondrial release of endonuclease G and apoptosis-inducing factor. Society for Neuroscience, Atlanta, GA, Meeting Planner.

Wheeler, K. T., Wang, L. M., Wallen, C. A., Childers, S. R., Cline, J. M., Keng, P. C. and Mach, R. H. (2000). "Sigma-2 receptors as a biomarker of proliferation in solid tumours." British Journal of Cancer **82**(6): 1223-1232.

Wu, Z. and Bowen, W. D. (2008). "Role of sigma-1 receptor C-terminal segment in inositol 1,4,5-trisphosphate receptor activation: constitutive enhancement of calcium signaling in MCF-7 tumor cells." Journal of Biological Chemistry **283**(42): 28198-28215.

Xu, J. B., Zeng, C. B., Chu, W. H., Pan, F. H., Rothfuss, J. M., Zhang, F. J., Tu, Z. D., Zhou, D., Zeng, D. X., Vangveravong, S., Johnston, F., Spitzer, D., Chang, K. C., Hotchkiss, R. S., Hawkins, W. G., Wheeler, K. T. and Mach, R. H. (2011). "Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site." Nature Communications **2**.

Zeng, C., Rothfuss, J., Zhang, J., Chu, W., Vangveravong, S., Tu, Z., Pan, F., Chang, K. C., Hotchkiss, R. and Mach, R. H. (2012). "Sigma-2 ligands induce tumour cell death by multiple signalling pathways." British Journal of Cancer **106**(4): 693-701.

Zeng, C., Rothfuss, J. M., Zhang, J., Vangveravong, S., Chu, W., Li, S., Tu, Z., Xu, J. and Mach, R. H. (2014). "Functional assays to define agonists and antagonists of the sigma-2 receptor." Analytical Biochemistry **448**: 68-74.

Zeng, C. B., Vangveravong, S., Jones, L. A., Hyrc, K., Chang, K. C., Xu, J. B., Rothfuss, J. M., Goldberg, M. P., Hotchkiss, R. S. and Mach, R. H. (2011). "Characterization and Evaluation of Two Novel Fluorescent Sigma-2 Receptor Ligands as Proliferation Probes." Molecular Imaging **10**(6): 420-433.

Chapter 6: General Conclusions and Discussion

Since their discovery in 1990, sigma-2 receptors have been of increasing interest to researchers for a wide variety of applications. While a growing number of dedicated researchers have demonstrated functions for the sigma-2 receptor in a diverse array of disease states, a vast body of knowledge about the structure, endogenous activators, physiological function, and many other aspects of this receptor remains lacking. In recent years, successful *in vitro*, *in vivo* animal, and human studies targeting sigma-2 receptors for cancer imaging and therapy, Alzheimer's disease, and other implications have expanded the promise with which this receptor is viewed as a therapeutic target. This dissertation focused on increasing the understanding of the role of sigma-2 receptors in cancer *in vitro*, from both a physiological and a therapeutic standpoint.

Activation of the sigma-2 receptor by synthetic agonists leads to cell death. This function for the sigma-2 receptor is widely accepted within the field, and has been demonstrated across a variety of agonists and partial agonists and in a wide range of cancer cell lines as well as *in vivo*. CM572, an isothiocyanate derivative of the well-characterized sigma-2 receptor-selective antagonist SN79, is one such partial agonist. CM572 irreversibly and selectively binds the sigma-2 receptor but not the sigma-1 receptor, lending specificity to its initial effect. While it is possible that CM572 may bind off-target proteins, screens using the parent compound SN79 did not reveal any non-sigma targets that demonstrated binding with significant affinity. CM572 induces apoptotic cell death, as demonstrated by cleavage of full length Bid into a pro-apoptotic fragment. This induction of apoptosis was consistent across neuroblastoma, pancreatic cancer,

and breast cancer cell lines, however significant levels of apoptosis were not achieved by treatment of non-cancerous melanocytes or mammary epithelial cells. This comparison highlights the specificity of the effect, and its potential use for selective killing of cancer cells as a chemotherapeutic agent.

Extrapolating the results of the investigation of CM572, a series of promising hypotheses takes form. The irreversible binding capability suggests two important implications for using CM572 in basic biology as well as translationally with a lens toward treating patients with cancer. First, the highly selective irreversible binding opens an avenue for the possible cloning of the sigma-2 receptor. CM572 can be used to selectively label sigma-2 receptors, the purification of which is the first challenge to the cloning process (see Chapter 7 for detailed discussion). Second, selective irreversible binding of sigma-2 receptors is beneficial in both the imaging and treatment of cancer. The formation of a covalent bond between CM572 and sigma-2 receptors results in washout-resistant induction of apoptosis. This result is significant as the repeated dosing of conventional chemotherapeutic agents is a burden to both patients and caregivers, and the irreversible nature of CM572 allows for a single dose to remain bound to and continually activate the sigma-2 receptor until the cell on which the ligand is bound undergoes apoptosis. This could lead to a less frequent dosing regimen, or possible even chemotherapy that requires only a single dose for non-solid tumors such as leukemias and lymphomas that do not need outer layer breakdown in order to access the tumor core.

Due to its inherent cancer cell selectivity based on differential expression, the sigma-2 receptor is a promising target for chemotherapy that may reduce off-target effects. This hypothesis is supported by the results of an investigation of sigma-2 receptor activity in triple negative breast cancer cell lines. Sigma-2 receptor agonists CM572 and SV119 were able to

potently induce cell death in three diverse types of triple negative breast cancer cells. While triple negative breast cancer is typically difficult to treat partially due to its highly resistant phenotype, sigma-2 receptor-targeted therapy demonstrated equal potency in triple negative breast cancer cell lines as in non-triple negative MCF-7 breast cancer cells. Significantly, these data demonstrate that typical mechanisms of drug resistance that prevent toxicity in triple negative breast cancer cells do not inhibit sigma-2 receptor-mediate cell death, as the viability of triple negative breast cancer cells was reduced with equal potency as in compared to the non-resistant MCF-7 cells. These results indicate that chemotherapy targeting the sigma-2 receptor may be successful in highly drug-resistant and recurrent aggressive types of cancer. This hypothesis is further supported by the caspase-nonessential mechanism that is employed in sigma-2 receptor-mediated cell death in these cell lines, as caspase inactivation is a common method of gaining apoptotic resistance in cancer cells. Furthermore, there was a large therapeutic window between the doses of CM572 and SV119 that were able to induce significant levels of cell death in MCF-7 and triple negative breast cancer cells as compared to non-cancerous primary breast tissue. This further supports the idea that sigma-2 receptor-targeted chemotherapy may be highly selective for cancerous tissue, without significant damage to the surrounding healthy cells.

While the promise that sigma-2 receptors hold as a target for selective and effective chemotherapy is substantial, this cell death-inducing function is unlikely to be the natural or endogenous role for this receptor. Proteins that are upregulated in cancer, as is the sigma-2 receptor, are generally associated with providing a pro-survival benefit to the cells on which they are expressed. Further, the endogenous ligand for sigma-2 receptors, which would exemplify its natural function, has yet to be elucidated. Activation of sigma-2 receptors by the SN79 derivative

CM764 demonstrates a novel alternate and previously unknown function mediated by the sigma-2 receptor. Treatment of SK-N-SH neuroblastoma cells with CM764 potently induces a variety of metabolically stimulative effects, the compilation of which indicates the upregulation of glycolysis. This is the first non-zero, non-cytotoxic functional role for sigma-2 receptors that has been reported.

Increased glycolytic phenotype in cancer cells in normoxic conditions, also called the “Warburg Effect”, has a variety of suspected pro-survival benefits. As cancer cells often have highly mutated DNA, it is possible that the switch to glycolysis, as opposed to oxidative phosphorylation, as the energy source for the cells may allow for energy production while allowing for the speed of DNA synthesis that includes many mistakes in faithful replication. As glycolysis requires far fewer functional enzymes and cofactors than oxidative phosphorylation, the large number of improperly folded and mistranscribed proteins in cancer cells would be less likely to inhibit this process. Another possible benefit of overdependence on glycolysis in cancer cells is the increased production of basic building blocks that are used in the pentose phosphate pathway for synthesis of nucleotides and proteins required for increased rates of replication. However, this is unlikely to be the root cause of the sigma-2 receptor-mediated CM764-induced increase in glycolytic hallmarks, as an increase in neither protein concentration nor proliferation was observed in response to treatment. An alternative hypothesis concerns the hypoxic core of a tumor, in which a lowered dependence on oxygen through reduced oxidative phosphorylation and concurrent increase in glycolytic flux may allow tumor cells to survive in an environment with lowered oxygen content, such as this hypoxic interior of a tumor.

A canonical marker of increased glycolysis in normoxia is stabilization of hypoxia-inducible factor 1- α (HIF1 α). This was observed in CM764-treated SK-N-SH neuroblastoma

cells, and an increase in protein level of a transcriptional target of functional HIF1 α , vascular endothelial growth factor (VEGF), was also induced. The HIF1 α /VEGF signaling mechanism is an important part of the angiogenetic process by which both neoplasms and metastasizing tumors establish new blood supply. This endpoint may represent a physiologically relevant and possibly endogenous role for sigma-2 receptors, as sigma-2 receptors are upregulated in both aggressively metastasizing cancer cells as well as non-cancerous but highly proliferative tissues. Further, this hypothesized role is consistent with the downregulation of sigma-2 receptors in senescent and overconfluent cancer cells, which could correspond to established tumors that do not require the genesis of new vasculature to sustain them. All of these hypothesized roles for the CM764-induced metabolically stimulative function of sigma-2 receptors are possible candidates for the endogenous role of sigma-2 receptors in the human body, and further study is required to assess the viability of each role.

While CM764 was the first compound shown to induce a sigma-2 receptor-mediated non-zero, non-cytotoxic function, it is not the only ligand with such capability. A detailed structure-activity relationship study of single-moiety changes to the core structure of SN79, the parent compound to CM764, revealed five additional structurally related compounds that are able to induce an increase in reduction of MTT in treated SK-N-SH neuroblastoma cells as compared to untreated control cells. Further, all isothiocyanate compounds examined in this series were able to induce cell death in SK-N-SH neuroblastoma cells, consistent with the in-depth study of CM572. Ability to induce apoptosis appeared to be dependent on ability to bind irreversibly rather than electron withdrawing character of the isothiocyanate moiety, as a fluorine substitution in the same position did not yield a cytotoxic ligand. Variations to the SN79 core structure greatly altered sigma receptor affinity and subtype selectivity, providing insight into critical

areas of the receptor binding pocket and pharmacophore of these ligands. An expansion of this structure-activity relationship investigation may lead to a better understanding of regions of the sigma-1 and sigma-2 receptor binding pockets, with an eye towards leaving behind “guess-and-check” ligand synthesis and moving towards rational ligand design.

The work described here highlights the complexity of and delicate balance between pro-survival and cell death functions mediated by the sigma-2 receptor. For the first time, a non-zero, non-cytotoxic function mediated by the sigma-2 receptor has been described that is consistent with a potential physiological role for this receptor. Further, the detailed investigation of CM572 as well as other irreversible ligands that are able to induce cytotoxicity that were revealed in the structure-activity relationship study highlight the potential for the use of sigma-2 receptors in the imaging and treatment of a variety of cancers. These two opposing functions for the sigma-2 receptor, each induced by structurally related yet distinct classes of ligands, suggest that the classification of ligands and function for sigma-2 receptors is in need of updating. Currently, the term “agonist” is used to describe a sigma-2 receptor ligand that induces cell death upon binding and activation. An “antagonist” is able to block agonist activity, although the term has also been used ubiquitously (and perhaps erroneously) to describe a sigma-2 receptor-binding ligand that does not induce cell death.

Initially, two possibilities emerge for the characterization of the CM764-induced metabolically stimulative sigma-2 receptor activity that has been discovered. First, it is possible that CM764 can be incorporated without disruption into the current definitions of ligands as an “inverse agonist”. This would suggest that the endogenous activity of sigma-2 receptors is depressive, and that blocking this basal activity therefore removes the depressive function and metabolism is increased as a byproduct. This proposal is analogous to releasing a tonically

engaged break on a car, whereby the car is no longer tonically stopped but neither is the gas pedal engaged. By this account, “agonist” activity (i.e. CM572, CB-64D, SV119, etc.) would act as the gas pedal (inducing cell death) and antagonists (i.e. SN79) would block the gas pedal from being engaged without stopping tonic breaking (endogenous activity). CM764 and other metabolically stimulative ligands, as inverse agonists, would release the tonically engaged break and allow the car to begin rolling (i.e. increase MTT reduction).

The alternative explanation is far more disruptive to the current classification of sigma-2 receptor ligands and function. Disregarding current ligand classifying definitions, it is likely that the endogenous function of sigma-2 receptors imparts pro-survival benefit to the cells on which they are expressed. It is therefore more likely that the endogenous function of sigma-2 receptors is in line with the metabolically stimulative function induced by CM764 binding than with the cell death function induced by CM572, SV119, or CB-64D binding. Therefore, “agonist” activity would be defined as that which induces the endogenous function, namely metabolic stimulation. In this proposal, CM764 and other stimulative ligands would be classified as “agonists” and ligands that block metabolic stimulation (i.e. BD1047, SN79, etc.) would maintain their designations as “antagonists”.

The complexity in this second proposal is maximized when cytotoxic ligands are considered. These ligands could be hypothesized to function as “inverse agonists”, blocking a metabolic function that is required for cell survival and thus inducing cell death as a byproduct. In this instance, cell death might resemble autophagic and apoptotic pathways that typically result from withdrawal of growth factors, with a variety of mechanisms at play. This is consistent with the wide array of factors, pathways, and types of cell death with which sigma-2 receptor “activation” is currently associated. Indeed, the same ligand in different tissues may induce

different types of cell death or different cell death pathways, or even the same ligand in the same cell line yet at different doses. Autophagy, necrosis, and apoptosis have all been demonstrated to result from treatment of cancer cells with traditionally-defined sigma-2 receptor “agonists” such as SV119, siramesine, and CB-64D.

With continued consistency, although a factor may be implicated in a sigma-2 receptor-induced cell death pathway, blocking this factor is often unable to fully protect cells against death induced by the same agonist. A canonical example is the implication of caspases in many apoptotic mechanisms induced by a variety of sigma-2 receptor activating compounds. Pretreatment of cells with the pan-caspase inhibitor Z-VAD-FMK is often unable to fully protect cells against death induced by the same activating ligand by which caspases were initially implicated, suggesting that alternative mechanisms are available. Further, this proposal provides an explanation for why irreversible ligands induce cell death so potently, while this function cannot be replicated by isoelectronically-substituted analogs. While a definite conclusion will require elucidation of the endogenous ligand for the sigma-2 receptor, it does appear that the metabolically stimulative function discovered in response to sigma-2 receptor activation by CM764 reveals a novel, pro-survival function for the sigma-2 receptor that is more theoretically consistent with the distribution of these receptors within cancers and the human body. While additional hypothesis including the existence of sigma-2 receptor subtypes or differential intracellular binding partners have not yet been directly supported, neither can they be ruled out based on the available data.

Chapter 7: Future Directions

The discovery of a non-zero, non-cytotoxic function mediated by sigma-2 receptors coupled to the ever-expanding body of research implicating sigma-2 receptors in a variety of diseases demonstrates the diverse expanse of directions that could be explored in future projects. Efforts to understand several of these topics are already underway, while others are nascent in their investigations. Below are several avenues for exploration that directly result from the findings described in this dissertation.

7.1 Multidrug Resistance

The study of sigma-2 receptor-mediated cytotoxicity in triple-negative breast cancer cell lines suggested that sigma-2 receptor-mediated therapy is not subject to traditional mechanisms of drug resistance associated with many conventional chemotherapies. Thus, this receptor may provide a target for highly resistant cancers for which there are few alternative therapeutic options available. While the results presented in chapter 3 provide preliminary evidence indicating the potential success of such therapy, a detailed analysis including a variety of types of cancer and the generation of resistant cell lines that are challenged with sigma-2 receptor ligands would provide a more substantial proof of concept.

As multidrug resistance can result from a variety of mechanisms, a multifaceted approach to this investigation would be most likely to be comprehensive. An initial question that must be

answered surrounds whether or not resistance to sigma-2 receptor-mediated therapy itself can be generated. This could be investigated by prolonged exposure to increasing doses of agonist, selecting surviving clones upon each round of treatment. Resistance could be quantified by comparison of agonist EC_{50} with cells that have not been pre-exposed, with a rightward shift in the dose-response curve indicating resistance. It would also be important to investigate each class of compounds individually in order to determine whether or not it is a substrate for drug resistance pumps by comparing the potency of each prototypic agonist when dosed alone or in combination with pump inhibitors.

Avoidance of traditional mechanisms of resistance could be investigated by increasing and decreasing expression of canonical drug resistance proteins by transfection of cDNA or siRNA for each gene individually. For example, MCF-7 cells do not typically express multidrug resistance protein 1 (MRP1). These cells could be transfected with *MRP1* to overexpress the protein, and the EC_{50} of a sigma-2 receptor agonist could be compared in the transfected line to the wild-type MCF-7 line. If there were a rightward shift in the dose-response curve in the transfected cell line, it could be concluded that MRP1 overexpression reduces efficacy of sigma-2 receptor agonist. An unchanged EC_{50} would indicate that sigma-2 receptor agonist therapy is not subject to MRP1-mediated resistance, and may be an optimal target for cancers in which MRP1 resistance causes traditional chemotherapy to be unsuccessful. Overexpression of P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP) are also common causes of chemotherapeutic resistance, and would be ideal candidates for analysis.

This same approach could be employed in reverse for confirmation. siRNA could be used to knock down a resistance protein in a cell line that endogenously overexpresses it. If sigma-2 receptor agonist therapy were susceptible to this mechanism of resistance, a leftward shift in the

dose-response curve would be expected to result from siRNA knockdown. For example, knockdown of Pgp in SK-N-SH cells, which endogenously overexpress Pgp, would be expected to result in a leftward shift in the dose-response curve of sigma-2 agonist treatment if the agonist were a substrate for Pgp. Alternatively, if the agonist were not a Pgp substrate, then no change in EC_{50} would be expected. Using either approach, resistance could be confirmed using typical resistance-susceptible agents, such as vincristine or adriamycin.

There is evidence in the literature for the use of sigma-2 receptor ligands as adjuvants for traditional chemotherapeutics (Crawford and Bowen 2002; Garg et al. 2014; Hornick et al. 2010; Kashiwagi et al. 2009). It is possible that modulation of multidrug resistance pathways contributes to the success of such combination therapy. Initial results of proteomics analysis between CM764-treated and untreated control SK-N-SH neuroblastoma cells indicate significant changes in at least 20 proteins regulated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (sample processing and proteomics performed at the Lifespan CORO Proteomic Facility on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA) and facilitated by Steven Berardinelli and Art Salomon, analysis performed using the DAVID Bioinformatics Database 6.7 (NIAID, NIH, Bethesda, MD)). Confirming these results, western blot analysis of SK-N-SH neuroblastoma cells treated with 10 μ M CM764 demonstrated a decrease in Nrf2 protein expression with increasing exposure time as compared to untreated control cells (Figure 1). As Nrf2 is known to promote the increased synthesis and activity of a number of drug metabolizing enzymes (Shen and Kong 2009; Ma 2013), these data suggest a possible implication for CM764-induced activation of sigma-2 receptors in sensitization of cancer cells to chemotherapeutics. Decreased expression of Nrf2 could lead to decreased

expression and activity of drug metabolizing enzymes, thus potentiating the cells to the effects of antineoplastic drugs.

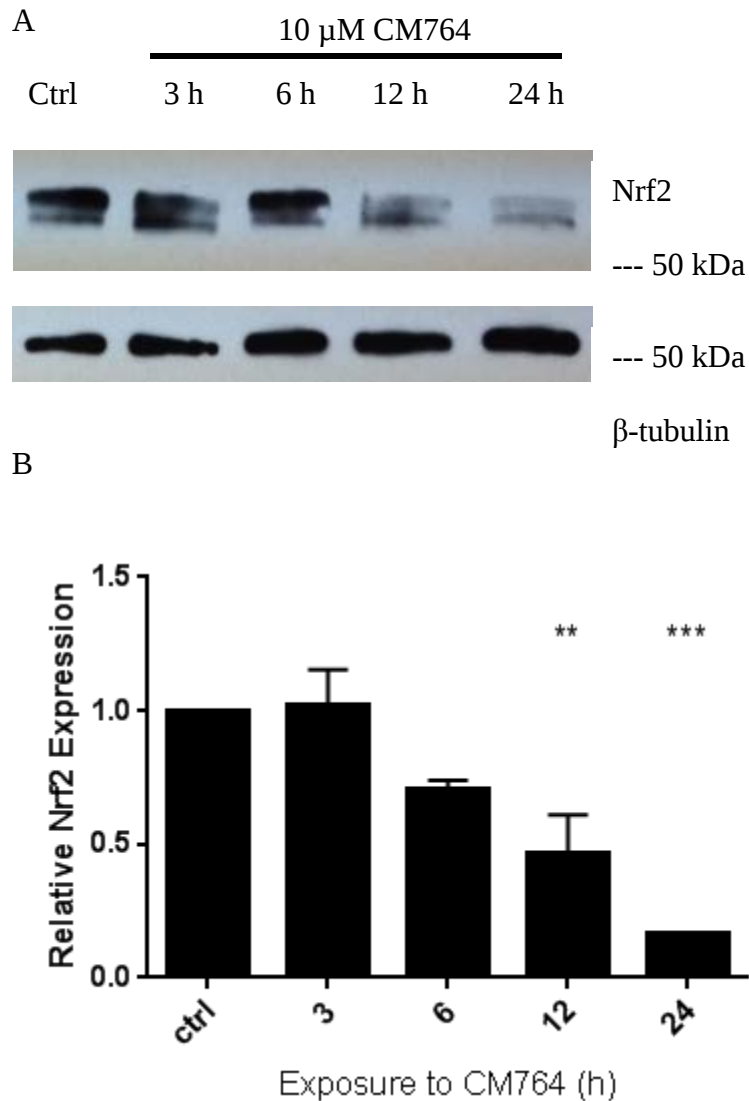


Figure 1. Effect of CM764 treatment on Nrf2 protein expression in SK-N-SH

neuroblastoma cells. Cell lysates of SK-N-SH neuroblastoma cells were made after treatment with 10 μ M CM764 for the indicated amount of time and were used for Western blotting for Nrf2 as described in Materials and Methods. Panel A: Nrf2 Western blot and β -tubulin loading control. Panel B: Independent blots were quantified and ratio of Nrf2/ β -tubulin determined and averaged for all experiments. Data is expressed as fold change in ratio relative to control treated without CM764. Treatment with CM764 resulted in a decrease in Nrf2 level 12 h after exposure

(one-way ANOVA $F=32.56$, Dunnett's test for multiple comparisons as compared to untreated control $**p<0.01$, $***p<0.001$). Results are an average of two independent experiments. A representative blot is shown.

The ability of CM764, and possibly other metabolically stimulative sigma-2 receptor ligands, to affect drug metabolizing enzyme expression and thus drug resistance suggests another possible implication for sigma-2 receptors in the treatment of cancer. Combination therapy involving pretreatment of cancer cells with CM764 followed by dosing with a conventional chemotherapeutic agent may allow for initial downregulation of DME proteins that would otherwise prevent the traditional chemotherapeutic agent from being efficacious. After initial confirmation of downregulation of specific resistance proteins by CM764, this combination therapy approach could be tested in a cell line that typically overexpresses that resistance protein. Changes in protein concentration could be compared by western blotting and increased efficacy could be measured by shifts in EC_{50} value, with concurrent measurement of the effect of CM764 without follow-up chemotherapeutic challenge monitored for control. Both the cytotoxic sigma-2 receptor-mediated therapeutic approach as well as the stimulative CM764-mediated combination therapy approach could have significant implications for the treatment of drug-resistant cancers for which there are currently few alternatives.

7.2 Hypoxia

Treatment of SK-N-SH neuroblastoma cells with CM764 results in upregulation of hallmarks of glycolysis, including stabilization of HIF1 α under normal oxygen conditions in which HIF1 α typically would be degraded rapidly. Further, the depression of reaction oxygen species may indicate that oxygen is not being processed or consumed at high rates in CM764-treated cells. These data suggest a possible implication for sigma-2 receptor function in conditions of hypoxia, where pretreatment with CM764 may shift cells to a metabolic program

that has a decreased dependence on oxygen. In this way, cells treated with CM764 may be able to withstand hypoxic conditions with increased resilience as compared to untreated cells.

Initially, the pretense of a CM764-induced depression in oxygen consumption could be investigated using a Seahorse XF Extracellular Flux Analyzer. Using this technique, the oxygen consumption rate of untreated and CM764-treated cells could be directly compared. This hypothesis could be tested further with the use of a hypoxic chamber. Cells could be pretreated with CM764 and then placed in the hypoxic chamber, and their survival over time could be compared to cells that were not pretreated but were otherwise subjected to the same conditions. If pretreatment with CM764 were able to reduce dependence on oxygen, then the pretreated cells would be expected to survive longer than untreated cells. Additionally, the experiment could be performed with a “rescue” design, in which cells could be exposed to hypoxic conditions briefly and then treated with CM764 prior to continued hypoxia exposure to determine whether the CM764-induced metabolic changes are able to help cells survive after initial hypoxic exposure. This “rescue” simulation could be extrapolated to have relevance for patient conditions of temporary hypoxia such as ischemia or stroke, during which treatment after initial exposure might prevent further tissue damage caused by prolonged exposure to hypoxia.

7.3 Angiogenesis

Similarly to the proposed investigation into the implications of CM764 treatment in hypoxia, CM764 may have implications in angiogenesis. Tumors in which hypoxia-inducible factor-1 α (HIF1 α) is constitutively active are generally more highly vascularized, presumably due to promotion of the transcriptional target VEGF (Maher and Kaelin 1997). Both HIF1 α and

vascular endothelial growth factor (VEGF) are upregulated in SK-N-SH cells in response to treatment with CM764, and activation of this signaling pathway is a well-characterized initiator of angiogenesis. As tumors grow, they need to establish their own blood supply in order to bring nutrients and oxygen to the cells. They often satisfy this need through VEGF expression, which causes endothelial cells to proliferate and form new blood vessels. This is another possible pro-survival benefit to cancer cells that upregulate sigma-2 receptors.

Preliminary data suggests that human umbilical vein endothelial cells (HUVEC) do not respond directly to CM764 (Figure 2). This might be explained by a lack of sigma-2 receptor expression, although this hypothesis requires confirmation by Scatchard analysis of [³H]DTG binding in HUVEC cell membrane homogenates. The possibility that CM764 may induce sigma-2 receptor-mediated angiogenesis could be investigated by the use of a transwell setup with SK-N-SH cells (or another cell line that responds well to CM764) over HUVEC cells. If CM764 treatment caused an increase in VEGF release from SK-N-SH cells that was adequate for initiation of angiogenesis, then the HUVEC cells would be expected to respond to the added VEGF in the media by forming tubules that could be imaged using a microscope. Similarly, the rate of proliferation in HUVEC cells responding to VEGF release from SK-N-SH cells treated with CM764 would be expected to increase in comparison to the same experimental setup without CM764 treatment. The validity of the experiment could be verified by the direct addition of VEGF to the media.

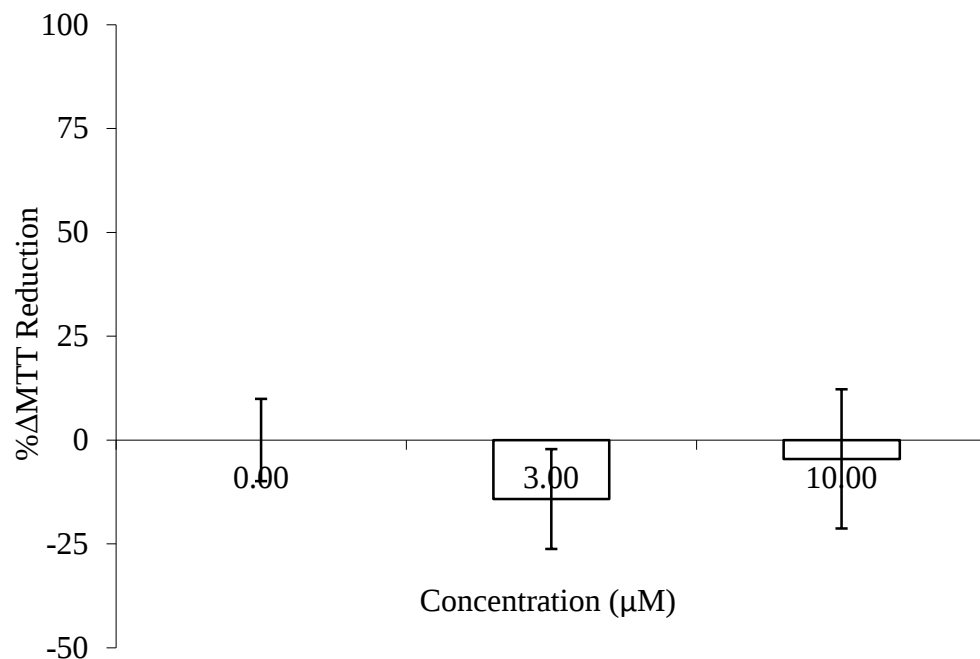


Figure 2. **Effect of CM764 on MTT reduction in HUVEC cells.** Cells were treated with CM764 at the indicated doses for 24 h prior to MTT assay as described in Methods. Treatment with CM764 did not induce significant changes in reduction of MTT in HUVEC cells. This could be due to a lack of sigma-2 receptor expression in HUVEC cells or failure of sigma-2 receptors to become activated in response to CM764 at the indicated dose after 24 h. Results are shown as an average percent change in MTT reduction $\pm$ S.D. as compared to an untreated control in each cell line for 5 replicates in a single preliminary experiment.

The specificity of this effect could be investigated in two ways: 1) SN79 could be used to block sigma-2 receptor activation, indicating specificity to the sigma-2 receptor, and 2) anti-VEGF antibodies could be used to block this signaling pathway in the HUVEC cells, indicating VEGF specificity. If CM764 treatment were able to induce downstream angiogenesis, this could add to an explanation of a possible physiological role for the sigma-2 receptor. This function also would be consistent with the dynamic expression of sigma-2 receptors based on proliferative status, as quiescent tumors would not require the establishment of new vasculature.

7.4 Ceramide

There is literature evidence that implicates ceramide formation in the apoptotic pathway induced by sigma-2 receptor activation in both MCF-7 breast cancer cells and SK-N-SH neuroblastoma cells (Crawford et al. 2002; Bowen et al. 2001; Bowen et al. 2000). As described in Chapter 1, ceramide is able to promote both cell death and cell survival, dependent on circumstance and pathway. The results in the preceding chapters demonstrate the ability of the sigma-2 receptor to promote cell death and potential cell survival functions, and the known connection to ceramide synthesis and metabolism suggests this may play a critical role in the determination of cell fate downstream of sigma-2 receptor activation.

While the evidence for ceramide playing a role in apoptosis initiated by sigma-2 receptor activation has been demonstrated previously, ceramide has not yet been connected to the sigma-2 receptor-mediated changes in cellular metabolism canonically induced in response to treatment with CM764. Preliminary data indicates that fumonisins B1, an inhibitor of ceramide synthase, is able to attenuate the increase in MTT reduction in SK-N-SH neuroblastoma cells induced by

CM764 treatment (Figure 3). This suggests that synthesis of ceramide plays an integral role in the increase in MTT reduction induced by CM764. To confirm this hypothesis, siRNA against ceramide synthase could be used to knock down the protein and measure the response in MTT reduction to CM764 treatment. If ceramide synthase were an integral part of this mechanism, then the response to CM764 would be expected to be reduced or eliminated in cells with ceramide synthase knocked down.

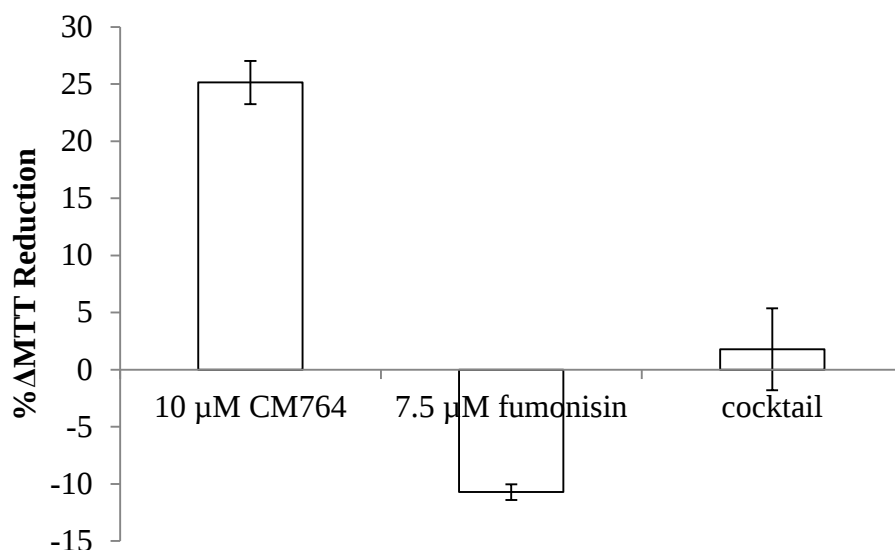


Figure 3. **Attenuation of CM764-induced stimulation of MTT reduction by fumonisin B1 in SK-N-SH neuroblastoma.** Cells were treated with CM764, fumonisin B1, or a cocktail of the two compounds at the indicated doses for 24 h prior to MTT assay as described in Methods. Treatment with fumonisin B1 alone promoted only very modest changes in reduction of MTT in neuroblastoma cells, however combined treatment with fumonisin B1 and CM764 eliminated the increase in MTT reduction observed in cells treated with CM764 alone beyond the additive effect of the two compounds independently. These results suggest that inhibition of ceramide synthase by fumonisin B1 reduces the ability of CM764 to stimulate MTT reduction through sigma-2 receptors, and may implicate ceramide synthase in CM764-induced metabolic changes. Results are shown as an average percent change in MTT reduction $\pm$ S.D. as compared to an untreated control in each cell line for compiled results from two independent experiments, with each experiment having 5 replicates per condition.

The involvement of ceramide in sigma-2 receptor-induced cellular processes could be further investigated using an ELISA to measure ceramide levels in response to CM764 treatment. Ceramide levels could then be neutralized in permeabilized cells using anti-ceramide antibodies and response to CM764 could be measured using the MTT assay. Recently, the balance between ceramide and ceramide-1-phosphate was implicated in the balance between pro-survival and cytotoxic cellular mechanisms in A549 human lung adenocarcinoma cells (Mitra et al. 2007). This may provide an explanation for how sigma-2 receptor activation could result in both pro-survival pathways and cytotoxic pathways. Ceramide-1-phosphate is generally pro-survival, while accumulation of this lipid results in dephosphorylation by ceramide-1-phosphate phosphatase and formation of pro-apoptotic ceramide. This process can also be reversed by ceramide kinase, which creates ceramide-1-phosphate from ceramide.

Activation of ceramide kinase may reduce ceramide levels enhanced by ceramide synthase and create ceramide-1-phosphate, while overaccumulation of ceramide-1-phosphate may result in dephosphorylation to apoptotic ceramide. This hypothesis is consistent with the low-dose stimulation of MTT reduction by CM572, which becomes toxic upon increasing concentration (Chapter 2). CM764-induced reduction of ceramide would also be consistent with the decrease in reactive oxygen species that is observed (Chapter 4), as a reduction in ceramide might also reduce ROS caused by ceramide-induced mitochondrial outer membrane permeabilization. The importance of the balance between ceramide-1-phosphate and ceramide could be further investigated by measuring the effects of siRNA knockdown and small molecule inhibition of ceramide kinase and ceramide-1-phosphate phosphatase on CM764- and CM572-induced changes in SK-N-SH neuroblastoma. With the dose-dependent stimulation and

cytotoxicity induced by CM572 treatment, ceramide levels might be expected to initially decrease after low-dose treatment and then increase upon exposure to high doses of CM572.

7.5 Cloning the Sigma-2 Receptor

The development of CM572 as a sigma-2 receptor-selective, irreversibly binding ligand opens the door for a new possible approach to the cloning of the sigma-2 receptor. Using LC/MS, it is possible to obtain a unique “fingerprint” for the fragmentation of CM572 bound to a known control peptide. Rat liver membrane homogenates could be treated with CM572, washed extensively to remove non-covalently non-specifically bound compound, and run on a gel to select for size and digested with an enzyme such as trypsin to obtain peptide fragments. Selective ion monitoring for the ligand-bound “fingerprint” in CM572-treated rat liver membrane homogenate could then be used to isolate CM572 covalently bound to a fragment of the sigma-2 receptor. Tandem sequencing MS could be used to determine the sequence of the protein fragment, which could then be used to make PCR primers with which to isolate the sigma-2 receptor gene.

The potential for success in this approach could be enhanced by enrichment for CM572-labeled peptide by running HPLC on treated and untreated peptide digests, and selectively continuing the procedure only with the eluent in which a peak shift was observed between the treated and untreated samples (indicating the presence of CM572). A potential pitfall of this approach is the possibility that CM572 may bind other proteins, which could be assessed by performing a commercial screen of CM572 for likely proteins as has been performed for the parent compound, SN79. If CM572 is found to have off-target binding for proteins that are

largely different in size than the sigma-2 receptor, then it is not problematic as these proteins will be removed during the size exclusion in the gel. If the off-target binding is found to be proteins of similar size and character to the sigma-2 receptor, then these proteins may need to be masked during initial treatment of the rat livers with CM572 in order to increase specificity.

Chapter 8: Detailed Methodology

8.1 Cell Culture

8.1.1 General Cell Culture Reagents

Unless otherwise specified below, cells were cultured in Minimal Essential Media (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 1 mM sodium pyruvate, 1X MEM Non-Essential Amino Acids (Gibco, Grand Island, NY), 1X Penicillin-Streptomycin (Invitrogen, Carlsbad, CA), 10 mg/L human insulin (SAFC Biosciences, Lenexa, KS or Invitrogen, Carlsbad, CA), and 26.2 mM sodium pyruvate. The final pH of the media was adjusted to 7.2 prior to sterile filtration through a 22 μm filter. Cells were maintained in an incubator at 37°C with 5% CO₂ in a humidified atmosphere. All cells investigated in this dissertation were adherent. Full volume of media was changed every 2-3 days unless otherwise specified. Cells were passaged at 70% confluency using 2.5 mM ethylenediaminetetraacetic acid in Dulbecco's Phosphate Buffered Saline (pH 7.2 and sterile filtered through a 22 μm filter) to detach cells prior to centrifugation for 5 minutes at 223g at room temperature. Pelleted cells were resuspended in 5 mL media and passaged into 75 cm² flasks unless otherwise specified. For cell counting, a 10 μL aliquot of resuspended cells in media was counted using a hemocytometer. Cells were frozen in 5% dimethylsulfoxide in media and placed directly into a cryofreezer in liquid nitrogen or placed in a -80°C freezer for 1-3 days prior to relocation into the cryofreezer.

8.1.2 HEK293 T/17 Human Embryonic Kidney Cells

HEK293 T/17 cells were a generous gift from Dr. Elena Oancea at Brown University. These cells are derived from human fetuses and were grown in media as described in section 9.1.1. Cells were typically passaged at 1:2 or 1:3 to maintain growth rate for seeding every 2-3 days. HEK293 T/17 cells were not used above passage 15.

8.1.3 HEM Human Epidermal Melanocyte Cells

HEM cells were a generous gift from Dr. Elena Oancea at Brown University. These primary cells are derived from neonatal foreskin and were grown in Medium 254 (Life Technologies, Grand Island, NY) supplemented with 5% Human Melanocyte Growth Supplement 2 (Life Technologies, Grand Island, NY). Cells were proliferative and were typically passaged at 1:2 once per week. Passaging procedure was followed as described in section 9.1.1. HEM cells were not used above passage 10.

8.1.4 HMEC Human Mammary Epithelial Cells

Normal prestasis human mammary epithelial cells were provided by Dr. Martha Stampfer at Lawrence Berkeley National Laboratory at the University of California HMEC Bank funded by the Department of Defense, Department of Energy, and National Institutes of Health. These primary cells (HMEC 184 AG358) were unfrozen and initially cultured in 100 mm tissue culture treated petri dishes, then passaged into 75 cm² flasks. Unique media was used to culture these cells, consisting of 1:1 Medium171 (Invitrogen, Carlsbad, CA) : DMEM/F-12 (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.25% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 35 µg/mL bovine pituitary

extract (Lonza, Hopkinton, MA), 100 ng/mL human insulin (Invitrogen, Carlsbad, CA), 10 ng/mL insulin growth factor-1 (Sigma-Aldrich, St. Louis, MO), 5 μ M isoprenaline hydrochloride (Sigma-Aldrich, St. Louis, MO), 0.3 μ g/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 2.5 μ g/mL human apo-transferrin (Sigma-Aldrich, St. Louis, MO), 0.1 nM oxytocin (Bachem, Torrance, CA), 0.5 ng/mL cholera toxin from *vibrio cholerae* (Sigma-Aldrich, St. Louis, MO), 5 ng/mL epithelial growth factor recombinant human protein (Invitrogen, Carlsbad, CA), 0.5 nM β -estradiol (Sigma-Aldrich, St. Louis, MO), 5 nM 3,3',5-triiodo-L-thyronine (Sigma-Aldrich, St. Louis, MO), 0.10% AlbuMAX I Lipid Rich BSA (Invitrogen, Carlsbad, CA), and 1X Penicillin-Streptomycin (Invitrogen, Carlsbad, CA). Cells were proliferative and were typically passaged at 1:2 once or twice per week. For passaging, cells were washed once with trypsin-Versene (ethylenediaminetetraacetic acid) (Lonza, Hopkinton, MA) prior to trypsin-versene dissociation at 37°C for 5 minutes or until most cells were rounded. Cells were then centrifuged for 5 minutes at 223g, spent media was removed, and pellet was resuspended and seeded for plating or culture. HMEC cells were not used above passage 10.

8.1.5 MCF-7 Human Breast Adenocarcinoma Cells

MCF-7 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). These cells are derived from a pleural effusion metastasis of a mammary gland adenocarcinoma from a 69-year old female patient and were grown in media as described in section 9.1.1. Cells were typically passaged at 1:2 or 1:3 to maintain growth rate for seeding every 2-3 days. MCF-7 cells were not used above passage 30.

8.1.6 MG-63 Human Osteosarcoma Cells

MG-63 cells were a generous gift from Dr. Eric Darling at Brown University. These cells are derived from an osteosarcoma in the bone of a 14-year old male patient and were grown in media as described in section 9.1.1. Cells were typically passaged at 1:4 to maintain growth rate for seeding every 2-3 days. MG-63 cells were not used above passage 10.

8.1.7 PANC-1 Human Pancreatic Carcinoma Cells

PANC-1 cells were obtained from ATCC (Manassas, VA). These cells are derived from the pancreas of a 56-year old male patient and were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 1X Penicillin-Streptomycin (Invitrogen, Carlsbad, CA), and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Cells were typically passaged at 1:2 or 1:3 to maintain growth rate for seeding every 2-3 days. PANC-1 cells were not used above passage 40.

8.1.8 SK-N-SH Human Neuroblastoma Cells

SK-N-SH cells were obtained from ATCC (Manassas, VA). These cells are derived from a thigh bone marrow metastasis of an adrenal gland-derived neuroblastoma from a 4-year old female patient and were grown in media as described in section 9.1.1. Cells were typically passaged at 1:2 or 1:3 to maintain growth rate for seeding every 2-3 days. SK-N-SH cells were not used above passage 35.

8.1.9 Triple Negative Breast Cancer Cells

Three triple negative breast cancer cell lines were used: MDA-MB-231, MDA-MB-453, and MDA-MB-468. All TNBC cell lines were obtained from ATCC. MDA-MB-231 cells are derived from a pleural effusion metastasis of a mammary gland breast adenocarcinoma in a 51-year old female patient. MDA-MB-453 cells are derived from a pericardial effusion metastasis of a mammary gland breast carcinoma in a 48-year old female patient. MDA-MB-468 cells are derived from a pleural effusion metastasis of a mammary gland breast adenocarcinoma in a 51-year old female patient. All cells were grown in media as described in section 9.1.1. MDA-MB-231 and MDA-MB-453 cells were passaged at 1:4 or 1:5 to maintain growth rate for seeding every 2-3 days. MDA-MB-468 cells were passaged at 1:2 once or twice per week. All TNBC cells were used below passage 20.

8.4 Radioligand Binding Assays

8.4.1 Liver Membrane Preparation

Male Sprague Dawley rat livers (Biochemed Services, Winchester, VA or Pel-Freez Biologicals, Rogers, AR) were stored at -80°C upon receipt. For membrane preparation, livers were thawed slowly on ice and cut into ~ 0.5-1 cm³ pieces for homogenization. Liver pieces were put into a 55 mL glass and Teflon homogenizer in an ice bucket in ~3.5 g portions with 10 mL/g 10 mM Tris pH 7.4 at 4°C with 0.32 M sucrose and an electric drill was attached using the homogenizer piston-type pestle as the drill bit. Using ~1/3 power, the drill was used to spin the pestle while homogenizing the liver with 10-15 vertical strokes without removing the

pestle from below the level of liquid in the homogenizer while holding the homogenizer against the bottom of the ice bucket. Care was taken to avoid increasing the temperature of the liver through friction heat generated by the drill, as well as to avoid creating bubbles or foam. This procedure was repeated for all liver tissue in repeated ~3.5 g portions.

Liver homogenate was first centrifuged at 1000*g* for 10 minutes at 4°C to remove large blood vessels, fur, and other contaminants. Supernatant was then transferred to clean tubes and pellets were discarded. Supernatant was centrifuged at 31,000*g* for 15 minutes at 4°C, after which supernatant was discarded and one pellet was resuspended in ~2 mL total volume 10 mM Tris pH 7.4 at room temperature using a plastic transfer pipet. The resuspended homogenate was then transferred to another pellet-containing tube, and was used to resuspend the next pellet. This process was continued without adding additional volume until all pellets had been resuspended. Buffer (10 mM Tris pH 7.4 at room temperature) was then added to a final volume of 3 mL per gram of initial liver weight (including 2 mL 10 mM Tris pH 7.4 added to resuspend pellets but not including pellet volumes themselves).

Suspension was then allowed to rest 15 minutes at room temperature prior to centrifugation at 31,000*g* for 15 minutes at 4°C. Supernatant was discarded and a plastic transfer pipet was used to resuspend one pellet in 0.75 mL 10 mM Tris pH 7.4 at room temperature per gram of initial liver weight including pellet volume while avoiding formation of bubbles. Suspension was then transferred to next pellet-containing tube and pellet was resuspended without adding additional volume. This process was repeated until all pellets were resuspended in the same suspension. A 5 µL aliquot was reserved for a protein concentration assay (adjusted to 1:100-1:1000 dilution for standard curve extrapolation) (see section 8.5.2), and the rest of the homogenate was distributed into 750-1000 µL aliquots and stored at -80°C.

8.4.2 Cell Line Membrane Preparation

For whole cell lysate total membrane preparation, cells from ~10 175 cm² tissue culture flasks, each at ~70% confluency, were used per preparation. Cells were dissociated using 2.5 mM ethylenediaminetetraacetic acid in Dulbecco's Phosphate Buffered Saline and 5 flasks' worth of cells were combined into each of two 50 mL conical tubes for centrifugation for 5 minutes at 223g at room temperature. Supernatant was discarded and each pellet was resuspended in 5 mL Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY). A 10 µL aliquot was used for counting with a hemocytometer. Cells were then centrifuged again for 5 minutes at 223g at room temperature, supernatant was discarded, and pellet was resuspended in homogenization buffer (10 mM Tris pH 7.4 at 4°C with 0.32 M sucrose, with protease inhibitor cocktail (Pierce, Rockland, IL) added immediately prior to use for a final concentration of 1X) for a final concentration of 10⁷ cells/mL buffer using a 1000 mL micropipette tip with the very end cut off to widen the opening to avoid shearing of membranes.

Suspension was then transferred to a Teflon and glass homogenizer and hand-homogenized with 8-10 strokes while avoiding the formation of bubbles and foam. Homogenate was then centrifuged at 105,000g for 60 minutes at 4°C in tubes that were between 2/3 and 3/4 full, and supernatant was discarded. Pellet was resuspended in 10 mM Tris pH 7.4 at 4°C using a 1000 mL micropipette tip with the very end cut off or a plastic transfer pipet to a final concentration of 15-20 mg/mL (~150 µL buffer added). A 5 µL aliquot was reserved for a protein concentration assay (adjusted to 1:100-1:1000 dilution for standard curve extrapolation) (see section 8.5.2), and the rest of the homogenate was distributed into 750-1000 µL aliquots and stored at -80°C.

8.4.3 Scatchard Analysis

Whole cell lysate membrane preparations were prepared as described in section 8.4.2 for Scatchard analysis. For determination of sigma-1 receptor binding sites, [^3H](+)-pentazocine was used. For determination of sigma-2 receptor binding sites, [^3H]di-o-tolylguanidine (DTG) was used in the presence of non-radiolabeled (+)-pentazocine to mask sigma-1 receptor binding sites. In both cases, 10 μM haloperidol was used to block all sigma-1 and sigma-2 receptor binding sites for determination of non-specific radioligand binding. All Scatchard analysis experiments were performed in 50 mM Tris pH 8.0 at room temperature in duplicate for each independent experiment.

Radioligand working solutions were made (50 nM for [^3H]DTG, 30 nM for [^3H](+)-pentazocine) using 50 mM Tris pH 8.0 at room temperature for the diluent and concentrations were confirmed by scintillation counter measurement with 5 μL radioligand working solution in 4.5 mL Ecoscint H scintillation solution (National Diagnostics, Atlanta, GA), using the reported specific activity for each radioligand source vial for calculations. Each experimental condition was then prepared in 13 x 100 disposable borosilicate glass test tubes. For sigma-2 receptor binding analysis, all conditions were supplemented with a final concentration of 100 nM (+)-pentazocine to mask sigma-1 receptor binding sites, as DTG does not demonstrate high subtype selectivity. Non-specific binding (containing 10 μM haloperidol) and total binding were determined individually for each experiment, and all experiments used 250 μg membrane homogenate unless otherwise noted.

The hot/cold method of Scatchard analysis was used to limit extraneous use of radioligand. The concentration of [^3H]DTG was held constant at 5 nM or 10 nM, with increasing

concentrations of unlabeled DTG added to each condition from 20 nM to 340 nM. Thus, the final concentrations of total DTG (radiolabeled and unlabeled) ranged from 5 nM to 350 nM. When preparing each condition, buffer and unlabeled DTG were added first and radioligand was the second to last component added to each test tube, followed only by the membrane homogenate. For non-specific binding determination conditions, 10 μ M haloperidol was also added to tubes. For total binding determination, no unlabeled DTG was added. The final volume in all test tubes was 250 μ L. Once all components had been added for all conditions, test tubes were incubated in a water bath at 37°C for 120 minutes while shaking. During the final 30 minutes of the incubation, a Whatman Glass Microfiber Grade GF/B filter (Brandel, Gaithersburg, MD) was placed in 0.05% polyethyleneimine to soak.

During the last 5 minutes of the incubation, a Brandel 48-Sample Semi-Automated Cell Harvester (Brandel, Gaithersburg, MD) was primed for use by flushing three times with 10 mL distilled water per wash, then flushing three times with 10 mL 10 mM Tris pH 7.4 at 4°C. Extra care was taken to ensure that the buffer was pH 7.4 after it had been cooled to 4°C, as the pH of Tris buffers shifts with temperature. The pre-soaked filter was then placed into the cell harvester, the rack of test tubes containing the experimental conditions was placed onto the cell harvester, and the incubation was terminated by the addition of ~5 mL 10 mM Tris pH 7.4 at °C. The contents of each test tube were then aspirated and passed over the filter, followed by two additional washes of the test tubes with ~5 mL 10 mM Tris pH 7.4 at 4°C per wash and passing over the filter. The filter was then removed from the cell harvester and the area used for each experimental condition was cut out and transferred to a plastic scintillation vial. A 4.5 mL volume of Ecoscint H scintillation solution (National Diagnostics, Atlanta, GA) was then added to each scintillation vial containing an area of filter, and samples were incubated overnight at

room temperature. The cell harvester was cleaned by washing three times with distilled water. Radioactivity of the samples was measured the next day using a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter with each sample being measured for two minutes.

To calculate receptor occupancy and density, specific activity of all DTG added to each sample was calculated based on the dilution of radiolabeled DTG in the total amount of radiolabeled and unlabeled DTG. Measurements for duplicates of each condition were averaged. Radioactivity counts measured in the non-specific binding condition were subtracted from each reading, and the Scatchard equation was used to determine fmol DTG bound/mg protein as well as bound DTG/free DTG. These values were then plotted on the x- and y-axes, respectively, for each concentration of DTG using GraphPad Prism 6 (GraphPad Software, La Jolla, CA) to create a straight line. The affinity of DTG binding for its site (K_d) was determined by the negative inverse of the slope of the line, and the receptor density (B_{max}) was determined by the x-intercept. For data that did not immediately appear linear, the possibility of a two-site fit was also assessed.

8.4.4 Inferred Affinity (K_i) Determination by Competition Binding

Rat liver membrane homogenates prepared as described in section 8.4.1 were used to determine inferred affinity of ligands for sigma receptors based on competition with [3H](+)-pentazocine for sigma-1 receptor affinity and competition with [3H]DTG in the presence of unlabeled (+)-pentazocine to mask sigma-1 receptors for selective sigma-2 receptor binding affinity. As isothiocyanate compounds may bind Tris, HEPES buffer was used in place of Tris in isothiocyanate ligand determinations and, for consistency of comparison, for determination of

the structurally related ligands described in Chapter 5. Radioligand working solutions were made and radioactivity measured as described in section 8.4.3.

Test ligand was diluted in 10 mM HEPES pH 7.4 at room temperature to make stock solutions. Borosilicate glass test tubes were used to prepare experimental conditions, with each experiment having a non-specific binding condition and a total binding condition as described in section 8.4.3, and all conditions prepared in duplicate. Varied concentrations of test ligand between 0.1 and 50,000 nM to test tubes containing 10 mM HEPES pH 7.4 at room temperature such that the final volume of all test tubes with all components added was 500 μ L. A final concentration of 1 nM unlabeled (+)-pentazocine was added to all test tubes measuring sigma-2 receptor binding in order to mask sigma-1 receptors. Conditions for sigma-1 receptor binding did not contain unlabeled (+)-pentazocine. Radioligand was added second to last, and 150 μ g rat liver homogenate from a 5 mg/mL substock diluted in 10 mM HEPES pH 7.4 was added immediately prior to incubation. All experimental conditions were incubated in a 37°C water bath shaking for 120 minutes, and cell harvesting, filtration, and sample reading were performed as described in section 8.4.3.

Results from duplicates were averaged for each condition. In order to calculate binding affinity of the test ligand, radioactivity resultant from non-specific binding conditions was subtracted from all conditions. The percentage of [3 H]DTG or [3 H](+)-pentazocine that remained bound to the receptor in the presence of each concentration of test ligand was then calculated by the ratio of radioactivity in each test condition to radioactivity in the total binding condition. The concentration of test ligand was then plotted against the percentage of total radioligand binding remaining on the x- and y-axes, respectively, and GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was then used to determine the K_i value using a one-site competition binding fit.

8.4.5 Irreversible Binding Analysis

Analysis of irreversible binding capability was assessed using a variation of the Scatchard Analysis procedure described in section 8.4.3. Rat liver membrane homogenate was diluted to 0.30 mg/mL in room temperature 20 mM HEPES pH 7.4 and was incubated with the test ligand at varying doses for 60 minutes with gentle shaking in a water bath at 25°C. Samples were then diluted to 0.018 mg/mL with ice cold 20 mM HEPES pH 7.4 at 4°C and centrifuged at 37,000g for 10 minutes. Supernatant was discarded and pellet was resuspended to initial incubation volume with ice cold 20 mM HEPES pH 7.4 at 4°C and centrifuged again at 37,000g for 10 minutes. Pellet was then resuspended to initial incubation volume with room temperature 20 mM HEPES pH 7.4 and shaken gently in a water bath at 25°C for 60 minutes to allow for dissociation of any non-covalently bound test ligand. Sample was then centrifuged at 37,000g for 10 minutes and resuspended in 50 mM Tris pH 8.0 at room temperature to a concentration of 0.6 mg/mL. This preparation was then used in the binding assay as described in section 8.4.3 with varying concentrations of [³H]DTG (without unlabeled DTG) used to measure recovery of sigma-2 receptor binding sites in the presence of unlabeled (+)-pentazocine after the test ligand pretreatment and washing procedure. Completely recovery of sigma-2 receptor binding sites through the washing and dissociation procedure was confirmed using SN79, which does not irreversibly bind sigma-2 receptors, as the test ligand.

8.3 Cell Viability Assays

8.2.1 MTT Assay

The MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) was used to assess cell viability. This assay is based on the premise that healthy cells metabolize yellow MTT into purple formazan crystals, while dead cells do not. Therefore, increased absorbance in treated cells as compared to the untreated control group indicates an increase in MTT reduction, either through increased cell number and/or enhanced metabolic reduction. Conversely, decreased absorbance in treated cells as compared to the untreated control group indicates a loss of ability to reduce MTT, presumably due to cell death. Cells were plated in a clear, tissue culture treated, 96-well microplate (Fisher Scientific, Waltham, MA) in 100 μ L appropriate media at a cell density to achieve 70% confluency at the time of the assay (7,000 cells/well for MDA-MB-231, MDA-MB-453, MDA-MB-468; 10,000 cells/well for HEK 293, HMEC, MCF-7, MG-63, PANC-1; 15,000 cells/well for SK-N-SH, HEM) and allowed to attach overnight in a humidified atmosphere at 37°C and 5% CO₂. Media from plating was then removed and wells were dosed per experimental conditions with each well having a final volume of 100 μ L and each condition having 5 replicates. Every experiment also included a column of media without cells for background absorbance determination and a column of cells without test ligand for basal absorbance determination. Exposure was carried out for 24 or 48 h in a humidified atmosphere at 37°C and 5% CO₂, with well contents being emptied and replaced with fresh media with or without ligand after 24 h during 48 h exposures.

When desired exposure time was completed, 10 μ L MTT Reagent (Trevigen, Gaithersburg, MD) was added directly to the contents of each well and was allowed to be

metabolized for 3 h in a humidified atmosphere at 37°C and 5% CO₂ prior to the addition of 100 µL of MTT Detergent Reagent (Trevigen, Gaithersburg, MD) to each well. Detergent was allowed to solubilize cell membranes and formazan crystals for 2 h in a humidified atmosphere at 37°C and 5% CO₂. Absorbance was then read using a Victor V Platereader (PerkinElmer, Waltham, MA) at 570 nm for 1.0 second per well. Absorbance values for 5 replicates were averaged for each condition, and average absorbance of background wells (media only, no cells) were subtracted from all experimental conditions. Absorbance averages of treated groups were then compared to the untreated control group (media and cells, no ligand) for comparison.

8.2.2 LDH Assay

The Pierce LDH Cytotoxicity Assay Kit (Fisher Scientific, Waltham, MA) was used to confirm cell viability results. This assay is based on the premise that damaged and dying cells release lactate dehydrogenase (LDH) into their surrounding media, which converts pyruvate into lactate while consuming NAD⁺ and generating NADH. This cofactor consumption is coupled to the diaphorase-mediated conversion of 4-iodonitrotriazolium to formazan, the absorbance of which can be measured as it is colored. Therefore, an increase in absorbance in treated cells as compared to untreated control cells indicates more LDH release by damaged and dying cells, and therefore decreased cell viability. For this assay, cells were plated and treated as described in section 8.2.1 with one additional column of cells plated to be used as total lysis control in addition to background and basal absorbance determination columns. When 45 minutes were remaining in the exposure time, 10 µL 10X lysis buffer (RIPA buffer or Lysis Buffer from LDH kit) was added to each total lysis control well and mixed by gentle tapping without generating bubbles.

Upon completion of the exposure time, a 50 μ L aliquot of each sample medium was transferred to a new clear 96-well microplate for LDH assay. A 50 μ L aliquot of Reaction Mix was added to each well and mixed by pipetting without generating bubbles. The microplate was then wrapped in aluminum foil to protect it from light and incubated for 30 minutes at room temperature. After incubation, 50 μ L of Stop Solution was added to each well and mixed by gentle tapping without generating bubbles. Absorbance was then read using a Victor V Platereader (PerkinElmer, Waltham, MA) at 490 nm for 1.0 second per well. Absorbance values for all 5 replicates were averaged for each condition, and average absorbance for total lysis control was normalized to 100% cytotoxicity.

8.4 Calcium Release Assay

Fura-2,AM (Invitrogen, Carlsbad, CA) stock was made by the addition of 10 μ L pure dimethylsulfoxide to individual vials containing 50 μ g lyophilized fura-2,AM per vial. This stock solution was stored at -20°C in the dark. SK-N-SH neuroblastoma cells were plated at 20,000 cells/well in a black-sided, black-bottomed 96-well plate (PerkinElmer, Waltham, MA) and allowed to attach overnight in media. As fura-2,AM is sensitive to light, preparation for this assay including loading cells with dye was performed in a sterile hood without lights on. Cells were washed twice with 100 μ L/well Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY), then loaded with 100 μ L/well 2.47 μ M fura-2,AM in 0.065% pluronic acid in HBSS and incubated for 60 minutes at 37°C in the dark. Fura-2,AM solution was then removed and cells were washed once with 100 μ L/well HBSS, then 100 μ L/well HBSS was added to all columns. HBSS was then removed from cell blank condition column and appropriate amount of fresh HBSS was added such that total volume including intended injection volume would equal

100 μ L/well (i.e. if intended injection volume is 20 μ L, cell blank condition column would have 80 μ L HBSS added).

To prime 2-Channel Injector (PerkinElmer, Waltham, MA) addition to the Victor V Platereader (PerkinElmer, Waltham, MA), pumps were flushed with 0.1% acetic acid, emptied, flushed with deionized water, emptied, flushed with HBSS, emptied, and then filled with HBSS. The 96-well plate was then transferred to the platereader and “Calcium Release Assay” protocol was run. Fura-2,AM is a ratiometric dye, with excitation at 380 or 340 nm, depending on whether fura-2 is free or bound to calcium, respectively, and emission at 510 nm regardless of bound vs. free state. Twenty-five emission readings were taken over the course of ~42 seconds to establish baseline levels of calcium prior to injection. Injections were made individually at speed 3, using no more than 30 μ L per injection with all final volumes being 100 μ L after injection. One hundred readings were taken after injection over the course of ~4.5 minutes.

To change injection solutions, current solution was emptied and pumps were flushed once with 0.1% acetic acid, emptied, flushed with deionized water, emptied, flushed with HBSS, emptied, and then filled with the new injection solution to be used (i.e. ligand in HBSS). In order to ensure proper functioning of the injector and platereader systems as well as proper fura-2,AM loading and health of the plated cells, 1 mM carbacholamine chloride (Sigma-Aldrich, St. Louis, MO) and 5 μ M calcium ionophore (A23187) (Sigma-Aldrich, St. Louis, MO) were used as positive controls. For each condition, baseline level of calcium (determined by an average of 25 initial reading prior to injection) was removed from each post-injection reading. Data is presented as a change in calcium level from baseline, which is normalized to zero. Each experiment was performed using 4 wells per condition, with readings from all wells averaged for

each measurement to create an individual experimental condition trace. Unless otherwise indicated, representative traces are shown.

8.5 Western Blotting

8.5.1 Lysate Preparation

For treated lysate preparation, ~500,000 cells were plated in a tissue culture treated 35 mm petri dish (Celltreat Scientific, Shirley, MA) and allowed to attach overnight. Media was aspirated from dish and fresh media with or without ligand was replaced for a final volume of 1 mL per dish, with each experiment having an untreated control dish prepared with media without ligand concurrently. Cells were treated for desired amount of time and then media was aspirated, plate was washed once with 1X PBS, and 100 μ L RIPA buffer containing 1X Halt Protease and Phosphatase Inhibitor Cocktail with 1X EDTA (Thermo Scientific, Waltham, MA) was added. Samples were incubated with lysis buffer for 30 minutes while shaking at 4°C prior to dislodging any remaining adherent cells and proteins and transferring to a microcentrifuge tube. Samples were then spun down for 10 minutes at 10,000g at 4°C, supernatant was transferred to a new tube, and pellet was discarded. Processed samples were stored at -20°C.

8.5.2 Protein Assay

The Pierce BCA Protein Assay Kit (Fisher Scientific, Waltham, MA) was used to determine protein concentration of lysates for western blotting as well as concentration of membrane preparations for radioligand binding assays. BCA Protein Assay Reagent B was

added to BCA Protein Assay Reagent A in a 1:50 ratio to create the working solution. An 80 μL aliquot of working solution was added to each well in a clear 96-well plate such that every sample to be tested could be done in triplicate, in addition to triplicates of a 9-sample BSA standard curve and 3 wells of only working solution that would not be assayed but would be used to assess contamination. The BSA standard curve samples were created by the dilution of a 2000 $\mu\text{g/mL}$ BSA stock into RIPA buffer to generate samples ranging from 0 (RIPA without BSA) to 2000 (straight BSA stock) $\mu\text{g/mL}$.

For protein concentration detection, a 10 μL aliquot of each standard and test sample was added to each of three wells containing working solution for every sample, such that each condition was assayed in triplicate. The microplate was then set on top of a test tube rack in a 37°C water bath so that the microplate was not touching the water in the bath and was incubated for 30 minutes. After this incubation, the working solution contamination determination wells were observed and if no traces of purple color were present, the assay was determined to be uncontaminated. The bottom of the microplate was then blotted dry with paper towels to remove condensation, and absorbance was read at using a Victor V Platereader (PerkinElmer, Waltham, MA) at 570 nm for 1.0 second per well. Triplicates of each condition were averaged, and the absorbance average of the 0 $\mu\text{g/mL}$ BSA standard (RIPA buffer alone) was subtracted from each measurement. Absorbance averages for the standard curve were plotted on the x-axis against protein concentration on the y-axis, and a linear equation was generated passing through the origin (as background absorbance was removed from each reading). This equation was used to extrapolate protein concentration values for the test samples.

8.5.3 Casting Gels

Hand-cast and pre-cast gels were both used for western blotting without significant differences observed in results. Pre-cast 4-15% TGX gels (Biorad, Hercules, CA) were stored at 4°C until used. Hand-cast gels were made up to three days in advance of use with the Mini-PROTEAN Tetra handcast system (Biorad, Hercules, CA), and were stored at 4°C if not used immediately. Acrylamide concentrations of hand-cast gels were varied between 10-15% depending on the size of the protein of interest, with higher acrylamide concentrations being used for better resolution of smaller proteins. Glass gel-casting plates of 1.5 mm depth were used in conjunction with 10-well combs to create gels used for western blotting.

8.5.4 Western Blotting Protocol

Acrylamide gels were set into a Mini-PROTEAN electrophoresis system (with a plastic buffer dam if only one gel) (Biorad, Hercules, CA) and the system was filled with 25 mM Tris, 192 mM glycine, 0.1% SDS running buffer. The protein concentrations of all lysates to be used in the experiment were standardized to the lowest concentration using Laemmli sample buffer (homemade or ordered from Biorad) containing β -mercaptoethanol prior to heating for 5 minutes in a 100°C heating block and loading 20 μ L of each standardized sample in the gel. One lane of ladder was loaded on one or both sides of the samples in each gel, and remaining empty wells were filled with sample buffer. Electrophoresis was run at 150 V until the dye front reached the bottom of the gel, typically ~1.5 hours. The proteins in the gel were then wet transferred onto nitrocellulose paper at 40 V for 2 hours at 4°C using the Mini Trans-Blot Cell system (Biorad, Hercules, CA) with 25 mM Tris, 192 mM glycine, 20% methanol transfer buffer.

After transfer was complete, the nitrocellulose blot was blocked in 10% milk/TBS/0.1% Tween-20 for 1 hour while shaking at room temperature. Blot was then washed three times for 5 minutes per wash in ~10 mL TBS/0.1% Tween-20 for each wash. Primary antibody was diluted in 10% milk/TBS/0.1% Tween-20 or 5% BSA/TBS/0.1% Tween-20 according to manufacturer's specifications, and 2-3 mL diluted primary antibody was used to incubate each blot in a plastic sachet sealed with a heat sealer while shaking overnight at 4°C. The following morning, the sachet containing the blot in diluted primary antibody was moved to room temperature and incubated for an additional hour while shaking. The blot was then washed three times for 5 minutes per wash in ~10 mL TBS/0.1% Tween-20 for each wash and 2-3 mL appropriate species secondary antibody (Santa Cruz, Dallas, TX) diluted in 10% milk according to manufacturer's specifications was applied and sealed in a plastic sachet using a heat sealer. Blot was incubated with diluted secondary antibody for 1 hour while shaking at room temperature. The blot was then washed three times for 5 minutes per wash in ~10 mL TBS/0.1% Tween-20 for each wash.

For chemiluminescence detection, the SuperSignal West Pico Chemiluminescent substrate was used (Thermo Scientific, Waltham, MA) and film exposures were taken. The blot was incubated with 1:1 Stable Peroxide solution and Luminol/Enhancer solution for 5 minutes shaking at room temperature. Excess solution was then dabbed onto a Kimwipe and the blot was wrapped in clear plastic wrap and taped into a cassette. Blue UltraCruz autoradiography film (Santa Cruz, Dallas, TX) was exposed for varying time lengths until a suitable level of detection was achieved upon development. Protein bands were then quantified from the film using ImageJ software (NIH, Bethesda, MD) and standardized by comparison to a loading control (i.e. actin, tubulin, or GAPDH). The results were then normalized to the level of the untreated control protein detection level for comparison.

8.5.5 Stripping Blots for Reprobing

For repeated use of a single blot, antibodies were stripped and the blot was reprobed for another protein. The blot was washed twice with ~10 mL mild stripping solution containing 200 mM glycine, 3.5 mM SDS, and 1% Tween-20 at pH 2.2 for 10 minutes per wash. Next, the blot was washed twice for 10 minutes per wash in ~10 mL 1X PBS, and then washed two more times for 5 minutes per wash in ~10 mL TBS/0.1% Tween-20. The Western blotting protocol described in section 8.5.4 was then continued beginning with reblocking the blot in 10% milk/TBS/0.1% Tween-20.

8.6 Cell Proliferation Assays

8.6.1 CyQUANT Cell Proliferation Assay

The CyQUANT Cell Proliferation Assay (Thermo Scientific, Waltham, MA) measures nucleic acid content in cells. As the amount of DNA in a single cell is constant within a given cell type, an increase in DNA can be attributed to an increase in cell number. Cells were plated in a black-bottomed, black-sided 96-well plate (PerkinElmer, Waltham, MA) at the same seeding density as was used for the MTT cell viability assay (section 8.2.1), allowed to attach overnight, and then treated with or without ligand for the desired amount of time up to 96 hours. An extra column of cells was plated in each experiment to be used as an untreated control for comparison to treatment conditions. If an absolute DNA concentration was desired, as opposed to a relative comparison between conditions, a DNA standard curve could be included. All experiments were carried out with 5 replicates per condition per experiment. For treatments

longer than 24 h, ligand and media were removed and fresh media and ligand were added after every 24 h period. Upon completion of desired exposure time, the solution in all wells was aspirated and cells were washed once with 100 μ L 1X PBS per well. The microplate was then inverted and residual solution was gently blotted onto a Kimwipe. The edges of the microplate were then wrapped in Parafilm and the microplate was frozen at -80°C for a minimum of 12 hours.

For DNA content detection, CyQUANT GR dye/cell lysis buffer solution was prepared with all components diluted to 1X in water according to manufacturer's specifications immediately prior to use. The microplate was thawed to room temperature and 200 μ L CyQUANT GR dye/cell lysis buffer solution was added to each well and briefly shaken to mix prior to being incubated for 5 minutes at room temperature with the microplate wrapped in aluminum foil to protect it from light. Fluorescence of the dye was then measured at 480 nm excitation/520 nm emission for 1 second per well using a Victor V Platereader (PerkinElmer, Waltham, MA). Fluorescence (RFU) was averaged for all 5 replicates for each condition, and then could either be directly compared between the untreated control group and the treated conditions or an absolute DNA concentration could be extrapolated from the standard curve.

8.6.2 BrdU Incorporation Cell Proliferation ELISA

The BrdU Cell Proliferation Chemiluminescent Assay Kit (Cell Signaling, Danvers, MA) measured incorporation of 5-bromo-d'-deoxyuridine (BrdU) into DNA during cellular proliferation. A higher level of BrdU detection using an HRP-linked anti-BrdU antibody indicates a higher level of BrdU incorporation, and therefore a higher rate of DNA replication and cellular proliferation. TMB substrate was used for absorbance-based detection of BrdU

antibody, with a higher level of absorbance corresponding to a higher level of anti-BrdU antibody and thus a higher level of BrdU incorporation and DNA replication. Cells were plated in a clear 96-well plate at the same seeding density as was used for the MTT cell viability assay (section 8.2.1), allowed to attach overnight. An extra column of cells was plated in each experiment to be used as an untreated control for comparison to treatment conditions, as well as a column of media without cells for detection of background absorbance. Cells were then dosed with the desired treatment condition, and 10 μ L 10X BrdU was added to each well. Cells were incubated for 24 h with ligand (or media without ligand for untreated control condition) and BrdU at 37°C and 5% CO₂ in a humidified atmosphere.

At the end of the exposure time, solution was aspirated from all wells and replaced with 100 μ L Fixing/Denaturing Solution per well. Cells were fixed for 30 minutes at room temperature, then solution was aspirated and replaced with 100 μ L 1X Detection Antibody Solution diluted in Detection Antibody Diluent per well and incubated at room temperature for an additional 1 hour. Detection Antibody Solution was then removed and cells were washed 3 times with 1X Wash Buffer or PBS. A 100 μ L volume of 1X Anti-mouse IgG HRP-linked Antibody Solution diluted in HRP-linked Antibody Diluent was then added to each well and incubated for 30 minutes at room temperature, prior to 3 more washes of the wells with 1X Wash Buffer or PBS. TMB substrate (100 μ L/well) was then added to each well and allowed to be metabolized for 30 minutes at room temperature, followed by the addition of 100 μ L STOP solution to each well. Absorbance was then read at 450 nm for 1.0 second per well using a Victor V Platereader (PerkinElmer, Waltham, MA). Absorbances values were averaged for all 5 replicates for each condition, and directly compared between the untreated control group and the

treated conditions with average absorbance level of the background condition (media only, no cells) being subtracted from all other absorbance averages.

8.6.3 DAPI Staining

Fluorescent 4',6-diamidino-2-phenylindole (DAPI) binds A-T rich regions of DNA, and therefore most prominently stains the nuclei of cells. The nuclei of DAPI-stained cells can be counted, therefore giving an indication of cell number. Cells were plated in a black-bottomed, black-sided 96-well plate (PerkinElmer, Waltham, MA) at the same seeding density as was used for the MTT cell viability assay (section 8.2.1), allowed to attach overnight, and then treated with or without ligand for 24 hours. An extra column of cells was plated in each experiment to be used as an untreated control for comparison to treatment conditions. All experiments were carried out in triplicate for each condition in each experiment. Upon completion of exposure time, the solution in all wells was aspirated and cells were washed 3 times with 100 μ L 1X PBS per well per wash. To fix cells, 200 μ L 10% formalin (Fisher Scientific, Waltham, MA) was added to each well and incubated to 30 minutes at room temperature.

After fixing, cells were washed 3 more times with 100 μ L 1X PBS per well per wash, and then immediately stained with 100 μ L 1000 ng/mL DAPI (Fisher Scientific, Waltham, MA) in PBS per well and allowed to stain for 30 minutes at room temperature covered in foil to protect the microplate from light. Well contents were then aspirated, cells were washed 3 times with 100 μ L 1X PBS per well per wash, and fluorescence images were taken in 4 random locations in each well using a BioTek Cytation 3 microplate reader (BioTek, Winooski, VT) at 377 nm excitation/447 nm emission. Number of cells in each image was then quantified using

CellProfiler software (Broad Institute, Cambridge, MA) and cell counts from all images were averaged for each condition.

8.7 Metabolic Factor Analysis Assays

8.7.1 NAD^+/NADH Quantification Assay

The NAD^+/NADH Quantification Colorimetric Kit (BioVision, Milpitas, CA) was used to directly measure total NAD ($\text{NADH} + \text{NAD}^+$) and NADH levels, from which NAD^+ levels also could be inferred. Cells were plated in 35 mm tissue culture treated petri dishes (Celltreat Scientific, Shirley, MA) at 200,000 cells/dish and allowed to attach overnight. Media was aspirated from dish and fresh media with or without ligand was replaced for a final volume of 1 mL per dish, with each experiment having an untreated control dish prepared with media without ligand concurrently. Cells were treated for 24 h and then media and ligand were aspirated, plate was washed once with 1X PBS, and 2.5 mM EDTA in DPBS was used to dissociate cells. Cells were then pelleted by centrifugation at 223g for 5 minutes, supernatant was discarded, and samples were extracted using 400 μL Extraction Buffer in two freeze/thaw cycles of 20 minutes on dry ice/10 minutes at room temperature. Extraction was vortexed for 10 seconds, pelleted at 37,500g for 5 minutes, and supernatant was collected for further processing. Pellet was discarded.

Extraction supernatant was divided into two portions: one portion for total NAD determination and one portion for NADH only determination. In order to degrade NAD^+ in the NADH only determination portion of the extraction supernatant, the sample was heated for 30

minutes in a heating block at 60°C and then cooled on ice. A 50 µL aliquot of each sample was then added to each of three wells of a clear 96-well microplate such that each condition could be assayed in triplicate. A 1 nmol/µL NADH standard was also added to separate wells at varying volumes to create a standard curve ranging from 0-100 pmol/well NADH, with the volume in each well brought to µL with Extraction Buffer. A 1:50 dilution of Enzyme Mix in Cycling Buffer was then made and 100 µL was added to each well. The microplate was then shaken gently to mix and incubated for 5 minutes at room temperature. A 10 µL aliquot of Developer was then added to each well and incubated for 4 hours.

Absorbance was read every hour at 450 nm using a Victor V Platereader (PerkinElmer, Waltham, MA). A linear extrapolation from the standard curve was used to determine concentrations of NADt and NADH with background absorbance of the 0 pmol/well standard subtracted from each measurement, and NAD⁺ level was determined by the difference between the NADt and NADH direct measurements. These concentrations were then standardized to the protein concentration in each sample, as determined by BCA assay (section 8.5.2). The timepoint used in data presentation was determined by the cleanest standard curve generated, both in terms of linearity and R² value.

8.7.2 ATP Quantification Assay

The ATP Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA) was used to measure ATP level in *in vitro* samples. Cells were plated in 35 mm tissue culture treated petri dishes (Celltreat Scientific, Shirley, MA) at 600,000 cells/dish and allowed to attach overnight. Media was aspirated from dish and fresh media with or without ligand was replaced for a final volume of 1 mL per dish, with each experiment having an untreated control dish

prepared with media without ligand concurrently. Cells were treated for 24 h and then media and ligand were aspirated, plate was washed once with 1X PBS, and cells were lysed in 100 μ L Assay Buffer and contents were transferred to a microcentrifuge tube. Samples were then deproteinized by the addition of 33.3 μ L ice cold 4 M perchloric acid to each tube and were vortexed briefly prior to 5 minute incubation on ice and then centrifugation for 2 minutes at 16,000*g*. Supernatant was then transferred to new tubes and neutralized by the addition of an equal volume of ice cold 2 M potassium hydroxide per volume of supernatant. Samples were vortexed briefly, vented to prevent excessive CO₂ buildup, and centrifuged for 15 minutes at 16,000*g*.

For measurement of ATP content, 50 μ L of each sample was added to each of two wells in a clear 96-well microplate such that each condition could be assayed in duplicate. For absolute ATP concentration determination, a 1 mM ATP solution was used to create a standard curve ranging from 0.01-0.1 mM ATP (for fluorometric assay) and each well was brought to 50 μ L total volume with Assay Buffer. For comparative results, no standard curve was required. Reaction Mix was then prepared with 2 μ L/well each of Converter and Developer, 0.2 μ L/well ATP Probe (protected from light), and 45.8 μ L/well Assay Buffer. A 50 μ L aliquot of Reaction Mix was then added to each assay well and incubated for 30 minutes at room temperature with the microplate wrapped in aluminum foil to protect it from light. Fluorescence was then read at 535 nm excitation/587 nm emission for 1.0 second per well using a Victor V Platerreader (PerkinElmer, Waltham, MA). Measurements of duplicate conditions were averaged and then standardized to the protein concentration in each sample, as determined by BCA assay (section 8.5.2). Results were either compared relatively between conditions or absolute ATP concentrations were extrapolated from the standard curve.

8.7.3 Reactive Oxygen Species Detection Assay

Reactive oxygen species were detected using 2',7'-dichlorofluorescein diacetate (DCFDA) fluorescent stain. Cell-permeable DCFDA enters cells and is deacetylated by esterases into an intermediate compound that can be oxidized by reactive oxygen species into fluorescent 2',7'-dichlorofluorescein (DCF). Thus, increased fluorescence corresponds to increased levels of reactive oxygen species. Cells were plated in a black-bottomed, black-sided 96-well plate (PerkinElmer, Waltham, MA) at 25,000 cells/well, with three extra columns of cells plated in each experiment to be used as positive, negative, and untreated controls. An additional column of media without cells was used to measure background fluorescence. All experiments were carried out with 4 replicates for each condition in each experiment. Cells were allowed to attach overnight and were then washed once with 100 μ L/well HBSS prior to staining with 50 μ M DCFDA (Abcam, Cambridge, MA) in HBSS for 45 minutes at 37°C wrapped in aluminum foil to protect the dye from light.

After staining, cells were washed once with 100 μ L/well HBSS and phenol red-free media with or without ligand was added per desired experimental conditions. Positive control cells were treated with 500 μ M *tert*-butyl hydrogen peroxide, negative control cells were treated with 200 μ g/mL α -tocopherol, and untreated control cells received phenol red-free media without ligand. Cells were treated for 4 hours, and then fluorescence was measured at 485 nm excitation/535 nm emission using a Victor V Platerreader (PerkinElmer, Waltham, MA). Background fluorescence of media without cells was subtracted from each measurement, and fluorescence from replicates were averaged for each condition and compared to the average fluorescence of the untreated control condition, which was normalized to 100%.

8.7.4 JC-1 Mitochondrial Potential Assay

The JC-1 mitochondrial potential assay kit (Abcam, Cambridge, MA) was used to measure mitochondrial depolarization. Tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye accumulates and aggregates in mitochondria with high membrane potential. This aggregate form of JC-1 emits fluorescence at 530 nm. When mitochondrial membrane potential is low, JC-1 does not aggregate in the mitochondria and rather exists primarily in its monomeric form, which emits fluorescence at 590 nm. Thus, an increase in mitochondrial membrane potential (hyperpolarization) is marked by an increase in aggregate formation, and thus an increase in fluorescence emission at 530 nm. Conversely, a decrease in mitochondrial membrane potential (depolarization) is marked by an increase in monomeric JC-1, and thus an increase in fluorescence emission at 590 nm.

Cells were plated in a black-bottomed, black-sided 96-well plate (PerkinElmer, Waltham, MA) at the same seeding density as was used for the MTT cell viability assay (section 8.2.1), allowed to attach overnight, and then treated with or without ligand in phenol red-free media for 24-40 hours. For MDA-MB-231 cells, 70 μ M verapamil hydrochloride (Sigma-Aldrich, St. Louis, MO) was added to cells 1 h prior to exposure to ligand and added to ligand treatment to prevent multidrug resistance pump activity from expelling JC-1 dye upon exposure. Three extra columns of cells were plated in each experiment to be used as controls for background fluorescence (non-stained cells), vehicle treatment (stained cells with media, no ligand), and positive control for loss of mitochondrial membrane potential (75 μ M valinomycin (Calbiochem, Darmstadt, Germany)). All experiments were carried out in triplicate for each condition in each experiment. Upon completion of desired exposure time, the solution in all wells was aspirated and cells were washed once with 100 μ L 1X PBS per well. Biosafety cabinet lights were then

turned off to reduce light exposure, and cells were stained with 100 μ L 10 μ M JC-1 dye in 1X Dilution Buffer per well for 15 minutes at 37°C wrapped in aluminum foil.

After staining, cells were washed twice with 100 μ L 1X PBS per well per wash and then 100 μ L 1X PBS per well was added for assay reading. Fluorescence was measured at 485 nm excitation/595 nm emission for 1.0 second per well for measurement of JC-1 aggregate and 485 nm excitation/535 nm emission for 1.0 second per well for measurement of JC-1 monomer.

Measurements from triplicate wells were averaged for each condition and average background fluorescence measured from non-stained cells was removed from each treatment average. The average fluorescence of the stained, untreated cells was normalized to 100% and all measurements were compared relatively. The ratio of aggregate:monomer was also calculated for each condition, with an increase in the aggregate:monomer ratio indicating hyperpolarization of the mitochondria and a decrease in this ratio indicating depolarization.

References

- Aanonsen, L M, and V S Seybold. 1989. "Phencyclidine and Sigma Receptors in Rat Spinal Cord: Binding Characterization and Quantitative Autoradiography." *Synapse* 4 (1): 1–10.
- Alonso, G, V Phan, I Guillemain, M Saunier, A Legrand, M Anoal, and T Maurice. 2000. "Immunocytochemical Localization of the sigma(1) Receptor in the Adult Rat Central Nervous System." *Neuroscience* 97 (1): 155–70.
- Aydar, E, C P Palmer, V A Klyachko, and M B Jackson. 2002. "The Sigma Receptor as a Ligand-Regulated Auxiliary Potassium Channel Subunit." *Neuron* 34 (3): 399–410.
- Bem, W T, G E Thomas, J Y Mamone, S M Homan, B K Levy, F E Johnson, and C J Coscia. 1991. "Overexpression of Sigma Receptors in Nonneural Human Tumors." *Cancer Research* 51 (24): 6558–62.
- Bermack, J E, and G Debonnel. 2005. "The Role of Sigma Receptors in Depression." *Journal of Pharmacological Sciences* 97 (3): 317–36.
- Bouchard, P, and R Quirion. 1997. "[3H]1,3-di(2-Tolyl)guanidine and [3H](+)pentazocine Binding Sites in the Rat Brain: Autoradiographic Visualization of the Putative sigma1 and sigma2 Receptor Subtypes." *Neuroscience* 76 (2): 467–77.
- Bowen, W D. 2007. "Sigma-2 Receptors: Regulation of Cell Growth and Implications for Cancer Diagnosis and Therapeutics." In *Sigma Receptors: Chemistry, Cell Biology and Clinical*

Implications, edited by R R Matsumoto, W D Bowen, and T-P Su, 215–35. New York: Springer Science+Business Media.

Bowen, W D, C M Bertha, B J Vilner, and K C Rice. 1995. “CB-64D and CB-184: Ligands with High Sigma 2 Receptor Affinity and Subtype Selectivity.” *European Journal of Pharmacology* 278 (3): 257–60.

Bowen, W D, K W Crawford, and A Coop. 2001. “Sigma-2 Receptors May Activate Sphingolipid-Ceramide N-Deacylase (SCDase) as a Mechanism to Regulate Cell Growth.” In *Society for Neuroscience Annual Meeting Proceedings*, 364.1.

Bowen, W D, K W Crawford, S Huang, and J W Walker. 2000. “Activation of Sigma-2 Receptors Causes Changes in Ceramide Levels in Neuronal and Non-Neuronal Cell Lines.” In *Society for Neuroscience Annual Meeting Proceedings*, 226.11.

Bowen, W D, B R de Costa, S B Hellewell, J M Walker, and K C Rice. 1993. “[3H]-(+)-Pentazocine: A Potent and Highly Selective Benzomorphan-Based Probe for Sigma-1 Receptors.” *Molecular Neuropharmacology* 3: 117–26.

Bowen, W D, B Jin, E Blann, B J Vilner, and B D Lyn-Cook. 1997. “Sigma Receptor Ligands Modulate Expression of the Multidrug Resistance Gene in Human and Rodent Brain Tumor Cell Lines.” In *Proceedings of the American Association for Cancer Research*, 3206.

Cagnotto, A, A Bastone, and T Mennini. 1994. “[3H](+)-Pentazocine Binding to Rat Brain Sigma-1 Receptors.” *European Journal of Pharmacology* 1 266: 131–38.

“Cancer Statistics.” 2015. *National Cancer Institute*. <http://www.cancer.gov/about-cancer/what-is-cancer/statistics>.

Cande, C, I Cohen, E Daugas, L Ravagnan, N Larochette, N Zamzami, and G Kroemer. 2002.

“Apoptosis-Inducing Factor (AIF): A Novel Caspase-Independent Death Effector Released from Mitochondria.” *Biochimie* 84 (2-3): 215–22.

Cassano, G, G Gasparre, M Niso, M Contino, V Scalera, and N A Colabufo. 2009. “F281,

Synthetic Agonist of the Sigma-2 Receptor, Induces Ca²⁺ Efflux from the Endoplasmic Reticulum and Mitochondria in SK-N-SH Cells.” *Cell Calcium* 45 (4): 340–45.

Chu, U, T A Mavlyutov, M-L Chu, H Yang, A Schulman, C Mesangeau, C R McCurdy, L-W

Guo, and A E Ruoho. 2015. “The Sigma-2 Receptor and Progesterone Receptor Membrane Component 1 Are Different Binding Sites Derived From Independent Genes.” *EBioMedicine* 2 (11): 1806–13.

Cobos, E J, J M Entrena, F R Nieto, C M Cendan, and E Del Pozo. 2008. “Pharmacology and Therapeutic Potential of sigma1 Receptor Ligands.” *Current Neuropharmacology* 6 (4): 344–66.

Connor, M A, and C Chavkin. 1991. “Focal Stimulation of Specific Pathways in the Rat

Hippocampus Causes a Reduction in Radioligand Binding to the Haloperidol-Sensitive Sigma Receptor.” *Experimental Brain Research*.

———. 1992. “Ionic Zinc May Function as an Endogenous Ligand for the Haloperidol-Sensitive Sigma-2 Receptor in Rat Brain.” *Molecular Pharmacology* 42: 471–79.

- Contreras, P C, D a DiMaggio, and T L O'Donohue. 1987. "An Endogenous Ligand for the Sigma Opioid Binding Site." *Synapse* 1 (1): 57–61.
- Crawford, K W, and W D Bowen. 2002. "Sigma-2 Receptor Agonists Activate a Novel Apoptotic Pathway and Potentiate Antineoplastic Drugs in Breast Tumor Cell Lines." *Cancer Research* 62 (1): 313–22.
- Crawford, K W, A Coop, and W D Bowen. 2002. "Sigma(2) Receptors Regulate Changes in Sphingolipid Levels in Breast Tumor Cells." *European Journal of Pharmacology* 443 (1-3): 207–9.
- Daven-Hudkins, D L, F Y Ford-Rice, J T Allen, and R L Hudkins. 1993. "Allosteric Modulation of Ligand Binding to [3H]-(+)-Pentazocine-Defined Sigma Recognition Sites by Phenytoin." *Life Sciences* 53: 41–48.
- De Costa, B R, W D Bowen, S B Hellewell, J M Walker, A Thurkauf, A E Jacobson, and K C Rice. 1989. "Synthesis and Evaluation of Optically Pure [3H]-(+)-Pentazocine, a Highly Potent and Selective Radioligand for Sigma Receptors." *Federation of European Biochemical Societies Letters* 251 (1-2): 53–58.
- Dussossoy, D, P Carayon, S Belugou, D Feraut, A Bord, C Goubet, C Roque, et al. 1999. "Colocalization of Sterol Isomerase and sigma(1) Receptor at Endoplasmic Reticulum and Nuclear Envelope Level." *European Journal of Biochemistry / FEBS* 263 (2): 377–86.

- Eisenberg, R M. 1985. "Plasma Corticosterone Changes in Response to Central or Peripheral Administration of Kappa or Sigma Opiate Agonists." *Journal of Pharmacology and Experimental Therapeutics* 223: 863–69.
- Elmore, S. 2007. "Apoptosis: A Review of Programmed Cell Death." *Toxicologic Pathology* 35 (4): 495–516.
- Ganapathy, M E, P D Prasad, W Huang, P Seth, F H Leibach, and V Ganapathy. 1999. "Molecular and Ligand-Binding Characterization of the Sigma-Receptor in the Jurkat Human T Lymphocyte Cell Line." *Journal of Pharmacology and Experimental Therapeutics* 289 (1): 251–60.
- Ganapathy, V, M E Ganapathy, and K Inoue. 2007. "Cloning of Sigma-1 Receptor and Structural Analysis of Its Gene and Promoter Region." In *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications*, edited by R Matsumoto, W D Bowen, and T-P Su, 99–112. New York: Springer Science+Business Media.
- Garg, G, S Vangveravong, C Zeng, L Collins, M Hornick, Y Hashim, D Piwnica-Worms, et al. 2014. "Conjugation to a SMAC Mimetic Potentiates Sigma-2 Ligand Induced Tumor Cell Death in Ovarian Cancer." *Molecular Cancer* 13 (1): 50.
- Ge, D, Q Jing, N Meng, L Su, Y Zhang, S Zhang, J Miao, and J Zhao. 2011. "Regulation of Apoptosis and Autophagy by Sphingosylphosphorylcholine in Vascular Endothelial Cells." *Journal of Cellular Physiology* 226 (11): 2827–33.

- Gebreselassie, D, and W D Bowen. 2004. "Sigma-2 Receptors Are Specifically Localized to Lipid Rafts in Rat Liver Membranes." *European Journal of Pharmacology* 493 (1-3): 19–28.
- Ghavami, S, M Hashemi, S R Ande, B Yeganeh, W Xiao, M Eshraghi, C J Bus, et al. 2009. "Apoptosis and Cancer: Mutations within Caspase Genes." *Journal of Medical Genetics* 46 (8): 497–510.
- Gomes, L C, and L Scorrano. 2013. "Mitochondrial Morphology in Mitophagy and Macroautophagy." *Biochimica et Biophysica Acta - Molecular Cell Research* 1833 (1): 205–12.
- Gundlach, A L, B L Largent, and S H Snyder. 1986. "Autoradiographic Localization of Sigma Receptor Binding Sites in Guinea Pig and Rat Central Nervous System with (+)3H-3-(3-Hydroxyphenyl)-N-(1-Propyl)piperidine." *Journal of Neuroscience* 6 (6): 1757–70.
- Hanner, M, F F Moebius, A Flandorfer, H G Knaus, J Striessnig, E Kempner, and H Glossmann. 1996. "Purification, Molecular Cloning, and Expression of the Mammalian sigma1-Binding Site." *Proceedings of the National Academy of Sciences of the United States of America* 93 (15): 8072–77.
- Harada, Y, H Hara, and T Sukamoto. 1994. "Characterization of the Specific (+)-[3H]N-Allylnormetazocine and [3H]1,3-di(2-Tolyl)guanidine Binding Sites in Porcine Gastric Fundic Mucosa." *Journal of Pharmacology and Experimental Therapeutics* 269: 905–10.

- Hayashi, T, T Maurice, and T-P Su. 2000. "Ca²⁺ Signaling via Sigma-1 Receptors: Novel Regulatory Mechanism Affecting Intracellular Ca²⁺ Concentration." *Journal of Pharmacology and Experimental Therapeutics* 293: 788–98.
- Hayashi, T, and T-P Su. 2001. "Regulating Ankyrin Dynamics: Roles of Sigma-1 Receptors." *Proceedings of the National Academy of Sciences of the United States of America* 98 (2): 491–96.
- . 2003a. "Intracellular Dynamics of Sigma-1 Receptors in NG108-15 Cells." *Journal of Pharmacology and Experimental Therapeutics* 306: 718–25.
- . 2003b. "Sigma-1 Receptors (sigma(1) Binding Sites) Form Raft-like Microdomains and Target Lipid Droplets on the Endoplasmic Reticulum: Roles in Endoplasmic Reticulum Lipid Compartmentalization and Export." *Journal of Pharmacology and Experimental Therapeutics* 306 (2): 718–25.
- . 2007. "Subcellular Localization and Intracellular Dynamics of Sigma-1 Receptors." In *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications*, edited by R R Matsumoto, W D Bowen, and T-P Su, 151–64. New York: Springer Science+Business Media.
- Heading, C. 2001. "Siramesine H Lundbeck." *Current Opinion in Investigational Drugs* 2 (2): 266–70.

- Heiskanen, K M, M B Bhat, H W Wang, J Ma, and A L Nieminen. 1999. "Mitochondrial Depolarization Accompanies Cytochrome c Release during Apoptosis in PC6 Cells." *Journal of Biological Chemistry* 274 (9): 5654–58.
- Hellewell, S B, and W D Bowen. 1990. "A Sigma-like Binding Site in Rat Pheochromocytoma (PC12) Cells: Decreased Affinity for (+)-Benzomorphans and Lower Molecular Weight Suggest a Different Sigma Receptor Form from that of Guinea Pig Brain." *Brain Research* 527 (2): 244–53.
- Hellewell, S B, A Bruce, G Feinstein, J Orringer, W Williams, and W D Bowen. 1994. "Rat Liver and Kidney Contain High Densities of σ_1 and σ_2 Receptors: Characterization by Ligand Binding and Photoaffinity Labeling." *European Journal of Pharmacology: Molecular Pharmacology* 268 (1): 9–18.
- Hjorth, S A, A Carlsson, H Wikstrom, P Lindberg, D Sanchez, U Hacksell, L E Arvidsson, I Svensson, and J L G Nilsson. 1981. "3-PPP, a New Centrally Acting Dopamine Receptor Agonist with Selectivity for Autoreceptors." *Life Sciences* 28 (1225-1238).
- Horan, Bryan, Andrew N Gifford, Kiyoshi Matsuno, Shiro Mita, and Charles R Ashby. 2002. "Effect of SA4503 on the Electrically Evoked Release of [3]H-Acetylcholine from Striatal and Hippocampal Rat Brain Slices." *Synapse* 46 (1): 1–3.
- Hornick, J R, S Vangveravong, D Spitzer, C Abate, F Berardi, P Goedegebuure, R H Mach, and W G Hawkins. 2012. "Lysosomal Membrane Permeabilization Is an Early Event in Sigma-2 Receptor Ligand Mediated Cell Death in Pancreatic Cancer." *Journal of Experimental & Clinical Cancer Research* 31: 41.

- Hornick, J R, J Xu, S Vangveravong, Z Tu, J B Mitchem, D Spitzer, P Goedegebuure, R H Mach, and W G Hawkins. 2010. "The Novel Sigma-2 Receptor Ligand SW43 Stabilizes Pancreas Cancer Progression in Combination with Gemcitabine." *Molecular Cancer* 9: 298.
- Jiang, X, and X Wang. 2004. "Cytochrome C-Mediated Apoptosis." *Annual Review of Biochemistry* 73: 87–106.
- Jonhede, S, A Petersen, M Zetterberg, and J-O Karlsson. 2010. "Acute Effects of the Sigma-2 Receptor Agonist Siramesine on Lysosomal and Extra-Lysosomal Proteolytic Systems in Lens Epithelial Cells." *Molecular Vision* 16: 819–27.
- Kashiwagi, H, J E McDunn, P O Simon, P S Goedegebuure, S Vangveravong, K Chang, R S Hotchkiss, R H Mach, and W G Hawkins. 2009. "Sigma-2 Receptor Ligands Potentiate Conventional Chemotherapies and Improve Survival in Models of Pancreatic Adenocarcinoma." *Journal of Translational Medicine* 7: 24.
- Kashiwagi, H, J E McDunn, P O Simon, P S Goedegebuure, J Xu, L Jones, K Chang, et al. 2007. "Selective Sigma-2 Ligands Preferentially Bind to Pancreatic Adenocarcinomas: Applications in Diagnostic Imaging and Therapy." *Molecular Cancer* 6: 48.
doi:10.1186/1476-4598-6-48.
- Kaushal, N, M J Robson, H Vinnakota, S Narayanan, B A Avery, C R McCurdy, and R R Matsumoto. 2011. "Synthesis and Pharmacological Evaluation of 6-Acetyl-3-(4-(4-(4-Fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-One (SN79), a Cocaine Antagonist, in Rodents." *AAPS Journal* 13 (3): 336–46.

- Kaushal, N, M J Seminerio, M J Robson, C R McCurdy, and R R Matsumoto. 2013. "Pharmacological Evaluation of SN79, a Sigma (σ) Receptor Ligand, against Methamphetamine-Induced Neurotoxicity in Vivo." *European Neuropsychopharmacology* 23 (8): 960–71.
- Kavanaugh, M P, B C Tester, M W Scherz, J F W Keana, and E Weber. 1988. "Identification of the Binding Subunit of the Sigma-Type Opiate Receptor by Photoaffinity Labeling With 1-(4-Azido-2-methyl[6-3H]phenyl)-3-(d-methyl[4,6-3H]phenyl)guanidine." *Proceedings of the National Academy of Sciences of the United States of America* 85: 2844–48.
- Keats, A S, and J Telford. 1964. "Narcotic Antagonists as Analgesics." In *Molecular Modification in Drug Design*, edited by F.W. Schueler, 45th ed., 170–76. American Chemical Society.
- Kekuda, R, P D Prasad, Y J Fei, F H Leibach, and V Ganapathy. 1996. "Cloning and Functional Expression of the Human Type 1 Sigma Receptor (hSigmaR1)." *Biochemical and Biophysical Research Communications* 229 (2): 553–58.
- Kennedy, C, and G Henderson. 1989. "An Examination of the Putative Sigma-Receptor in the Mouse Isolated Vas Deferens." *British Journal of Pharmacology* 98: 429–36.
- Khazan, N, G A Young, E E El-Fakany, O Hong, and D Calligaro. 1984. "Sigma Receptors Mediated the Psychotomimetic Effects of N-Allylnormetazocine (SKF-10,047), but Not Its Opioid Agonistic-Antagonistic Properties." *Neuropharmacology* 23 (8): 983–87.

- Kolesnick, R N, and M Krönke. 1998. "Regulation of Ceramide Production and Apoptosis." *Annual Review of Physiology* 60: 643–65.
- Kroemer, G, L Galluzzi, and C Brenner. 2007. "Mitochondrial Membrane Permeabilization in Cell Death." *Physiological Reviews* 87 (1): 99–163.
- Lemasters, J J. 2014. "Variants of Mitochondrial Autophagy: Types 1 and 2 Mitophagy and Micromitophagy (Type 3)." *Redox Biology* 2 (1): 749–54.
- Li, L Y, X Luo, and X Wang. 2001. "Endonuclease G Is an Apoptotic DNase When Released from Mitochondria." *Nature* 412: 90–94.
- Linn, S C, A H Honkoop, K Hoekman, P van der Valk, H M Pinedo, and G Giaccone. 1996. "p53 and P-Glycoprotein Are Often Co-Expressed and Are Associated with Poor Prognosis in Breast Cancer." *British Journal of Cancer* 74: 63–68.
- Lupardus, P J, R A Wilke, E Aydar, C P Palmer, Y Chen, A E Ruoho, and M B Jackson. 2000. "Membrane-Delimited Coupling between Sigma Receptors and K⁺ Channels in Rat Neurohypophyseal Terminals Requires Neither G-Protein nor ATP." *The Journal of Physiology* 526 Pt 3: 527–39.
- Ma, Q. 2013. "Role of nrf2 in Oxidative Stress and Toxicity." *Annual Review of Pharmacology and Toxicology* 53: 401–26.
- Mach, R H, C R Smith, I Al-Nabulsi, B R Whirrett, S R Childers, and K T Wheeler. 1997. "Sigma-2 Receptors as Potential Biomarkers of Proliferation in Breast Cancer." *Cancer Research* 57 (1): 156–61.

- Mach, R H, C Zeng, and W G Hawkins. 2013. "The Sigma-2 Receptor: A Novel Protein for the Imaging and Treatment of Cancer." *Journal of Medicinal Chemistry* 56: 7137–60.
- Maher, E R, and W G Kaelin. 1997. "Von Hippel-Lindau Disease." *Medicine* 76 (6): 381–91.
<http://www.ncbi.nlm.nih.gov/pubmed/9413424>.
- Martin, W R, C G Eades, J A Thompson, R E Huppler, and P E Gilbert. 1976. "The Effects of Morphine- and Nalorphine-like Drugs in the Nondependent and Morphine-Dependent Chronic Spinal Dog." *Journal of Pharmacology and Experimental Therapeutics* 197 (3): 517–32.
- Matsumoto, R R. 2007. "Sigma Receptors: Historical Perspective and Background." In *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications*, edited by R R Matsumoto, Wayne D Bowen, and T-P Su, 1–24. New York: Springer Science+Business Media.
- . 2009. "Targeting Sigma Receptors: Novel Medication Development for Drug Abuse and Addiction." *Expert Review of Clinical Pharmacology* 2 (4): 351–58.
- Matsumoto, R R, K A McCracken, M J Friedman, B Pouw, B R De Costa, and W D Bowen. 2001. "Conformationally Restricted Analogs of BD1008 and an Antisense Oligodeoxynucleotide Targeting sigma1 Receptors Produce Anti-Cocaine Effects in Mice." *European Journal of Pharmacology* 419 (2-3): 163–74.
- Matsumoto, R R, K A McCracken, B Pouw, Y Zhang, and W D Bowen. 2002. "Involvement of Sigma Receptors in the Behavioral Effects of Cocaine: Evidence from Novel Ligands and Antisense Oligodeoxynucleotides." *Neuropharmacology* 42 (8): 1043–55.

- Maurice, T. 2007. "Cognitive Effects of Sigma Receptor Ligands." In *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications*, edited by R R Matsumoto, W D Bowen, and T-P Su, 237–72. New York: Springer Science+Business Media.
- Maurice, T, and B P Lockhart. 1997. "Neuroprotective and Anti-Amnesic Potentials of Sigma Receptor Ligands." *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 21 (1): 69–102.
- Maurice, T, R Martin-Fardon, P Romieu, and R R Matsumoto. 2002. "Sigma(1) Receptor Antagonists Represent a New Strategy against Cocaine Addiction and Toxicity." *Neuroscience & Biobehavioral Reviews* 26 (4): 499–527.
- Maurice, T, A Urani, V L Phan, and P Romieu. 2001. "The Interaction between Neuroactive Steroids and the sigma1 Receptor Function: Behavioral Consequences and Therapeutic Opportunities." *Brain Research* 37 (1-3): 116–32.
- Mavlyutov, Timur A, and Arnold E Ruoho. 2007. "Ligand-Dependent Localization and Intracellular Stability of Sigma-1 Receptors in CHO-K1 Cells." *Journal of Molecular Signaling* 2: 8.
- McCann, D J, and T-P Su. 1990. "Haloperidol-Sensitive (+)[3H]SKF-10,047 Binding Sites (sigma Sites) Exhibit a Unique Distribution in Rat Brain Subcellular Fractions." *European Journal of Pharmacology* 188 (4-5): 211–18.

- . 1991. “Solubilization and Characterization of Haloperidol-Sensitive (+)-[3H]SKF-10,047 Binding Sites (sigma Sites) from Rat Liver Membranes.” *Journal of Pharmacology and Experimental Therapeutics* 257 (2): 547–54.
- McCurdy, C R, C Mesangeau, R R Matsumoto, J H Poupaert, B A Avery, and A H A Abdelazeem. 2014. Highly selective sigma receptor ligands. US8686008 B2, issued 2014.
- McLean, S, and E Weber. 1988. “Autoradiographic Visualization of Haloperidol-Sensitive Sigma Receptors in Guinea-Pig Brain.” *Neuroscience* 25: 259–69.
- Mendelsohn, L G, V Kalra, B G Johnson, and G A Kerchner. 1985. “Sigma Opioid Receptor: Characterization and Co-Identity with the Phencyclidine Receptor.” *Journal of Pharmacology and Experimental Therapeutics* 233 (3): 597–602.
- Mesangeau, C, S Narayanan, A M Green, J Shaikh, N Kaushal, E Viard, Y T Xu, et al. 2008. “Conversion of a Highly Selective Sigma-1 Receptor-Ligand to Sigma-2 Receptor Preferring Ligands with Anticocaine Activity.” *Journal of Medicinal Chemistry* 51 (5): 1482–86.
- Mitra, P, M Maceyka, S G Payne, N Lamour, S Milstien, C E Chalfant, and S Spiegel. 2007. “Ceramide Kinase Regulates Growth and Survival of A549 Human Lung Adenocarcinoma Cells.” *FEBS Letters* 581 (4): 735–40. <http://www.ncbi.nlm.nih.gov/pubmed/17274985>.
- Moebius, F F, G G Burrows, M Hanner, E Schmid, J Striessnig, and H Glossmann. 1993. “Identification of a 27-kDa High Affinity Phenylalkylamine-Binding Polypeptide as the

Sigma 1 Binding Site by Photoaffinity Labeling and Ligand-Directed Antibodies.”

Molecular Pharmacology 44 (5): 966–71.

Moebius, F F, R J Reiter, M Hanner, and H Glossmann. 1997. “High Affinity of Sigma 1-

Binding Sites for Sterol Isomerization Inhibitors: Evidence for a Pharmacological

Relationship with the Yeast Sterol C8-C7 Isomerase.” *British Journal of Pharmacology* 121

(1): 1–6.

Monnet, F P. 2007. “Intracellular Signaling and Synaptic Plasticity.” In *Sigma Receptors:*

Chemistry, Cell Biology and Clinical Implications, edited by R R Matsumoto, W D Bowen,

and T-P Su, 165–94. New York: Springer Science+Business Media.

Monnet, F P, and T Maurice. 2006. “The sigma1 Protein as a Target for the Non-Genomic

Effects of Neuro(active)steroids: Molecular, Physiological, and Behavioral Aspects.”

Journal of Pharmacological Sciences 100 (2): 93–118.

Morin-Surun, M P, T Collin, M Denavit-Saubié, E E Baulieu, and F P Monnet. 1999.

“Intracellular sigma1 Receptor Modulates Phospholipase C and Protein Kinase C Activities

in the Brainstem.” *Proceedings of the National Academy of Sciences of the United States of*

America 96 (14): 8196–99.

Mtchedlishvili, Z, and J Kapur. 2003. “A Presynaptic Action of the Neurosteroid Pregnenolone

Sulfate on GABAergic Synaptic Transmission.” *Molecular Pharmacology* 64 (4): 857–64.

- Nagornaia, L C, N N Samovilova, N V Korobov, and V A Vinogradov. 1988. "Partial Purification of Endogenous Inhibitors of (+)-[3H]SKF-10,047 Binding with Sigma Opioid Receptors of the Liver." *Biulleten Eksperimentalnoi Biologii Meditsiny* 106: 314–17.
- Nguyen, E C, K A McCracken, Y Liu, B Pouw, and R R Matsumoto. 2005. "Involvement of Sigma Receptors in the Acute Actions of Methamphetamine: Receptor Binding and Behavioral Studies." *Neuropharmacology* 49 (5): 638–45.
- Novakova, M, C Ela, J Barg, Z Vogel, Y Hasin, and Y Eilam. 1995. "Inotropic Action of Sigma Receptor Ligands in Isolated Cardiac Myocytes from Adult Rats." *European Journal of Pharmacology* 286 (1): 19–30.
- Ogretmen, B, and Y A Hannun. 2004. "Biologically Active Sphingolipids in Cancer Pathogenesis and Treatment." *Nature Reviews Cancer* 4 (8): 604–16.
- Ola, M S, P Moore, A El-Sherbeny, P Roon, N Agarwal, V P Sarthy, P Casellas, V Ganapathy, and S B Smith. 2001. "Expression Pattern of Sigma Receptor 1 mRNA and Protein in Mammalian Retina." *Brain Research* 95 (1-2): 86–95.
- Orlati, S, A M Porcelli, S Hrelia, A Lorenzini, and M Rugolo. 1998. "Intracellular Calcium Mobilization and Phospholipid Degradation in Sphingosylphosphorylcholine-Stimulated Human Airway Epithelial Cells." *Biochemical Journal* 334 (Pt 3): 641–49.
- Ostenfeld, M S, N Fehrenbacher, M Høyer-Hansen, C Thomsen, T Farkas, and M Jäättelä. 2005. "Effective Tumor Cell Death by Sigma-2 Receptor Ligand Siramesine Involves Lysosomal Leakage and Oxidative Stress." *Cancer Research* 65 (19): 8975–83.

- Pasternak, G W, M Carroll-Buatti, and K Spiegel. 1981. "The Binding and Analgesic Properties of a Sigma Opiate, SKF 10,047." *Journal of Pharmacology and Experimental Therapeutics* 219 (1): 192–98.
- Patterson, T A, M A Connor, and C Chavkin. 1994. "Recent Evidence for Endogenous Substance(s) for Sigma Receptors." In *Sigma Receptors*, edited by Y Itzhak, 171–89. San Diego: Academic Press.
- Pearl, J, and L S Harris. 1966. "Inhibition of Writhing by Narcotic Antagonists." *Journal of Pharmacology and Experimental Therapeutics* 154 (2): 319–23.
- Prokop, A, T Wieder, I Sturm, F Essmann, K Seeger, C Wuchter, W D Ludwig, G Henze, B Dorken, and P T Daniel. 2000. "Relapse in Childhood Acute Lymphoblastoma Leukemia Is Associated with a Decrease of the Bax/Bcl-2 Ratio and Loss of Spontaneous Caspase-3 Processing in Vivo." *Leukemia* 14 (1606-1613).
- Samovilova, N N, and V A Vinogradov. 1992. "Subcellular Distribution of (+)-[3H]SKF 10,047 Binding Sites in Rat Liver." *European Journal of Pharmacology* 225: 69–74.
- Seminario, M J, M J Robson, A H Abdelazeem, C Mesangeau, S Jamalapuram, B A Avery, C R McCurdy, and R R Matsumoto. 2012. "Synthesis and Pharmacological Characterization of a Novel Sigma Receptor Ligand with Improved Metabolic Stability and Antagonistic Effects against Methamphetamine." *AAPS Journal* 14 (1): 43–51.

- Seth, P, Y J Fei, H W Li, W Huang, F H Leibach, and V Ganapathy. 1998. "Cloning and Functional Characterization of a Sigma Receptor from Rat Brain." *Journal of Neurochemistry* 70 (3): 922–31.
- Shen, G, and A-N Kong. 2009. "Nrf2 Plays an Important Role in Coordinated Regulation of Phase II Drug Metabolism Enzymes and Phase III Drug Transporters." *Biopharmaceutics & Drug Disposition* 30 (7): 345–55.
- Shioda, N, K Ishikawa, H Tagashira, T Ishizuka, H Yawo, and K Fukunaga. 2012. "Expression of a Truncated Form of the Endoplasmic Reticulum Chaperone Protein, Sigma-1 Receptor, Promotes Mitochondrial Energy Depletion and Apoptosis." *Journal of Biological Chemistry* 287 (28): 23318–31.
- Sircar, R, R Nichtenhauser, J R Ieni, and S R Zukin. 1986. "Characterization and Autoradiographic Visualization of (+)-[3H]SKF10,047 Binding in Rat and Mouse Brain: Further Evidence for Phencyclidine/'sigma Opiate' Receptor Commonality." *Journal of Pharmacology and Experimental Therapeutics* 237 (2): 681–88.
- Søby, K K, J D Mikkelsen, E Meier, and C Thomsen. 2002. "Lu 28-179 Labels a sigma(2)-Site in Rat and Human Brain." *Neuropharmacology* 43 (1): 95–100.
- Spitzer, D, P O Simon, H Kashiwagi, J Xu, C Zeng, S Vangveravong, D Zhou, et al. 2012. "Use of Multifunctional Sigma-2 Receptor Ligand Conjugates to Trigger Cancer-Selective Cell Death Signaling." *Cancer Research* 72 (1): 201–9.

- Su, T-P. 1982. "Evidence for the Sigma Opioid Receptor: Binding of [3H]SKF-10047 to Etorphine-Inaccessible Sites in Guinea-Pig Brain." *Journal of Pharmacology and Experimental Therapeutics* 223: 284–90.
- Su, T-P, and T Hayashi. 2001. "Cocaine Affects the Dynamics of Cytoskeletal Proteins via sigma(1) Receptors." *Trends in Pharmacological Sciences* 22 (9): 456–58.
- Su, T-P, T Hayashi, T Maurice, S Buch, and A E Ruoho. 2010. "The Sigma-1 Receptor Chaperone as an Inter-Organelle Signaling Modulator." *Trends in Pharmacological Sciences* 31 (12): 557–66.
- Su, T-P, E D London, and J H Jaffe. 1988. "Steroid Binding at Sigma Receptors Suggests a Link between Endocrine, Nervous, and Immune Systems." *Science* 240 (4849): 219–21.
- Su, T-P, and D B Vaupel. 1988. "Further Characterization of an Endogenous Ligand ('SIGMAPHIN') for Sigma Receptors in the Brain." In *Society for Neuroscience Annual Meeting Proceedings*, 14:545.
- Su, T-P, A D Weissman, and S Y Yeh. 1986. "Endogenous Ligands for Sigma Opioid Receptors in the Brain ('sigmaphin'): Evidence from Binding Assays." *Life Sciences* 38 (24): 2199–2210.
- Takebayashi, M, T Hayashi, and T-P Su. 2004. "Sigma-1 Receptors Potentiate Epidermal Growth Factor Signaling towards Neuritogenesis in PC12 Cells: Potential Relation to Lipid Raft Reconstitution." *Synapse* 53 (2): 90–103.

- Tam, S W. 1983. "Naloxone-Inaccessible Sigma Receptor in Rat Central Nervous System." *Proceedings of the National Academy of Sciences of the United States of America* 80 (21): 6703–7.
- Tam, S W, and L Cook. 1984. "Sigma Opiates and Certain Antipsychotic Drugs Mutually Inhibit (+)-[3H] SKF 10,047 and [3H]haloperidol Binding in Guinea Pig Brain Membranes." *Proceedings of the National Academy of Sciences of the United States of America* 81 (17): 5618–21.
- Teitz, T, J M Lahti, and V J Kidd. 2001. "Aggressive Childhood Neuroblastomas Do Not Express Caspase-8: An Important Component of Programmed Cell Death." *Journal of Molecular Medicine* 79 (8): 428–36.
- Torrence-Campbell, C, and W D Bowen. 1996. "Differential Solubilization of Rat Liver Sigma-1 and Sigma-2 Receptors: Retention of Sigma-2 Sites in Particulate Fractions." *European Journal of Pharmacology* 304 (210-210).
- Ueda, H, M Inoue, A Yoshida, K Mizuno, H Yamamoto, J Maruo, K Matsuno, and S Mita. 2001. "Metabotropic Neurosteroid/ Sigma-Receptor Involved in Stimulation of Nociceptor Endings of Mice." *Journal of Pharmacology and Experimental Therapeutics* 298: 703–10.
- Ukai, M, H Maeda, Y Nanya, T Kameyama, and K Matsuno. 1998. "Beneficial Effects of Acute and Repeated Administrations of Sigma Receptor Agonists on Behavioral Despair in Mice Exposed to Tail Suspension." *Pharmacology Biochemistry and Behavior* 61: 247–52.

- Urani, A, P Romieu, F J Roman, K Yamada, Y Noda, H Kamei, H Manh Tran, T Nagai, T Nabeshima, and T Maurice. 2004. "Enhanced Antidepressant Efficacy of sigma1 Receptor Agonists in Rats after Chronic Intracerebroventricular Infusion of Beta-Amyloid-(1-40) Protein." *European Journal of Pharmacology* 486 (2): 151–61.
- Vilner, B J, and W D Bowen. 2000. "Modulation of Cellular Calcium by Sigma-2 Receptors: Release from Intracellular Stores in Human SK-N-SH Neuroblastoma Cells." *Journal of Pharmacology and Experimental Therapeutics* 292 (3): 900–911.
- Vilner, B J, B R de Costa, and W D Bowen. 1995. "Cytotoxic Effects of Sigma Ligands: Sigma Receptor-Mediated Alterations in Cellular Morphology and Viability." *Journal of Neuroscience* 15 (1 Pt 1): 117–34.
- Vilner, B J, C S John, and W D Bowen. 1995. "Sigma-1 and Sigma-2 Receptors Are Expressed in a Wide Variety of Human and Rodent Tumor Cell Lines." *Cancer Research* 55 (2): 408–13.
- Wang, W-F, K Ishiwata, M Kiyosawa, K Kawamura, K Oda, T Kobayashi, K Matsuno, and M Mochizuki. 2002. "Visualization of sigma1 Receptors in Eyes by Ex Vivo Autoradiography and in Vivo Positron Emission Tomography." *Experimental Eye Research* 75 (6): 723–30.
- Wang, X, and W D Bowen. 2006. "Sigma-2 Receptors Mediate Apoptosis in SK-N-SH Neuroblastoma Cells via Caspase-10-Dependent Bid Cleavage and Mitochondrial Release of Endonuclease G and Apoptosis-Inducing Factor." In *Society for Neuroscience Annual Meeting Proceedings*, 90.1. Atlanta.

- . 2009. “Sigma-2 Receptor-Mediated Apoptosis in Pancreatic Cancer Cells.” In *Proceedings of the American Association for Cancer Research*, 50:102.
- Wang, Y, J Xu, X Xia, M Yang, S Vangveravong, J Chen, R H Mach, and Y Xia. 2012. “SV119-Gold Nanocage Conjugates: A New Platform for Targeting Cancer Cells via Sigma-2 Receptors.” *Nanoscale* 4 (C): 421.
- Weber, E, M Sonders, and J F Keana. 1987. Sigma brain receptor ligands and their use. 4709094, issued 1987.
- Weber, E, M Sonders, M Quarum, S McLean, S Pou, and J F W Keana. 1986. “1,3-Di(2-[5-³H]tolyl)guanidine: A Selective Ligand That Labels Sigma-Type Receptors for Psychotomimetic Opiates and Antipsychotic Drugs.” *Proceedings of the National Academy of Sciences of the United States of America* 83: 8784–88.
- Werling, L L, A E Derbez, and S J Nuwayhid. 2007. “Modulation of Classical Neurotransmitter Systems by Sigma Receptors.” In *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications*, edited by R R Matsumoto, W D Bowen, and T-P Su, 195–214. New York: Springer Science+Business Media.
- Wheeler, K T, L M Wang, C A Wallen, S R Childers, J M Cline, P C Keng, and R H Mach. 2000. “Sigma-2 Receptors as a Biomarker of Proliferation in Solid Tumours.” *British Journal of Cancer* 82 (6): 1223–32.

- Wolfe, S A, B K Ha, B B Whitlock, and P Saini. 1997. "Differential Localization of Three Distinct Binding Sites for Sigma Receptor Ligands in Rat Spleen." *Journal of Neuroimmunology* 72 (1): 45–58.
- Wolfe, S A Jr, S G Culp, and E B de Souza. 1989. "Sigma-Receptors in Endocrine Organs: Identification, Characterization, and Autoradiographic Localization in Rat Pituitary, Adrenal, Testis, and Ovary." *Endocrinology* 124: 1160–72.
- Wong, E H, A R Knight, and G N Woodruff. 1988. "[3H]MK-801 Labels a Site on the N-Methyl-D-Aspartate Receptor Channel Complex in Rat Brain Membranes." *Journal of Neurochemistry* 50 (1): 274–81.
- Wu, Z, and W D Bowen. 2008. "Role of Sigma-1 Receptor C-Terminal Segment in Inositol 1,4,5-Trisphosphate Receptor Activation: Constitutive Enhancement of Calcium Signaling in MCF-7 Tumor Cells." *Journal of Biological Chemistry* 283 (42): 28198–215.
- Wu, Z, W Peti, and W D Bowen. 2006. "Delineation of Functional Domains of the Sigma-1 Receptor." In *Society for Neuroscience Annual Meeting Proceedings*, 713.13. Atlanta.
- Xu, J, Z Tu, L A Jones, S Vangveravong, K T Wheeler, and R H Mach. 2005. "[3H]N-[4-(3,4-Dihydro-6,7-Dimethoxyisoquinolin-2(1H)-Yl)butyl]-2-Methoxy-5-Methyl Benzamide: A Novel Sigma-2 Receptor Probe." *European Journal of Pharmacology* 525 (1-3): 8–17.
- Xu, J, C Zeng, W Chu, F Pan, J Rothfuss, Z Fanjie, Z Tu, et al. 2011. "Identification of the PGRMC1 Protein Complex as the Putative Sigma-2 Receptor Binding Site." *Nature Communications* 2 (380).

Zeng, C, J M Rothfuss, J Zhang, S Vangveravong, W Chu, S Li, Z Tu, J Xu, and R H Mach.

2014. "Functional Assays to Define Agonists and Antagonists of the Sigma-2 Receptor."

Analytical Biochemistry 448 (1): 68–74.

Zeng, C, J Rothfuss, J Zhang, W Chu, S Vangveravong, Z Tu, F Pan, K C Chang, R Hotchkiss,

and R H Mach. 2012. "Sigma-2 Ligands Induce Tumour Cell Death by Multiple Signalling

Pathways." *British Journal of Cancer* 106 (4): 693–701.

Zeng, C, S Vangveravong, J Xu, K C Chang, R S Hotchkiss, K T Wheeler, D Shen, Z P Zhuang,

H F Kung, and R H Mach. 2007. "Subcellular Localization of Sigma-2 Receptors in Breast

Cancer Cells Using Two-Photon and Confocal Microscopy." *Cancer Research* 67 (14):

6708–16.

Zukin, S R, K T Brady, B L Slifer, and R L Balster. 1984. "Behavioral and Biochemical

Stereoselectivity of Sigma opiate/PCP Receptors." *Brain Research* 294 (1): 174–77.