SIGMA-2 RECEPTOR-MEDIATED CYTOTOXICITY AND CALCIUM SIGNALING: EVIDENCE FOR BIFURCATING PATHWAYS.

BY

DIOSCARIS R. GARCIA

B.S. UNIVERSITY OF RHODE ISLAND, 2004

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE DEPARTMENT OF MOLECULAR PHARMACOLOGY, PHYSIOLOGY, AND BIOTECHNOLOGY AT BROWN UNIVERSITY

PROVIDENCE, RHODE ISLAND

MAY 2012
This dissertation by Dioscaris R. Garcia is accepted in its present form by Department of Molecular Pharmacology, Physiology, and Biotechnology as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date_________________  
Dr. Wayne Bowen, Advisor

Recommended to the Graduate Council

Date_________________  
Dr. John Marshall, Reader

Date_________________  
Dr. Anita Zimmerman, Reader

Date_________________  
Dr. Anatoly Zhitkovich, Reader

Date_________________  
Dr. Rae Matsumoto, Reader

Approved by the Graduate Council

Date_________________  
Dr. Peter M. Weber, Dean of the Graduate School
CURRICULUM VITAE

DIOSCARIS R. GARCIA
26 CEDARBROOK ROAD, PAWTUCKET, RI 02861
DIOSCARISGARCIA@GMAIL.COM (401) 368-2574

PROFESSIONAL PROFILE

Sigma receptors are a unique pharmacologically defined class of drug-binding proteins comprised of the sigma-1 and sigma-2 subtypes differentiated by their pharmacological profile, functionality and size (25kd and 18kd respectively). To date, sigma receptors have been found in the CNS and peripheral organs such as liver, kidneys and pancreas and are highly expressed in tumor cells. Sigma receptors have been implicated neuronal processes such as synaptic plasticity, schizophrenia and drug abuse as well as cellular processes ranging from modulation of calcium release and neurotransmitters, growth and apoptosis and regulation of movement and posture. My work has focused on characterizing the signal transduction pathway of the sigma-2 receptor and the effects receptor-targeted ligands via calcium-release and cytotoxicity assays.

EDUCATION

2005-2011 Brown University Providence, RI
- PhD Medical Science
  - Sigma-2 Receptor Pharmacology
- AAAS Member
- SfN Member
- Pharmacia Scholar
- Sigma Xi Scholar

2000-2004 University Of Rhode Island Kingston, RI
- B.S. Microbiology
- Minor in Chemistry
- USDA Scholar
- DiMaio Scholar
- National Scholars Honor Society
- Outstanding Students Honor Society
- Member of American Society for Microbiologists
EXPERIENCE

2004 – 2005 Amgen, Inc. West Greenwich, RI

- Process Development – Purification (Enbrel manufacturing process)
- Ultrafiltration/Diafiltration Systems
- AKTA Liquid Chromatography Systems
- Characterization of Bench-Top Purification Systems
- Well versed in cGMPs
- Generation and Optimization of protocols

2003-2004 University of Rhode Island Kingston, RI

Studied the *Micromonospora* *Sp.* population in the marine sediment of Fisher’s Island Sound through the use of phylogenetic studies. Also, assayed for the production of bioactive secondary metabolites from Gram-positive marine bacteria through novel screening methods.

- Developed Gram positive selective medium
- High volume culture growth and purification
- Designed proliferation schemes for several microorganisms
- Developed genetic and morphological Identification scheme
- Extraction methods of culture broths with various organic solvents
- Developed DNA extraction methods for different types of cell envelope composition.

2002-2003 Hybrigene LLC. West Kingston, RI

Tested various experimental bi-directional promoters using the genetic model *Arabidopsis thaliana*. Also, assisted in projects related to the production and analysis of transgenic turf grass and rice.

- Plant tissue culture
- Gene expression analysis in plants
- Gus assay
- Gene bombardment using the Sanford Gene Gun
PUBLICATIONS AND PRESENTATIONS:


TECHNICAL SKILLS

- Experience using advanced instrumentation in the discovery of drugs and protein purification including:
  - RotaVapor
  - HPLC
  - AKTA Systems
  - Advanced Molecular Biology techniques:
    - Knockdowns
    - Western Blotting
    - DNA Extraction
    - PCR
  - Expert in cell culture techniques.
  - Advanced Pharmacological techniques:
    - Cytotoxicity and Proliferation Assays
    - Calcium release
    - Ligand Binding
    - Signal Transduction Characterization
  - Advanced Microbiological Techniques
  - Experience using Electron Microscopes
  - Fluent speaker and writer of English and Spanish
Abstract

Sigma receptors are a unique pharmacologically defined class of drug-binding proteins comprised of the sigma-1 and sigma-2 subtypes. These subtypes are differentiated by their pharmacological profile, functionality and size (25kDa and 21.5kDa respectively). To date, sigma receptors have been found in the CNS and peripheral organs such as liver, kidneys and pancreas and are highly expressed in tumor cells. Sigma receptors have been implicated neuronal processes such as synaptic plasticity, schizophrenia and drug abuse as well as cellular processes ranging from modulation of calcium release and neurotransmitters, growth and apoptosis and regulation of movement and posture.

The sigma-2 selective ligand CB-64D has shown the ability to produce a biphasic calcium signal consisting of a rapid, transient rise followed by a delayed, prolonged calcium signal. In cytotoxicity studies, CB-64D has a dose-dependent cytotoxic effect with an EC50 of 6+/-2μM under normal conditions in the SK-N-SH neuroblastoma cell line. In addition, the ability of the CB-64D analog CB-184 to induce a dose-dependent formation of Sphingosylphosphorylcholine (SPC) and ceramide, has provided additional insight on the possible second messengers responsible for modulating the apoptotic and non-cytotoxic signals. Focusing on the non-cytotoxic signal, we characterized Sphingosylphosphorylcholine in parallel with CB-64D, testing their ability to induce cell survival, proliferation and DNA-synthesis. While CB-64D showed no induction of the aforementioned effects, SPC appeared to induce cell proliferation in conditions favoring cell death.

Due to the absence of an endogenous ligand or cloning of the receptor, we’ve employed the use of synthetic ligands as our primary tool for elucidating the signaling pathway of the sigma-2 receptor. Characterizing the signal transduction of the sigma-2
receptor has led to a bifurcating pathway leading to both apoptosis and non-cytotoxic signals in the neuronal SK-N-SH cell line. This bifurcation has allowed for the proposal of a sigma-2 receptor pathway model based on the signals observed from characterization of the various synthetic ligands tested to date.
Acknowledgements

Firstly, I’d like to thank my parents, without your countless sacrifices and continued support I would not be where I am today. I’d also like to extend my deepest gratitude to the “Gold Team 1996” at Central Falls High School for seeing in me attributes I didn’t see in myself, and teaching an otherwise misguided urban youth how to embrace my talents and branding in me the love for science that started it all. Mrs. Carol Silver from Central Falls High school for dragging me out of football practice and forcing me to write my college essays in that makeshift desk in the guidance office. I simply cannot forget Deborah Grossman-Garber for selecting me to be a recipient of the USDA scholarship at URI and providing me with the means to attend college. Additionally, I’d like to thank Dr. David Rowley for encouraging me to apply to graduate school and for being a great mentor during my days at URI.

Needless to say none of this would be possible without the guidance and support of my advisor and mentor, Dr. Wayne Bowen. You gave me the independence to flourish as a scientist and teacher to my undergrads, while constantly pushing me to strive to be the best I can be. You will always be the voice inside my head questioning if we have explored every possible experiment and keeping my head out of the “ostrich hole”. I’d also like to acknowledge my committee for their continued support and guidance. Dr. Anita Zimmerman for always being available to answer all of my questions and having hands-down the best snacks in BMC. I greatly appreciate your help and guidance in my early days at Brown. Dr. Anatoly Zhitkovich, for heading one of the most interesting courses I’ve ever taken and your vast knowledge in the field of cancer. I’d like to extend great appreciation to Dr. John Marshall for always being available whenever I needed you and Dr. Rae Matsumoto for graciously agreeing to be my outside reader.
Last but not least, I’d like to extend much overdue gratitude to my fiancé Rossy, for putting up with my late nights in lab and in my office.
# Table of Contents

## Chapter I

- Introduction ........................................................................................................ 2
- Sigma-2 Receptor Signaling .............................................................................. 3
- Sphingolipid Signaling ..................................................................................... 11
- Calcium Signaling ............................................................................................. 13
- A Bifurcating Pathway ..................................................................................... 14

## Chapter II

- Materials and Methods .................................................................................... 20

## Chapter III

- Sphingolipids as Signaling Molecules ............................................................. 26
- Metabolism and Downstream Targets of Sphingosylphosphorylcholine .......... 27
- Results and Discussion ................................................................................... 28
- Calcium Signaling ............................................................................................. 31
- Effect of the Non-Cytotoxic Signal .................................................................. 31
- Conclusion ......................................................................................................... 44

## Chapter IV

- Bifurcating Signaling via the Sigma-2 Receptor .............................................. 47
- Results .............................................................................................................. 50
- Effects of the Putative Sigma-2 Receptor Antagonist AC927 on the Calcium and Cytotoxic Signal of CB-64D in the SK-N-SH Neuroblastoma Cell Line .......... 55
- Effects of SN79 on the Sigma-2 Receptor System ............................................ 57
- UMB Series ....................................................................................................... 64
- MV1036A Blocks CB-64D's Cytotoxic Effect .................................................... 64
- Discussion ......................................................................................................... 73

## Chapter V

- Summary and Conclusion ............................................................................... 82

## References ........................................................................................................ 88
List of Tables

Chapter III

**Table 1.** Broad range of effects of SPC on intracellular calcium........................30

Chapter IV

**Table 2.** Binding affinity of the putative sigma-2 receptor antagonists..............51

**Table 3.** Effects of various sigma-2 receptor ligands.......................................78
List of Illustrations

Chapter I

Figure 1. Molecular structure of CB-64D and CB-184 ........................................6
Figure 2. Cytotoxic effect of 24 hour exposure of CB-64D on neuroblastoma SK-N-SH cells .................................................................7
Figure 3. Biphasic calcium signal elicited by 100μM CB-64D on the SK-N-SH neuroblastoma cell line .................................................................8
Figure 4. Dose-dependent production of sphingosylphosphorylcholine elicited by CB-184 in the SKBr3 breast tumor cell line ........................................9
Figure 5. Production of ceramide elicited by CB-184 in the MCF-7/Adr breast tumor cell line .................................................................10
Figure 6. CB-184-dependent production of SPC in the SKBr3 breast tumor cell line is attenuated by the putative sigma-2 receptor antagonist AC927 ........................................16
Figure 7. Preliminary proposed sigma-2 receptor signaling pathway in the SK-N-SH neuroblastoma cell line .................................................................17

Chapter III

Figure 8. Known metabolism of sphingosylphosphorylcholine ........................................29
Figure 9. Transient calcium signal elicited by 30μM CB-64D on the SK-N-SH neuroblastoma cell line .................................................................35
Figure 10. Transient calcium signal elicited by 10μM SPC on the SK-N-SH neuroblastoma cell line .................................................................36
Figure 11. Effect of 24 hour SPC exposure on the SK-N-SH neuroblastoma cell line .................................................................37
Figure 12. Effect of SPC on SK-N-SH neuroblastoma cells in normal 10% FBS growing conditions in the absence of insulin over 72 hours .....................38
Figure 13. Effect of CB-64D on SK-N-SH neuroblastoma cells under normal 10% FBS conditions in the absence of insulin over 72 hours ....................39
Figure 14. CB-64D has no effect on the doubling time of serum-starved SK-N-SH neuroblastoma cell line .................................................................40
Figure 15. SPC has no effect in the doubling time of serum starved SK-N-SH neuroblastoma cell line .................................................................41
Figure 16. Effect of CB-64D on the induction of DNA-synthesis in serum-starved SK-N-SH neuroblastoma cell line .................................................................42
Figure 17. Effect of SPC on the induction of DNA-synthesis in serum-starved SK-N-SH neuroblastoma cell line .................................................................43
Figure 18. Molecular structure of AC927. .................................................................49
Chapter IV

Figure 19. Molecular structure of SN79. ..........................................................52

Figure 20. Effect of 24 hour exposure of AC927 on the SK-N-SH neuroblastoma cell line..........................................................54

Figure 21. Calcium signal elicited by 30µM AC927 in the SK-N-SH neuroblastoma cell line. ..........................................................56

Figure 22. Antagonism of the calcium signal induced by 50µM CB-64D by 50µM AC927..........................................................59

Figure 23. Antagonism of the cytotoxicity of 10µM CB-64D by a variable concentration range of AC927 over a 24 hour period in the SK-N-SH neuroblastoma cell line. ..........................................................60

Figure 24. Effect of SN79 on the SK-N-SH neuroblastoma cell line over 24 hours. ........................................................................61

Figure 25. Augmentation of the cytotoxic effect of 3µM CB-64D by SN79 in the SK-N-SH neuroblastoma cell line over a 24 hour exposure.................62

Figure 26. Antagonistic effect of 30µM SN79 on 30µM CB-64D compared to the effects of 30µM SN79 and 30µM CB-64D. ..........................................................63

Figure 27. Molecular structures of UMB408 and UMB414. .........................66

Figure 28. Effect of UMB408 over 24 hours in the SK-N-SH neuroblastoma cell line. ........................................................................67

Figure 29. Effect of UMB414 over 24 hours in the SK-N-SH neuroblastoma cell line. ........................................................................68

Figure 30. Comparison of the calcium signals elicited by 30µM UMB408 and 30µM UMB414 compared to 30µM CB-64D..................................................69

Figure 31. 30µM UMB408 has no antagonistic effect on the calcium signal elicited by 30µM CB-64D. ..........................................................70

Figure 32. 30µM UMB414 has no antagonistic effect on the calcium signal elicited by CB-64D..........................................................71

Figure 33. Molecular Structure of MV1036A. .............................................72

Figure 34. Effect of MV1036A over 24hr in the SK-N-SH neuroblastoma cell line over 24 hours..........................................................75

Figure 35. Attenuation of the cytotoxicity of 3µM CB-64D by MV1036A in the SK-N-SH neuroblastoma cell line..........................................................76

Figure 36. Calcium signal elicited by 30µM MV1036A in the SK-N-SH neuroblastoma cell line..........................................................77
Figure 37. Proposed sigma-2 signaling pathway based on the actions of CB-64D and CB-184 and the putative sigma-2 antagonists which have shown activity to date.
CHAPTER I

Background and Introduction
Introduction

Sigma receptors are a unique, pharmacologically defined class of drug-binding proteins comprised of the sigma-1 and sigma-2 receptor subtypes, differentiated by their pharmacological profile, functionality and size. Sigma receptors have been implicated in several pathological and physiological processes ranging from schizophrenia and drug abuse to synaptic plasticity as well as cell processes such as Ca\(^{2+}\) release, cell growth and survival, and apoptosis. Systematically, sigma receptors have been found in a wide array of tissues ranging from the CNS, to peripheral organs such as kidneys, liver and pancreas (Wolfe et al., 1989; Hellewell et al., 1994). Two distinct subtypes of sigma receptors have been elucidated, termed sigma-1 and sigma-2. Pharmacologically, sigma-1 and sigma-2 receptors are differentiated by their affinity for dextrorotatory benzomorphans and morphinans, where sigma-1 receptors have a high affinity for these compounds and sigma-2 receptors have a low to negligible affinity. The sigma-1 receptor has been cloned and consists of a 25 kDa protein whereas photoaffinity labeling suggest the sigma-2 receptor to be a 21.5 kDa protein (Hellewell et al., 1994) with close association to lipid rafts (Gebresselassie and Bowen, 2004). No endogenous ligand has been identified for these receptors, but data suggests a sterol is a likely candidate (Maurice, 2004). Recent reports, propose the PGMCR1 (progesterone receptor membrane component 1) to be the binding site for the sigma-2 receptor (Xu et al., 2012). Additionally, recent studies utilizing 2-photon and confocal microscopy have shown that sigma-2 receptors co-localize with fluorescent markers of the mitochondria, lysosomes, endoplasmic reticulum and the plasma membrane, as well as suggested that sigma-2 receptors are internalized (Zeng et al., 2007). This study strengthened previous observations regarding the potency of sigma-2 ligands in cytotoxic and calcium release
studies, in which ligands showed variable results under different cellular density conditions (Data not shown).

The lack of an identified endogenous ligand has complicated elucidating the biological function of these receptors. The development of the 5-phenylmorphan opiate derivative and highly selective sigma-2 receptor ligand CB-64D and its more selective chlorinated analog CB-184 (Figure 1) has been pivotal in characterizing the properties of the sigma-2 receptor (Bowen et al., 1995). Ligand binding assays showed CB-64D had a 185 fold higher binding affinity for sigma-2 over sigma-1 (Ki = 16.5 nM and 3,063 nM, respectively) and 554 fold higher for CB-184 (Ki = 13.4 nM and 7,436 nM, respectively) making them among the most selective sigma-2 ligands to date (Bowen et al., 1995).

Sigma-2 Receptor Signaling

The sigma-2 receptor has been found to be expressed in high density in some solid tumors (Thomas et al., 1996; Bem et al., 1990). Both sigma receptor subtypes are highly expressed in a variety of tumor cell lines derived from a variety of organs (Vilner et al., 1995). Sigma-2 receptors are upregulated in cells undergoing rapid proliferation (Mach et al., 1997; Al-Nabulsi et al., 1999), and expression has been linked to the ability of cells to progress through the cell cycle. These observations suggest that the sigma-2 receptor may be a trophic receptor. However, activation of the sigma-2 receptor results in cessation of cell division, changes in cell morphology and cell death, as first demonstrated in rat C6 glioma cells (Vilner et al., 1995). Treatment of breast tumor cells with CB-64D or CB-184 resulted in cell death. The mode of cell death was determined to be apoptotic, and occurred via a caspase-independent and p53-independent pathway (Crawford and Bowen, 2002). Subsequent studies have shown that sigma-2 receptor-mediated apoptosis can occur by either caspase-independent or caspase-dependent
pathways. Treatment of human SK-N-SH neuroblastoma cells with CB-64D for 24hrs, results in dose-dependent apoptosis, with an EC$_{50}$ of 6 µM +/- 2 (Figure 2). Cell death occurs via various mechanisms ranging from the indiscriminate necrosis, to the orchestrated apoptosis and cornification. Sigma-2 receptors have been shown to induce cell death by multiple signaling pathways (Zeng et al., 2012). Among these modes of cell death via sigma-2 receptors, apoptosis has been the most notarized. The sigma-2 receptor ligand siramesine showed the ability to induce caspase-independent cell death (Ostenfeld et al., 2012). Unlike the previously reported caspase-independent mode of cell death induced by CB-64D, siramesine was shown to destabilize lysosomes leading to autophagy (Ostenfeld et al., 2008). Although the mechanism by which siramesine triggers autophagy has not been fully characterized, the evidence supporting the induction of cell death by sigma-2 receptors indicates a broad spectrum by which sigma-2 receptors trigger cellular demise.

Sigma-2 receptors have been implicated in cellular calcium mobilization. Treatment of SK-N-SH neuroblastoma cells with CB-64D results in the release of Ca$^{2+}$ from thapsigargin-sensitive ER storage and mitochondrial pools, as described by a transient Ca$^{2+}$ increase (Figure 3), followed by a prolonged and sustained Ca$^{2+}$ signal (Vilner and Bowen, 2000). These signaling profiles have been typically associated with cell proliferation and apoptosis respectively (Berridge et al., 1998). Treatment of breast tumor cells with the more potent analog CB-184 has been shown to stimulate the formation of the sphingolipids sphingosylphosphorylcholine (SPC) (Figure 4) and ceramide (Figure 5) with a subsequent decline in sphingomyelin in a dose and time responsive manner (Crawford et al., 2000, 2002).

Similar results were obtained for ceramide in SK-N-SH neuroblastoma cells. In addition, the production of these products was shown to be blocked by the sigma-2
receptor antagonist AC927 (Crawford et al., 2000, 2002) further cementing the production of these molecules to be specific to the sigma-2 receptor. The production of SPC and ceramide occurred in the absence of sphingomyelinase (SMase) activity (Crawford et al., 2000, 2002) suggesting an alternate mode of ceramide production. Given the absence of SMase activation, there is no known mammalian enzyme with the ability to modulate the production of SPC and ceramide fitting the observed profile.
Figure 1. Molecular structure of CB-64D and CB-184.

C. Bertha
Figure 2. Cytotoxic effect of 24 hour exposure of CB-64D on neuroblastoma SK-N-SH cells. Low passage SK-N-SH neuroblastoma cells were incubated for 24hrs in MEM supplemented with 10%FBS and 10mg/L insulin. Cells are plated at 5,000 cells per well from 60-65% confluent flasks and incubated for 24hrs. This data represents the average of 6 experiments, each consisting of 5 duplicates. An average EC$_{50}$ of 6µM +/-2 is obtained under these conditions.
Figure 3. Biphasic calcium signal elicited by 100μM CB-64D on the SK-N-SH neuroblastoma cell line. Cells attached to cover slips were loaded with Indo-1 in calcium-containing DPBS, and mounted on Leuden cover slip dishes.

Vilner and Bowen, 2000. JPET.
Figure 4. Dose-dependent production of sphingosylphosphorylcholine elicited by CB-184 in the SKBr3 breast tumor cell line. Cells were seeded at 500,000 cells per well in 6-well culture dishes. Cells were incubated in the presence of $[^3]H$ palmitic acid for 48hrs. Cells were washed, replaced with normal DMEM, and incubated in the presence of CB-184. Data was analyzed by TLC.

Crawford and Bowen, Eur. J. Pharmacol, 2002
Figure 5. Production of ceramide elicited by CB-184 in the MCF-7/Adr breast tumor cell line. Cells were seeded at 500,000 cells per well in 6-well culture dishes. Cells were incubated in the presence of $[^3H]$ palmitic acid for 48hrs. Cells were washed, replaced with normal DMEM, and incubated in the presence of CB-184. Data was analyzed by TLC.

Crawford and Bowen, Eur. J. Pharmacol, 2002
Sphingolipid Signaling

Sphingolipids are a major component of lipid rafts (Simons & Ikonen, 1997) and have been reported to be involved in several cellular processes including differentiation, cellular senescence, apoptosis and intra and extracellular Ca$^{2+}$ mobilization, among many other functions (Ohanian and Ohanian, 2001). Sphingosylphosphorylcholine (SPC) has been implicated in a growing list of cell functions as a second messenger, including stimulation of cell death, proliferation, differentiation and mitogenesis (Ohanian and Ohanian, 2001; Xu, 2002). Ceramide on the other hand has been implicated in cell differentiation, apoptosis and proliferation (Guenther et al., 2008; Pettus et al., 2002) among many other cellular functions. Very little is known regarding the actions of SPC in neuronal systems. However, studies in other tissues, most notably muscle tissue, involve SPC in various functions relating to calcium signaling. In addition, no receptor has been identified as a target of SPC. Some studies suggest that SPC may be acting through some of the sphingosine-1-phosphate (S1P) family of receptors or the ER protein termed sphingolipid calcium-mediated protein of the endoplasmic reticulum (SCaMPER) (Kindman et al., 1994; Mao et al., 1996; Betto et al., 1997; Cavalli et al., 2003).

Sphingosine-1-Phosphate (S1P) is the most well characterized of the lysosphingolipids, with a cloned extensive receptor family termed S1P1-5 (Pyne & Pyne, 2000), GPR3, -6, -12 (Uhlenbrock et al., 2002) and GPR63 (Niedenberg et al., 2003). The S1P1,2,3 receptors are coupled to Gi/o which inhibits adenylyl cyclase, activates Ras, ERKs of MAPK family, phospholipase C (PLC), and Ca$^{2+}$ release (Alewijinse et al., 2004). The implication of S1P with GPCR signaling and SPC’s close association with the S1P family of receptors led us to investigate SPC’s role in IP3 signaling. Adding to their similarities, SPC and S1P have been evidenced to be involved in DNA synthesis in
endothelial and smooth muscle cells (Auge et al., 1998). The proliferative action of these sphingolipids has been reported to involve MAPK (Raines et al., 1993), and cytoprotective actions under serum starvation conditions by S1P do so via interaction with ERK (Kimura et al., 2001).

The production of SPC via sigma-2 receptor stimulation, along with the observations regarding the possible implications of sigma-2 receptors acting as a trophic receptor led to the characterization of SPC as a possible second messenger in the sigma-2 receptor system.

The other lipid produced via CB-184 stimulation, ceramide is most commonly associated with apoptosis (Nica et al., 2008; Guenther et al., 2008). Even though the mechanism of ceramide-induced apoptosis is not well understood, it’s been reported that it involves activation of the stress kinases p38 and C-Jun N-terminal (JNK) (Nica et al., 2008). Similar to the sigma-2 receptor-mediated apoptotic pathways, ceramide has been reported to induce apoptosis via both caspase-dependent and caspase-independent pathways (Nica et al., 2008; Guenther et al., 2008) with the involvement of caspase-8 in the caspase-dependent mechanism (Nica et al., 2008). In cells where ceramide plays a role in apoptosis, the apoptotic profile has been reported to involve a biphasic formation of ceramide. This consists of a rapid and transient increase thought to be the inducer phase, and a secondary effector phase consisting of a slow constant increase in ceramide formation (Pettus et al., 2002).

In SK-N-SH neuroblastoma cells, sigma-2 receptor activation by CB-64D results in activation of caspase-8 and caspase-10 (Wang and Bowen, 2006); this is consistent with the formation of ceramide, since ceramide can activate caspase-8 (Nica et al., 2008). Activation of caspases 8 and 10 is known to result in cleavage of the pro-
apoptotic protein Bid, a major substrate for these caspases. Bid cleavage results in formation of t-Bid, which depolarizes the mitochondria and causing release of apoptosis-inducing factor (AIF), endonuclease G, and cytochrome c, (Wang and Bowen, in preparation). This signaling pathway is proposed to link sigma-2 receptor activation to apoptosis, via ceramide formation.

**Calcium Signaling**

One of the hallmarks of the sigma-2 receptor is the ability to produce a calcium signal from thapsigargin-sensitive stores in the ER (Vilner and Bowen, 2000). The sigma-2 receptor selective agonist, CB-184 showed the ability to induce a dose-dependent production of the sphingolipid metabolites ceramide and SPC. Both SPC and ceramide are capable of producing calcium signals from the ER, which provide a clue to the potential effects of the sigma-2 receptor. Calcium is a ubiquitous molecule with a continuously increasing range of roles and effects in biological systems. Calcium signaling can be better understood in terms of spacio-temporal waves. While the ER is the cell’s main calcium storage, the mitochondrion, Golgi apparatus and nucleus also play a role in calcium storage, where calcium plays various roles as a signal for transcription and exocytosis, as well as a pro-differentiating factor, proliferative and pro-apoptotic signal. Aside from the various tissue-specific voltage-gated channels on the plasma membrane, calcium release can be elicited from ryanodine receptors, IP3 receptors, the SERCA pump, and the mitochondrial permeability transition pore, to name a few (Berridge, 2006). While calcium plays a different role depending on the source and the receptor from which it is released, the magnitude, frequency and duration of the signal are significant variables mediating the ultimate effect of the signal. Adding to these variables is the number of chaperones that act to further modulate the signal, namely, calcineurin, calmodulin and the calreticulin/calnexin cycle (Michalak et al, 2002).
As previously mentioned, SPC has been reported to share many of its reported functions and receptor binding with S1P (Meyer zu Heringdorf et al., 2002). However, the body of literature has demonstrated various distinctions, separating the signaling pathways used by SPC and S1P (Chen et al., 1998; Orlati et al., 1998). The Sphingolipid Ca^{2+} Mediating Protein of Endoplasmic Reticulum (SCaMPER) is a 181 amino protein which has been shown to be a target of SPC (Kindman et al., 1994; Mao et al., 1996; Betto et al., 1997; Cavalli et al., 2003), and as a result of SPC binding elicits Ca^{2+} release from the endoplasmic reticulum. Knockdown of SCaMPER with anti-sense RNA in the Xenopus oocyte model blocked the SPC induced Ca^{2+} release (Mao et al. 1996). Due to these reports, SCaMPER emerged as a protein of interest for SPC’s target receptor. Treatment with thapsigargin in Ca^{2+}-free media greatly reduced the Ca^{2+} signal mediated by SPC, indicating an ER source similar to the effects reported from CB-64D in SK-N-SH cells (Vilner and Bowen, 2000). For S1P2 and 3, PLC activation and Ca^{2+} mobilization are Ptx-sensitive/insensitive suggesting they possibly activate G_{i/o} and G_{q/11} respectively (Alewijnse et al., 2004). Due to the great similarity between these two sphingolipids, the possibility of SPC-induced Ca^{2+} release via an S1P receptor or SCaMPER is very likely, as is the possibility that SPC may be acting through a yet to be characterized receptor. The production of SPC has been linked to the sigma-2 receptor pathway by the ability of the sigma-2 selective antagonist AC927 to block the CB-184-induced formation of SPC (Figure 6).

A Bifurcating Pathway

Previous studies have shown that the transient calcium signal that is generated by sigma-2 agonists is not a trigger for apoptosis. If cells are stimulated acutely for time periods long enough to produce a complete transient calcium signal, and then the cells are washed free of the agonist, the cells remain healthy (unpublished observation).
Previous studies have also shown that the sigma antagonist AC927 has the ability to block the CB-64D-induced Ca\(^{2+}\) signal in SK-N-SH cells, and block the SM decrease and the SPC increase in breast tumor cells (Crawford et. al., 2002). However, AC927 was less able to block the increase in ceramide formation or the CB-64D induced cytotoxicity.

This proposed pathway is shown in Figure 7. Both pathways are the result of changes in sphingolipid second messengers, with the non-toxic pathway mediated by SPC and calcium and the apoptotic pathway mediated by ceramide. Augmentation of the cytotoxic effect of CB-64D by AC927 would be consistent with an opposing role of the calcium and ceramide signaling pathways, whereby attenuation of the calcium pathway leaves the apoptotic pathway unopposed. We hypothesize, that SPC is second messenger responsible for the possible non-cytotoxic effects of the sigma-2 receptor, which may be mediated by the transient calcium signal observed. In addition, the bifurcating signals observed via characterization of the putative sigma-2 receptor ligands is hypothesized to be mediated via multiple sigma-2 receptor subtypes, which mediate the cytotoxic and calcium signals.
Figure 6. CB-184-dependent production of SPC in the SKBr3 breast tumor cell line is attenuated by the putative sigma-2 receptor antagonist AC927. Cells were seeded at 500,000 cells per well in 6-well culture dishes. Cells were incubated in the presence of [3H] palmitic acid for 48hrs. Cells were washed, replaced with normal DMEM, and incubated in the presence of CB-184 and AC927. Data was analyzed by TLC.

Crawford and Bowen, Eur. J. Pharmacol, 2002
Figure 7. Preliminary proposed sigma-2 receptor signaling pathway in the SK-N-SH neuroblastoma cell line. Under this model, we identify the possible targets of sphingosylphosphorylcholine (SPC), focusing on the Sphingolipid Calcium Mediating Protein of the Endoplasmic Reticulum (S) and the IP3 receptor (IR) as the most likely calcium-mediating targets and survival as the most likely effect based on the reported effects of SPC. On the left side of the model, we focused on the apoptotic signals observed as a result of chronic stimulation and the proteins identified to play a role leading towards apoptosis.
CHAPTER II

MATERIALS AND METHODS
Cell Culture of SK-N-SH Neuroblastoma Cells (ATCC)

*Media Formulation*

MEM (Gibco) is supplemented with 10% FBS (Atlanta Biologicals), 10mg/L insulin (Gibco), 1mM sodium pyruvate (Gibco), 1mM non-essential amino acids (Gibco), 25,000Ui/L Pen/Strep (MP) and 2.2g/L sodium bicarbonate (Fisher).

*Reconstituting Frozen Stock*

SK-N-SH neuroblastoma cells (ATCC) were removed from nitrogen storage and placed in water bath (36°C) until thawed for approximately 2mins. Thawed cells were immediately reconstituted with 5ml of MEM media (Gibco) supplemented with 10% FBS (Atlanta Biologicals) and 10mg/L insulin and plated in 75cm² flasks (Corning) containing 5ml of media. Cells were incubated in a humidified atmosphere at 37°C with 5% CO² for 24hrs. After confirming cell attachment, media was changed and cells were observed for proper morphology, rate of proliferation and detachment time using 2.5mM EDTA (Sigma-Aldrich).

*Cell Propagation*

When cells reached 60-70% confluency, media was aspirated and cells were detached with 7ml 2.5mM EDTA (Sigma-Aldrich). Cells are placed in the incubator in the previously mentioned conditions for 5-15mins, periodically monitored for signs of detachment. Cells were detached with 5-7ml of media and centrifuged at 1,200Rpm for 5mins (Coulter Beckman Allegra X-15R). Supernatant was removed and cells were reconstituted using 5-7ml of media. Cell homogenate was plated in 75cm² flasks (Corning) at 40-50% confluency and incubated in previously mentioned conditions for 24-48hrs prior to usage.
Calcium Release Assay

Fura-2 Reconstitution

Fura-2AM (Invitrogen) stock is reconstituted by the addition of 10μl DMSO (Sigma-Aldrich) and pulsed vortexed until fully dissolved. Working solution was prepared by combining 5ml of HBSS (Gibco), 50μl 6.6% Pluronic acid solution (0.0652% final concentration) (BASF) and 2.5μl Fura-2 AM (2.47μM final concentration) in a 5ml falcon tube (Corning).

Cell Culture

Once cells reached 60-70%, cells were dissociated from cell culture flask by the addition of 7ml 2.5mM EDTA solution. Cell mixture was centrifuged at 1,200rpm for 5 minutes (Beckman Coulter Allegra X-15R Centrifuge). Supernatant was removed and cells were reconstituted in 5-10ml of media.

Plating

Plating was achieved by the addition of 100μl media into each 96-well black plate (Perkin-Elmer). Cells were plated at 15-20,000 cells per well and incubated for 24-48hrs at the previously mentioned conditions.

Fura-2AM Loading and Measurement

Media was aspirated from wells and cells were washed twice with HBSS (Gibco). 30μl Fura-2 mixture was added to each well, and cells were incubated in the dark for 60mins at the previously mentioned conditions. After incubation, Fura-2 AM mixture was
aspirated and the cells were washed twice with HBSS (Gibco). Calcium release was measured using a Victor 3V 1420 Multilabel Counter (Perkin Elmer).

**Cytotoxicity Assay (MTT, Trevigen)**

Cells were cultured in the previously mentioned conditions and plated from 60-70% confluent flaks into clear 96-well plates (Costar) at 10-15,000 per well. Assay was developed per the manufacturer's parameters for a 100µl volume. Assay was developed using the Victor 3V 1420 Multilabel Counter (Perkin Elmer) at 570nm.

**Serum Starvation Survival (MTT, Trevigen)**

Cells were cultured in the previously mentioned conditions and harvested at 95-100% confluency. Cells were plated at 50,000 cells per well under normal cell culture conditions in the absence of insulin and immediately exposed to CB-64D and SPC for 72hrs. Media and ligands were changed daily. Cell viability was assayed utilizing the MTT (Trevigen) Cell Proliferation assay and measured using a Victor 3V 1420 Multilabel Counter (Perkin Elmer) at 570nm.

**Cell Doubling Time Assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega)**

Cells were plated at 5,000 cells per well in a clear 96-well plate (Costar) and incubated in the previously mentioned conditions for 24hrs. After cells have attached, the media is replaced with MEM in the absence of FBS and incubated for 48hrs to induce quiescence. After 48hrs, the medium is replaced and cells are treated with their prospective ligand under serum starvation conditions for 24hrs. Assay is developed per the manufacturer's specifications utilizing a Victor 3V 1420 Multilabel Counter (Perkin Elmer) at 570nm.
**Induction of DNA Synthesis Assay (BrdU Cell Proliferation Assay, Cell Signaling)**

Preparation of the assay reagents are conducted per manufacturer’s instructions. Cell preparation is achieved by plating the cells at 5,000 cells per well in clear 96-well plates (Costar) and serum starved for 48hrs as previously mentioned. Cells are exposed to ligands for 12hrs. BrdU incorporation and assay development is conducted per the manufacturer’s instructions. The assay is read utilizing a Victor 3V 1420 Multilabel Counter (Perkin Elmer) at 450nm.

**Data Analysis**

The use of GraphPad Prism (GraphPad Software, CA) was utilized to determine EC\textsubscript{50} values in cytotoxicity assays. Excel (Microsoft, WA) was utilized to organize and present the data.

**Materials:**

SK-N-SH neuroblastoma cells were acquired from ATCC. Sphingosylphosphorylcholine was obtained from (Sigma-Aldrich, MO). The compounds used in this study were synthesized by: MV Compounds: Dr. Marcian Van Dort, Associate Professor of Radiology, Center for Molecular Imaging, Department of Radiology, University of Michigan Medical School, Ann Arbor, MI. AC927 and UMB Compounds: Dr. Andrew Coop, Professor and Chair, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD. SN79: Dr. Christopher R. McCurdy, Associate Professor of Medicinal Chemistry and Pharmacology, Co-Director, NIH COBRE CORE-NPN, Department of Medicinal Chemistry, School of Pharmacy, The University of Mississippi University, MS. CB-64D
and CB-184: Dr. Craig Bertha, IRTA Fellow, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD.
CHAPTER II

Parallel Characterization of CB-64D and Sphingosylphosphorylcholine (SPC) Provide Insights into SPC’s Role in the Sigma-2 Receptor Signaling Pathway.

All work and figures shown in this chapter were generated by the author unless otherwise specified.

CHAPTER III

Parallel Characterization of CB-64D and Sphingosylphosphorylcholine (SPC) Provide Insights into SPC’s Role in the Sigma-2 Receptor Signaling Pathway.
Sphingolipids as Signaling Molecules

Sphingolipids are integral molecules in the structure of the cell membrane. The last couple of decades have shed light on the additional function of these ubiquitous molecules, resulting in the discovery of their importance as second messengers. Of these sphingolipids, sphingosine-1-phosphate (S1P), ceramide and sphingosylphosphorylcholine (SPC) are the most characterized to date, resulting in a broad range of implications in cellular processes ranging from proliferation and cell death to differentiation. In addition, these sphingolipids have been found to play a major role in physiological processes, most notably vasoconstriction, vasodilation and wound healing. Although, much remains to be characterized regarding the sources and metabolism of sphingolipids, current knowledge reveals a very extensive tissue specific-effect and intricate metabolism (Figure 8).

Of these sphingolipids, sphingosine-1-phosphate (S1P), the product of sphingosine phosphorylation by sphingosine kinase and hydrolysis of SPC by autotoxin (Clair et al. 2003) has been the most characterized to date. S1P has a cloned, extensive receptor family termed S1P1-5 (Pyne & Pyne, 2000), GPR3, 6, 12 (Uhlenbrock et al., 2002) and GPR63 (Niedenberg et al., 2003). S1P has been shown to produce a calcium signal via GPCR and inositol-1, 4, 5-triphosphate (IP3) (Mathieson and Nixon, 2006). Also, S1P has been implicated in mitogenesis in a number of cell lines (Berger et al, 1996). S1P has been reported to stimulate the activity of the DNA-binding protein Activator Protein-1 (AP-1) in Swiss 3T3 cells (Berger et al. 1996), as well as MAPK (Wu et al. 1995).
Metabolism and Downstream Targets of Sphingosylphosphorylcholine

Alternately, sphingosylphosphorylcholine (SPC) has not been as well characterized as S1P. SPC is produced via sphingomyelin N-deacylation (Spiegel and Milstien, 1995) and is normally found in high-density lipoproteins (HDL). Little is known about SPC’s role in normal physiological conditions, but its involvement in the neuropathic disease Niemann-Pick Type A disease (Berger et al. 1995) has yielded many clues regarding its possible function. Although the levels of free-SPC in circulation are very low, patients with Niemann-Pick Type A disease show up to a 40-fold increase in the levels of SPC in the spleen, liver, and brain (Rodriguez-Lafrasse et al, 1994). Patients exhibiting this disease show acid sphingomyelinase deficiency, among other physiological effects which have been suggested to occur via destabilization of mitochondrial Ca\(^{2+}\) uptake (Strasberg and Callahan, 1988).

SPC has been shown to share many of S1P’s cellular effects as well as physiological processes. SPC has also been shown to bind with low affinity to the S1P family of receptors acting via GPCR signaling (Okajima and Kondo, 1995, Van Koppen et al, 1996). SPC has been reported to be a potent mitogen as well as stimulate proliferation and differentiation in various cell lines (Sugiyama et al, 1990). SPC also induces a calcium release from the endoplasmic reticulum, which has been shown to be both IP3-dependent and independent (Table 1).

Due to SPC’s low affinity for S1P’s family of receptors, its IP3-dependent calcium signal has been attributed to GPCR signaling via the S1P receptors. Although no receptor has been found for SPC, the Sphingolipid Calcium-mediating Protein of the Endoplasmic Reticulum (SCaMPER) has been proposed to be the receptor responsible for the calcium release effects of SPC (Mao et al, 1996, Cavalli et al, 2003). SCaMPER’s
elucidation proved to be the most attractive receptor for SPC, however, revised topology and localization of SCaMPER (Schnurbus et al, 2002) suggest that its role as an intracellular calcium mediator may be specific to cardiac tissue.

Results and Discussion

The induction of calcium release via thapsigargin-sensitive ER storage by sigma-2 ligands via a possible IP3-independent pathway was concurrent with the actions of the newly proposed effects of sphingolipid metabolites. This observation led to the hypothesis of sphingolipids playing a role in the sigma-2 receptor signaling pathway. The highly selective sigma-2 receptor ligand CB-184 was shown to produce a dose-dependent production of SPC (Figure 4) and the sphingolipid metabolite ceramide (Figure 5) (Crawford et al. 2000, 2002). The production of ceramide was concurrent with the observed cytotoxic effects as a result of chronic stimulation of the sigma-2 receptor. Sigma-2 receptor-mediated effects were found to be caspase-dependent and caspase-independent apoptosis. The production of SPC however, led to the hypothesis of non-cytotoxic effects being exerted by the sigma-2 receptor. This observation was cemented by the ability of the putative sigma-2 receptor antagonist AC927 to block the CB-184-dependent production of SPC (Figure 6). As a result of these observations, SPC’s reported proliferative and mitogenic activity became a prime target of study. SPC has been proposed as the second messenger responsible for the non-cytotoxic effects mediated by the sigma-2 receptor. To test this hypothesis, exogenously added D-erythro-sphingosylphosphorylcholine was used in parallel experiments with the more available sigma-2 selective ligand CB-64D, to explore the ability of SPC to mirror the observed effects of CB-64D.
Figure 8. Known metabolism of sphingosylphosphorylcholine.

Table 1: Broad range of effects of SPC on intracellular calcium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Intracellular calcium source</th>
<th>Intracellular calcium effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat aorta smooth muscle cells</td>
<td>Partially intracellular and extracellular</td>
<td>Insensitive to intracellular calcium</td>
</tr>
<tr>
<td>Human coronary artery smooth muscle cells</td>
<td>Insensitive to intracellular calcium</td>
<td>Insensitive to intracellular calcium</td>
</tr>
<tr>
<td>HEK-293 cells</td>
<td>Insensitive to extracellular calcium</td>
<td>Insensitive to intracellular calcium</td>
</tr>
<tr>
<td>Hela cervical epithelial cancer cells</td>
<td>Insensitive to extracellular calcium</td>
<td>Insensitive to intracellular calcium</td>
</tr>
<tr>
<td>Rat brain microglia</td>
<td>Insensitive to extracellular calcium</td>
<td>Insensitive to intracellular calcium</td>
</tr>
<tr>
<td>Human foreskin fibroblasts</td>
<td>Insensitive to extracellular calcium</td>
<td>Insensitive to intracellular calcium</td>
</tr>
<tr>
<td>Swiss 3T3 fibroblasts</td>
<td>Insensitive to extracellular calcium</td>
<td>Insensitive to intracellular calcium</td>
</tr>
</tbody>
</table>

Intracellular stores would be expected to occur via an intracellular action of SPC.
**Calcium Signaling**

The first step in determining whether SPC is the second messenger responsible for the hypothesized, non-cytotoxic effects of sigma-2 receptors was to test the ability of SPC to produce a calcium signal. SPC was hypothesized to be responsible for the ability of the sigma-2 receptor agonist CB-64D to produce a transient calcium signal, which has been associated with non-cytotoxic signals.

The release of a calcium signal in the sigma-2 receptor system occurs in a very narrow cell density between 15,000-30,000 cells per well. SPC was shown to produce a transient calcium signal from fura-2-loaded neuronal SK-N-SH system (**Figure 10**), which is consistent with the calcium signal induced by CB-64D (**Figure 9**). The signal is approximately 4 minutes in duration and 300nM in magnitude, which is comparable to the signal elicited by CB-64D consisting of a duration of 4 minutes and 350nM in magnitude.

**Effect of the Non-Cytotoxic Signal**

The ability of sigma-2 ligands to stimulate cell death (**Figure 2**) has been extensively characterized and hypothesized to be connected to the delayed-prolonged calcium signal elicited by sigma-2 ligands. It’s been demonstrated that washing off the ligand after a short period does not result in cell death (**Data not shown**). This observation led to the investigation of the role of the transient calcium signal in the sigma-2 system. According to the literature, transient calcium signals are commonly associated with mitogenic, proliferative, pro-survival and differentiating cell processes (Berridge et al., 1998) among a host of other tissue specific functions. Treatment of SK-N-SH cells with SPC for a 24hr period resulted in no cytotoxicity (**Figure 11**) until 30μM where 18+/− 2 percent cytotoxicity is observed. In accordance with our initial hypothetical
model, SPC was tested in parallel with CB-64D to test their ability to confer survival onto the SK-N-SH neuroblastoma cells under conditions favoring cell death. Under these conditions, cells are harvested from 75cm² flasks at 90-100% confluency and plated at 50,000 cells per well into clear 96-well plates. At the cell density plated, the cells are at 100% confluency, which under normal MEM media conditions, containing 10% FBS and 10mg/ml insulin have been observed to detach and die within 24-48hrs. Cells were observed for 72hrs, with daily media and ligand change. MTT (Trevigen) was used to assay cell viability, which shown as a function of the untreated cells. Under these conditions, SPC was shown to confer what appeared to be a proliferative effect in a dose-dependent manner over 72hrs (Figure 12). Alternatively, CB-64D did not show a proliferative effect, but instead exhibited cytotoxic effects at the same concentration range shown under normal conditions (Figure 13). These results are concurrent with our proposed sigma-2 receptor signaling model, in which SPC is hypothesized to be responsible for the non-cytotoxic effects of the sigma-2 receptor, while CB-64D is able to activate both the apoptotic and non-apoptotic signal cascades. Cells were also tested under the same cell density conditions, with the added parameter of serum and insulin withdrawal, cells became quiescent, as reflected by the lack of dieback in the absence of ligand treatment conditions (Data not shown).

In order to further characterize the possible proliferative effects of SPC, as well as continue to elucidate the effect of the transient calcium signal produced by CB-64D, the use of the more sensitive CellTiter 96 Proliferation Assay (Promega) was employed. Knowing that SK-N-SH neuroblastoma cells become quiescent under serum starvation conditions, we tested the ability of SPC and CB-64D to induce proliferation on cells in the absence of serum and insulin. In this experiment, cells were forced into quiescence, by exposure to serum starvation conditions for 48hrs prior to ligand treatment. To
facilitate quantification of small changes in cell number, as well as encourage cell
growth, cells were plated at 10,000 cells per well in clear 96-well plates from 60-65%
confluent 75cm² flaks. These conditions are optimal for cell growth and sigma-2
receptor-induced cytotoxicity and calcium release, which would suggest that any
changes in cell density mediated through the sigma-2 receptor, would be most favorable
under these conditions. Since we know that CB-64D is able to confer cytotoxicity at
higher concentrations, we tested a low concentration range of both SPC and CB-64D
over 24hrs. Neither CB-64D nor SPC were able to trigger cell proliferation under serum
and insulin-free conditions, compared to the positive control consisting of MEM
containing 10%FBS and 10mg/ml insulin (Figures 14, 15) in the time frame and
conditions tested, which pointed our direction towards the possibility of the transient
calcium signal inducing DNA-synthesis. The lack of induction of proliferation under these
conditions and time frame, suggested that there may be a time dependence to the
actions of SPC and CB-64D signaling via the sigma-2 receptor.

Given the cell processes attributed to the actions of SPC, commensurate to the
physiological observations accredited to the sigma-2 receptor, we tested the ability of
SPC and CB-64D to stimulate DNA-synthesis via BrdU incorporation in the SK-N-SH
neuroblastoma cell line utilizing the BrdU Cell Proliferation Assay Kit (Cell Signaling).
Cells were forced into quiescence by incubation under serum starvation conditions for
48hrs prior to being exposed to any ligands. Cells were treated with a low concentration
range of CB-64D and SPC for 12hrs. While neither SPC nor CB-64D were shown to
confer DNA-synthesis in the SK-N-SH neuroblastoma cell line (Figure 16, 17), CB-64D
appeared to inhibit DNA-synthesis at non-cytotoxic concentrations. This observation
suggests that the cytotoxic cascade of the sigma-2 receptor may start earlier than
previously thought. Like the lack of effects on doubling time, these results suggest that
the non-cytotoxic signals may be specific to conditions of distress at a longer time frame than tested.
Figure 9. Transient calcium signal elicited by 30μM CB-64D on the SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of injection with 30μM CB-64D.
Figure 10. Transient calcium signal elicited by 10μM SPC on the SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells were incubated in MEM supplemented with 10%FBS and 10mg/L insulin for 24-48hrs. Cells are plated at 15,000 cells per well in a black 96-well plate from 60-65% confluent plates. Calcium release assay was conducted in calcium-containing HBSS in Fura-2 loaded cells. Syringe denotes the approximate location of the injection of SPC. Experiment is representative of 3 separate experiments consisting of 4 wells each.
Figure 11. Effect of 24 hour SPC exposure on the SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated in clear 96-well plates at 5,000-10,000 cells per well and incubated for 24hrs to allow for proper attachment and adaptation. Cells are treated with SPC and incubated for an additional 24hrs.
Figure 12. Effect of SPC on SK-N-SH neuroblastoma cells in normal 10%FBS growing conditions in the absence of insulin over 72 hours. SK-N-SH neuroblastoma cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 90% confluent. Cells are plated onto 96-well plates at 50,000 cells per well and incubated with SPC in the absence of insulin, in MEM supplemented with 10% FBS. Data displayed is the average of 3 experiments consisting of 5 repetitions each. Data shown has been normalized by subtracting time 0. Viability is established by setting the untreated values to “1” and calculating the rest of the values as ratios of the control.

Credit to: Jyotsna Mullur
Figure 13. Effect of CB-64D on SK-N-SH neuroblastoma cells under normal 10% FBS conditions in the absence of insulin over 72 hours. SK-N-SH neuroblastoma cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 90% confluent. Cells are plated onto 96-well plates at 50,000 cells per well and incubated in the presence of CB-64D, in MEM supplemented with 10% FBS in the absence of insulin. Data displayed is the average of 3 experiments consisting of 5 repetitions each. Data shown has been normalized by subtracting time 0. Viability is established by setting the untreated values to “1” and calculating the rest of the values as ratios of the control.

Credit to: Jyotsna Mullur
Figure 14. CB-64D has no effect on the doubling time of serum-starved SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin, until 60-65% confluent. Cells are plated at 10,000 cells per well in 96 well plates and are incubated in MEM in the absence of serum or insulin for 48hrs. Media is removed and cells are treated with CB-64D for 24 hours. Data represents the average of 5 replicates.
Figure 15. SPC has no effect in the doubling time of serum starved SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin, until 60-65% confluent. Cells are plated at 10,000 cells per well in 96 well plates and are incubated in MEM in the absence of serum or insulin for 48hrs. Media is removed and cells are treated with CB-64D for 24 hours. Data represents the average of 5 replicates.
Figure 16. Effect of CB-64D on the induction of DNA-synthesis in serum-starved SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are grown in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 5,000 cells per well, allowed to attach and serum starved in MEM for 48hrs. Cells are treated with CB-64D for 12hrs prior to being incubated with BrdU. The data represents the average of 5 repeats. Media containing 10% FBS and 10mg/L insulin is used as a positive control.
Figure 17. Effect of SPC on the induction of DNA-synthesis in serum-starved SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are grown in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 5,000 cells per well, allowed to attach and serum starved in MEM for 48hrs. Cells are treated with SPC for 12hrs prior to being incubated with BrdU. The data represents the average of 5 repeats. Media containing 10% FBS and 10mg/L insulin is used as a positive control.
Conclusion

While neither SPC nor CB-64 D showed the ability to induce proliferation or DNA-synthesis in the SK-N-SH neuroblastoma cell line under serum-starvation conditions, the data adds to the minute knowledge base of the actions of SPC in neuronal systems. Calcium is a ubiquitous molecule which plays a diverse role as an intracellular second messenger, and whose true extent as a second messenger is dynamically expanding. Despite the fact that the transient calcium signal elicited by CB-64D and SPC did not result in the stimulation of DNA synthesis or induction of proliferation in the time frame and conditions tested, the results observed from SPC under starvation conditions shed light into the possible effects of the receptor under physiological conditions. The very intricate metabolism of SPC is only recently being taken under consideration when attributing the effects thought to be caused by S1P or ceramide. The use of exogenously added SPC was a factor we closely monitored when interpreting our data. It is likely that other factors may be involved in the modulation of the calcium signal. Among these, CaMKII, calcineurin and calmodulin are prime candidates, as their role is still concurrent with physiological observations played by the sigma-2 receptor in the immune system and possible implications in memory. Elucidating the effect of the transient calcium signal with respect to the stimulation of SPC and sigma-2 receptors, will help determine the physiological role of the sigma-2 receptor and its use as a potential therapeutic target. The evidence showing SPC’s ability to elicit a transient Ca\textsuperscript{2+} release from the SK-N-SH neuroblastoma cell line, as well as various reported cell lines (Nixon et al., 2002) indicate that SPC is a very attractive second messenger, whose reported effects are concurrent with our observations. The Ca\textsuperscript{2+} release profile of SPC is consistent with the transient Ca\textsuperscript{2+} release observed in the first phase of the CB-64D-induced signal. Given this body of evidence, SPC is
hypothesized to be the second messenger responsible for sigma-2 receptor induced calcium release.
CHAPTER IV

Characterization of Putative Sigma-2 Receptor Antagonists Provide Evidence for the Possibility of Multiple Sigma-2 Receptor Subtypes.

All work and figures shown in this chapter were generated by the author unless otherwise specified.
Bifurcating Signaling via the Sigma-2 Receptor

Sigma-2 receptors have been implicated in a host of cellular processes and physiological functions. Among these cellular processes is the induction of calcium release from thapsigargin-sensitive ER storage, consisting of a rapid, transient signal followed by a delayed, prolonged calcium signal (Vilner and Bowen, 2000). In addition, sigma-2 receptor agonists are cytotoxic at chronic doses in the SK-N-SH neuroblastoma cell line and MCF-7, T47D and SKBr3 breast cancer cell lines. In acute doses, coinciding with the timeframe for the release of the transient calcium signal, sigma-2 agonists are not cytotoxic. Studies in the SKBr3 cell line utilizing [3H] serine and [3H] palmitic acid resulted in the dose-dependent production of the sphingolipids ceramide and sphingosylphosphorylcholine (SPC) by the selective sigma-2 agonist CB-184 (Crawford et al. 2000, 2002). The production of ceramide and SPC in conjunction with the transient and prolonged calcium release by CB-64D prompted the hypothesis of a signaling pathway leading to cytotoxic and non-cytotoxic signals via the sigma-2 receptor.

As a whole, stimulation of the sigma-2 receptor results in both cytotoxic and non-cytotoxic signals. The production of the sphingolipid SPC, a molecule which has been increasingly associated with cell proliferation and mitogenesis, suggests that the sigma-2 receptor is acting as a trophic receptor. This observation is concurrent with the transient calcium signal and its implication in cellular differentiation, proliferation, mitogenesis and a host of other signals. The notion of the sigma-2 receptor being a trophic receptor is coexisting with previous observations of the sigma-2 receptor being found to be expressed in high density in some tumor cell lines (Thomas et al., 1996, Bem et al., 1990), upregulated in cells undergoing rapid proliferation (Mach et al., 1997, Al-Nabulsi
et al., 1999) and the linkage of the expression of the sigma-2 receptor to the ability of cells to progress through the cell cycle. Contrary to those signals is the release of a prolonged calcium signal emanating from thapsigargin-resistant ER storages, which has been linked to mitochondrial destabilization and apoptosis. Additional evidence contrary to the sigma-2 receptor being a trophic receptor is the dose-dependent production of the sphingolipid metabolite ceramide, which has been characterized to generally be a cytotoxic signal in both caspase-dependent and caspase-independent manner. Finally, the chronic stimulation of cells with sigma-2 agonists results in both caspase-dependent and caspase-independent apoptosis. These signals suggest the presence of a possible bifurcating signaling pathway which is able to activate both a non-cytotoxic signal and a cytotoxic signal.

Since the sigma-2 receptor has not been cloned, and with the lack of selective sigma-2 antagonists, the elucidation of the sigma-2 pathway has proven to be a very difficult task. Using the putative sigma-2 receptor antagonist AC927 (N-phenethylpiperidine oxalate) (Figure 18) has shown to block methamphetamine-induced neurotoxic effects, including, release of dopamine and generation of reactive oxygen species (Kaushal et al. 2011), attenuation of hyperthermia and serotonin damage (Seminario et al. 2011) as well as block the aforementioned CB-184-dependent formation of SPC (Crawford et al. 2002). AC927 has also shown the ability to attenuate the convulsive, locomotor and neurotoxic effects of cocaine (Matsumoto et al. 2011).
Figure 18. Molecular structure of AC927.

A. Coop
As the only ligand to show antagonistic effects on the sigma-2 receptor, AC927 provided a blueprint for testing and characterizing putative sigma-2 antagonists. Under this blueprint, putative sigma-2 receptor antagonists are tested on their ability to block sigma-2 receptor mediated cytotoxicity and calcium release. Since the potent, sigma-2 receptor agonist CB-184 is very scarce, its non-chlorinated analog and highly selective sigma-2 receptor agonist CB-64D (Figure 1) is used as the gold standard for testing these parameters. In addition, recent medicinal chemistry approaches have produced a host of compounds with high affinity for the sigma-1 and sigma-2 receptors (Table 2). The recent synthesis of the ligand SN79 (6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one) (Figure 19), has shown nanomolar affinity for sigma-receptors, with 28.03+/−3.39nM Ki for sigma-1 and 6.89+/− 0.09nM for the sigma-2 receptor. (Narayanan et al. 2007). SN79 was based on the synthesis of various chemical groups which showed high sigma-2 receptor affinity. SN79 has shown the ability to block cocaine-induced toxic and stimulant effects in Sprague-Dawley rats (Kaushal et al. 2011) suggesting it is acting as an antagonist in the sigma-1 receptor system and possibly sigma-2 system. Given the lack of selective antagonists for the sigma-2 receptor, the synthesis of SN79 and its possible antagonistic activity led us to characterize the effects in our SK-N-SH neuroblastoma system.

Results

The biphasic calcium signal observed as a result of sigma-2 receptor stimulation by CB-64D, (Figure 3) suggests the existence of two distinct signals being modulated by the receptor’s activation. The initial transient calcium signal was shown to originate from thapsigargin-sensitive ER storage, but the secondary delayed, prolonged calcium signal
Table 2. Binding affinity of the putative sigma-2 receptor antagonists. Constant of inhibition values for sigma-1 receptor are obtained via competition experiments with \[^{3}H\]+Pentazocine, while those for the sigma-2 receptor are conducted in the presence of \[^{3}H\] di-o-tolyl guanidine and unlabeled +Pentazocine as a masking agent. Ki values for the sigma-2 receptor were determined by Dr. Anthony Comeau. Values for SN79 were obtained from Kaushal et al, 2011.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sigma-1 Ki (nM)</th>
<th>Sigma-2 Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC927</td>
<td>30 +/- 2</td>
<td>138 +/- 18</td>
</tr>
<tr>
<td>UMB408</td>
<td>116 +/- 8</td>
<td>331 +/- 34</td>
</tr>
<tr>
<td>UMB414</td>
<td>18.7 +/- .09</td>
<td>101 +/- 5</td>
</tr>
<tr>
<td>MV1036A</td>
<td>28 +/- 8</td>
<td>194 +/- 36</td>
</tr>
<tr>
<td>SN79</td>
<td>28.03 +/- 3.39</td>
<td>6.89 +/- 0.09</td>
</tr>
</tbody>
</table>
Figure 19. Molecular structure of SN79.

C. McCurdy
was insensitive to thapsigargin and thought to originate from the mitochondria (Vilner and Bowen, 2000). The observation of a rapid, transient calcium signal suggests a cytoprotective signal (Berridge, 1998). My work demonstrated that the exact nature of this signal was not conferring starvation protection, induction of proliferation or stimulation of DNA synthesis suggesting the activation of an alternative effect.

It’s been shown that chronic stimulation of the sigma-2 receptor results in both a caspase-dependent and caspase independent mode of apoptosis. Additionally, acute stimulation of cells with sigma-2 receptor agonists followed by washout does not result in cell death (not shown). Finally, stimulation of the sigma-2 receptor by the more potent chlorinated CB-64D analog in the SKBr3 cell line results in the formation of the putative cytotoxic, sphingolipid metabolite ceramide as well as SPC. Ceramide formation has also been demonstrated in the SK-N-SH neuroblastoma cell line. Unlike ceramide, SPC is commonly associated with cytoprotective signals, which is concurrent with the observations resulting from transient calcium signals. The production of SPC was shown to be blocked by the putative sigma-2 receptor antagonist AC927 (Figure 6), which further cemented its connection to the sigma-2 receptor. We previously showed that SPC is able to produce a rapid transient calcium signal, which closely mirrors that of CB-64D. More so, chronic stimulation of SPC was shown to not be cytotoxic at the same conditions at which CB-64D showed cytotoxicity.

The duplicity of signals observed as a result of sigma-2 receptor stimulation suggests the presence of a bifurcating pathway with a yet-to-be characterized system. Since the sigma-2 receptor has not been cloned, and no endogenous ligand has been identified, further characterization of the duality of this signal has proven difficult. To further exacerbate characterization, there is a lack of suitable antagonists for the sigma-2 receptor.
Figure 20. Effect of 24 hour exposure of AC927 on the SK-N-SH neuroblastoma cell line. Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM, 10%FBS, 10mg/ml insulin conditions. AC927 is added and the cells were incubated for 24hrs. MTT assay was used to determine cell viability, which is expressed as a function of the untreated cells.
Effects of the Putative Sigma-2 Receptor Antagonist AC927 on the Calcium and Cytotoxic Signal of CB-64D in the SK-N-SH Neuroblastoma cell line.

The putative sigma-2 receptor antagonist AC927 (Figure 18) has shown the ability to block the CB-184-mediated production of SPC in the SKBr3 breast cancer cell line (Figure 6) (Crawford et al., 2000). When tested for activity in the SK-N-SH neuroblastoma cell line, AC927 shows little to no effect over a 24hr period, with a maximum 12% cytotoxicity at 100μM (Figure 20). When tested in our calcium assay in the presence of extracellular calcium, AC927 produces a small, immediate calcium signal of about 170nM that persists beyond the timeframe constraints of our equipment (Figure 21). Preliminary data suggests that AC927 blocks the calcium signal elicited by CB-64D, while producing a calcium signal of its own in a similar way to SN79 (Figure 22) AC927 showed no effect on the cytotoxicity of CB-64D (Figure 23). AC927 yielded the first piece of data towards the proposal of a sigma-2 receptor signaling model.

AC927’s ability to block the CB-184-induced production of SPC, while being unable to block the production of ceramide, suggested that the production of ceramide was via a different mechanism than the production of SPC. This piece of information became an important piece of information under the newly proposed sigma-2 receptor signaling model, where AC927 is classified as a partial antagonist of the sigma-2 receptor subtype responsible for the non-cytotoxic effects. Under this classification, AC927 is able to attenuate the production of SPC, while still producing a small calcium signal of its own and conferring little to no cytotoxic effects.
Figure 21. Calcium signal elicited by 30μM AC927 in the SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of injection with 30μM AC927. 30μM CB-64D is shown in blue as a comparison.
These observations suggest that AC927 is differentiating different phases of the sigma-2 receptor mediated signaling pathway. One phase is related to calcium release from the ER and downstream calcium signaling, which is sensitive to blockade by AC927. The ability of AC927 to also block SPC formation appears to link SPC to this pathway. This phase does not result in cell death, but rather may be coupled to a physiological response such as proliferation or survival. The other phase is related to the sigma-2 receptor mediated apoptotic effects, which are not sensitive to blockade by AC927. The failure of AC927 to block CB-184-induced ceramide formation is consistent with the linking of ceramide to the apoptotic pathway. Therefore, sigma-2 receptors may activate a bifurcating pathway with both non-cytotoxic and cytotoxic endpoints.

**Effects of SN79 on the Sigma-2 Receptor System**

The synthesis of SN79, *(Figure 19)* a ligand with high affinity for the sigma-2 receptor has shed light on the signaling pathway of the receptor system. SN79 has shown the ability to block the neurotoxic effects of methamphetamine *(Kaushal, Thesis)*. Chronic treatment of the SK-N-SH neuroblastoma cell line for 24hrs with up to 30μM SN79 resulted in no cytotoxicity *(Figure 24)*, suggesting that SN79 is acting as an antagonist. To test SN79’s role as an antagonist, we tested its activity on the cytotoxic effect and calcium signal induced by CB-64D. Previous data showed SN79 did not block micromolar doses of CB-64D, but data suggests enhancement of its cytotoxic effect *(Figure 25)*. This effect suggested that SN79 was not a pure antagonist, but may in fact be acting as a partial agonist. To further characterize SN79’s effects in the SK-N-SH neuroblastoma cell line, we tested its effects in our calcium assay. Once again, SN79 exhibited an unexpected effect by producing a transient calcium signal at 30μM *(Figure 26)*.
The signal differed from that of CB-64D in magnitude and kinetics, with a maximum concentration of 50nM calcium induced, and a much flatter and approximately 2 minutes and 20 seconds of duration prior to return to baseline. Pretreatment of SK-N-SH cells with 30µM SN79 for 20mins followed by stimulation with 30µM CB-64D resulted in an almost complete blockage of CB-64D’s-induced calcium signal, with the calcium signal induced more closely resembled that induced by SN79 alone. SN79’s action prompted the developments of a new sigma-2 receptor signaling model, since its effects were not dependent on acute or chronic exposure of the sigma-2 receptor.

The sigma-2 agonists CB-64D and CB-184 would bind to and activate both subtypes given their ability to produce a transient and sustained calcium signal, produce a cytotoxic effect, as well as stimulate the production of SPC and ceramide. Under this model, SN79 would bind to and block the receptor subtype responsible for the non-cytotoxic signals, which would in turn allow CB-64D’s cytotoxic signal to be more pronounced, while also blocking its calcium signal. This hypothesis is also concurrent with the lack of cytotoxicity from SN79 exposure.
Figure 22. Antagonism of the calcium signal induced by 50µM CB-64D by 50µM AC927. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of ligand injection. Cells are exposed to 50µM AC927 for 25-30mins prior to injection with 50µM CB-64D. Data is expressed as increase over basal calcium levels.
Figure 23. Antagonism of the cytotoxicity of 10μM CB-64D by a variable concentration range of AC927 over a 24 hour period in the SK-N-SH neuroblastoma cell line. Cells are incubated in MEM supplemented with 10% FBS and 10mg/ml insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM, 10%FBS, 10mg/L insulin conditions. A dose range of AC927 is added plus 10μM CB-64D, and the cells were incubated for 24hrs. MTT assay was used to determine % cytotoxicity, which is expressed as a function of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 24. Effect of SN79 on the SK-N-SH neuroblastoma cell line over 24 hours.

Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM, 10%FBS, 10mg/L insulin conditions. AC927 is added and the cells were incubated for 24hrs. MTT assay was used to determine cell viability, which is expressed as a function of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 25. Augmentation of the cytotoxic effect of 3\(\mu\)M CB-64D by SN79 in the SK-N-SH neuroblastoma cell line over a 24 hour exposure. Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM supplemented with 10%FBS and10mg/L insulin conditions. A dose range of SN79 is added over 3\(\mu\)M CB-64D, determined to be the maximum, non-effective concentration and the cells were incubated for 24hrs. MTT assay is used to determine cell viability, which is expressed as a percentage relative to that of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 26. Antagonistic effect of 30µM SN79 on 30µM CB-64D compared to the effects of 30µM SN79 and 30µM CB-64D. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of ligand injection. Cells are exposed to 30µM SN79 for 25-30mins prior to injection with 30µM CB-64D.
UMB Series

The ability of the putative sigma-2 receptor antagonist AC927 to block the sigma-2 receptor-induced production of SPC prompted the synthesis of the compounds UMB408 and UMB414 (Figure 27). These compounds showed affinities for the sigma-1 and sigma-2 receptors of 116+/−8nM and 331+/−34nM for UMB408 and 18.7+/−0.09nM and 101+/−5nM for UMB414 respectively (Table 2). These compounds produced negligible calcium signals (Figure 30) and peak cytotoxicity levels of 37+/−4% at 30μM for UMB408 (Figure 28) and 28+/−2% at 30μM for UMB414 (Figure 29) over a 24hr period in normal 10%FBS-containing MEM. Neither of these compounds showed an effect on 3μM CB-64D’s cytotoxicity (Data not shown). In calcium antagonism experiments, neither UMB408 (Figure 31) nor UMB414 (Figure 32) demonstrated the ability to block the CB-64D-induced calcium signal. These two compounds closely mirrored the effects observed from AC927, differentiating themselves from AC927 in having a higher cytotoxicity level, yet much lower to negligible calcium signal. Their activities are consistent with our proposed sigma-2 receptor model, in that these two compounds could be acting as weak agonists of the sigma-2 receptor subtype proposed to be responsible for the apoptotic effects.

MV1036A Blocks CB-64D’s Cytotoxic Effect

To date, putative sigma-2 receptor antagonists had only shown the ability to attenuate CB-64D’s induced calcium signal and augment its cytotoxicity in a dose-dependent manner. The compound MV1036A (Figure 33) showed affinities for the sigma-1 and sigma-2 receptors of 28+/−8nM and 194+/−36nM (Table 2).
MV1036A showed little to no cytotoxicity in the SK-N-SH neuroblastoma cell line (Figure 34) over a 24hr incubation period, with MV1036A showing virtually no cytotoxicity up to 100μM. Testing of MV1036A for antagonist activity against CB-64D’s cytotoxic effect resulted in MV1036A exhibiting a significant attenuation of the cytotoxic signal, becoming the first compound to date to show such effects (Figure 35). MV1036A produced a very robust calcium signal under normal calcium-containing HBSS (Figure 36), but the calcium signal never returned to basal, preventing further treatment with CB-64D.

MV1036’s ability to block the CB-64D-induced cytotoxic effect suggest that it is an antagonist of the cytotoxic sigma-2 receptor subtype, while at the same time being an agonist of the non-cytotoxic subtype, given its ability to produce a very robust transient calcium signal, while having no cytotoxic effects of its own.
Figure 27. Molecular structures of UMB408 and UMB414.

A. Coop
Figure 28. Effect of UMB408 over 24 hours in the SK-N-SH neuroblastoma cell line.

Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM, 10%FBS, 10mg/L insulin conditions. UMB408 is added and the cells were incubated for 24hrs. MTT assay was used to determine cell viability, which is expressed as a function of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 29. Effect of UMB414 over 24 hours in the SK-N-SH neuroblastoma cell line. Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM, 10%FBS, 10mg/L insulin conditions. UMB414 is added and the cells are incubated for 24hrs. MTT assay was used to determine cell viability, which is expressed as a function of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 30. Comparison of the calcium signals elicited by 30μM UMB408 and 30μM UMB414 compared to 30μM CB-64D. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of ligand injection.
Figure 31. 30μM UMB408 has no antagonistic effect on the calcium signal elicited by 30μM CB-64D. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of ligand injection. Cells are exposed to 30μM UMB408 for 25-30mins prior to injection with 30μM CB-64D.
Figure 32. 30μM UMB414 has no antagonistic effect on the calcium signal elicited by CB-64D. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of ligand injection. Cells are exposed to 30μM UMB414 for 25-30mins prior to injection with 30μM CB-64D.
Figure 33. Molecular Structure of MV1036A.

M. Van Dort
Discussion

To date, no purposed sigma-2 ligand has shown to be a pure antagonist in this system. The small subset of ligands characterized in the last few years as putative antagonists (Table 3) have shown characteristics more in line with those observed of partial antagonists in our system. The activities observed with these compounds in conjunction with the effects observed via CB-64D and CB-184 stimulation have shed light on the signal transduction pathway of the sigma-2 receptor. None of the compounds characterized to date has shown the ability to completely block all of the sigma-2 agonist-induced effects. The putative sigma-2 antagonist AC927 blocked the CB-184-induced dose-dependent production of SPC, yet had no effect on the cytotoxicity induced by CB-64D. Even though AC927 showed the ability to block the sigma-2 induced production of SPC and produced no cytotoxic effects in the SK-N-SH neuroblastoma cell line, it induced a slight, transient, intracellular calcium signal.

Preliminary data suggests AC927’s is able to completely block the CB-64D-induced calcium signal (Figure 22). The putative sigma-2 antagonist SN79 was shown to augment CB-64D’s cytotoxicity while having little to no cytotoxicity of its own. SN79 also showed the ability to produce a transient calcium signal and blocked CB-64D’s-induced calcium signal. MV1036A’s ability to block CB-64D’s cytotoxicity, represents the first compound to show antagonism on the cytotoxic signal of the sigma-2 receptor. MV1036A produced little to no cytotoxicity on its own, but induced a calcium signal whose recovery kinetics did not return to basal calcium signal within the time frame constraints of our system. CB-64D was unable to produce a calcium signal after pre-treatment with MV1036A, but it is difficult to attribute these antagonistic effects to
MV1036A, as we know that the sigma-2 receptor is unable to elicit a calcium signal under elevated calcium levels.

The effects of these compounds, along with the previously mentioned agonistic effects of the sigma-2 receptor ligands, strengthen our hypothesis of the presence of a bifurcating pathway leading towards apoptosis and a yet to be determined effect. The observations of these partial antagonists as well as the signals observed via CB-64D and CB-184 stimulation have allowed for the compilation of a model proposing an explanation of the different effects observed of the sigma-2 receptor (Figure 37). This model explains the ability of CB-64D and CB-184 to produce both cytotoxic and non-cytotoxic signals and the activities of the partial antagonists characterized to date.

The lack of pure antagonists and an endogenous ligand have greatly hindered the elucidation of the endogenous function of the sigma-2 receptor and characterization of its signal transduction pathway. The continuing efforts to synthesize ligands for this receptor system have slowly shed light on its signal transduction pathway. Although much remains to be elucidated, the potential of the sigma-2 receptor as a therapeutic target is becoming increasingly important with the synthesis and characterization of each new potential ligand.
Figure 34. Effect of MV1036A over 24hr in the SK-N-SH neuroblastoma cell line over 24 hours. Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM, 10%FBS, 10mg/L insulin conditions. MV1036A is added and the cells are incubated for 24hrs. MTT assay was used to determine cell viability, which is expressed as a function of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 35. Attenuation of the cytotoxicity of 3μM CB-64D by MV1036A in the SK-N-SH neuroblastoma cell line. Cells are incubated in MEM supplemented with 10% FBS and 10mg/L insulin until 60-65% confluent. Cells are plated at 10-15,000 cells per well in clear 96 well plates and incubated overnight in normal MEM supplemented with 10% FBS and 10mg/ml insulin conditions. A dose range of MV1036A is added in the presence of 3μM CB-64D and the cells are incubated for 24hrs. MTT assay is used to determine cell viability, which is expressed as a percentage relative to that of the untreated cells. Data presented represents the average of 3 experiments consisting of 5 duplicate wells for each concentration.
Figure 36. Calcium signal elicited by 30µM MV1036A in the SK-N-SH neuroblastoma cell line. SK-N-SH neuroblastoma cells are incubated until 60-65% confluent in MEM supplemented with 10% FBS and 10mg/L insulin. Cells are plated at 15,000 cells per well in black 96 well plates and incubated for 24-48hrs. Fura-2 loaded cells are treated in the presence of calcium-containing HBSS. Syringe denotes the approximate time of injection with 30µM MV1036A. 30µM CB-64D is shown as a comparison.
### Table 3. Effects of various sigma-2 receptor ligands.

This table outlines the effects and magnitude of the putative sigma-2 receptor antagonists relative to CB-64D.

<table>
<thead>
<tr>
<th>Effect</th>
<th>CB-64D</th>
<th>AC927</th>
<th>SN79</th>
<th>UMB408</th>
<th>UMB414</th>
<th>MV1036A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) Release</td>
<td>++++++</td>
<td>++</td>
<td>+</td>
<td>None</td>
<td>None</td>
<td>++++++</td>
</tr>
<tr>
<td>Cytotoxic Effect</td>
<td>++++++</td>
<td>+</td>
<td>None</td>
<td>++</td>
<td>++</td>
<td>None</td>
</tr>
<tr>
<td>Antagonism of CB-64D - induced Ca(^{2+}) Release</td>
<td>N/A</td>
<td>++++</td>
<td>++++</td>
<td>None</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>Effect on CB-64D Cytotoxicity</td>
<td>N/A</td>
<td>None</td>
<td>Enhanced</td>
<td>None</td>
<td>None</td>
<td>Block</td>
</tr>
</tbody>
</table>
Figure 37. Proposed sigma-2 signaling pathway based on the actions of CB-64D and CB-184 and the putative sigma-2 antagonists which have shown activity to date. This newly proposed sigma-2 receptor signaling pathway based on the properties of the sigma-2 receptor ligands tested to-date proposes the existence of multiple sigma-2 receptor subtypes, which bifurcate the apoptotic and non-cytotoxic effects observed via sigma-2 receptor stimulation. Under this model, each subtype is in close proximity to a known enzyme capable of acylating sphingosine (SPH) to ceramide in the apoptotic pathway, and hydrolyzes sphingomyelin (SM) to sphingosylphosphorylcholine (SPC) in the non-cytotoxic pathway, while mediating their respective effects independently of each other. Under this model, SN79 binds to a subtype of the sigma-2 receptor responsible for the non-cytotoxic signal, which explains its ability to produce a calcium signal while muting the non-cytotoxic signal allowing for the augmentation of CB-64’s cytotoxic effect. AC927 would also bind to this sub-site, which would explain its ability to block the production of SPC and calcium. MV1036A would bind to and block the subtype responsible for the cytotoxic signal observed, which would explain its ability to block CB-64D’s cytotoxic effect. UMB compounds would act as partial agonists at the cytotoxic subtype.
CHAPTER V

Summary and Conclusion
Summary and Conclusion

Sigma-2 receptors have been implicated in a broad range of physiological functions and cellular effects, which have been attributed to the modulation of intracellular calcium signaling. The development of the selective sigma-2 receptor ligands CB-64D and its chlorinated analog CB-184 (Figure 1) have allowed for the elucidation of a significant portion of the signaling mechanism and function of the sigma-2 receptor. The physiological observations in which the sigma-2 receptor has found to be upregulated in cells undergoing rapid proliferation and in high density in tumor cells, along with the production of SPC and ceramide, as well their prospective calcium signals have allowed us to propose a hypothetical signaling pathway for the sigma-2 receptor (Figure 7). Under this model, ceramide and SPC are proposed to play polarizing roles based on their attributed cytotoxic and non-cytotoxic roles respectively. Ceramide is proposed to be responsible for the cytotoxic effects observed as a result of chronic sigma-2 receptor stimulation. In the SK-N-SH neuroblastoma cell line, the cytotoxic effect is proposed to be initiated by the activation of caspase 8/10, which lead to mitochondrial depolarization. The mitochondrial depolarization is thought to lead to the observed delayed-prolonged calcium signal elicited by CB-64D treatment. Adding to the role of sphingolipids in cell death through mitochondrial involvement, recent data suggests that the sphingolipid products S1P and hexadecenal play a role in the activation of BAK and BAX (Chipuk et al., 2012). Sigma-2 receptors have been shown to induce apoptosis via a caspase-dependent and caspase-independent pathway, which is concurrent with the effects reported of ceramide. SPC on the other hand has been shown to produce a transient calcium signal and not be cytotoxic in our cell line. Although SPC is not as well characterized as ceramide, the present body of evidence shows that SPC is a potent mitogen and activator of transcription factors.
The highly potent sigma-2 selective ligand CB-184’s ability to induce the dose-dependent production of SPC and ceramide (Figures 4,5) followed by the putative sigma-2 receptor antagonist AC927’s ability to block the production of SPC (Figure 6) was pivotal in providing a major clue in elucidating the signal transduction of the sigma-2 receptor. The involvement of the sphingolipid metabolite ceramide provided a clue of the potential signaling mechanism of the cytotoxic signals observed, as a result of chronic stimulation of the sigma-2 receptor by its putative agonists. The production of SPC on the other hand, provided a clue as to the possible mechanism and cellular role of the transient calcium signal observed as a result of stimulation of the sigma-2 receptor. The limited characterization of the function of SPC’s signaling mechanism complicated characterizing its role in the sigma-2 receptor system. Even more so, the available literature was limited to cardiac muscle and SPC’s role in contraction and dilation of venous tissue. Based on the limited body of literature, we were able to deduce the possible roles that SPC was playing in our system, which would be commensurate with the physiological observations of the sigma-2 receptor's increased density in highly proliferating cells and in some tumor cell lines. We hypothesized that SPC was responsible for the transient calcium signal observed as a result of sigma-2 receptor stimulation by CB-64D. Based on published data, it is widely accepted that a transient calcium signal is a precursor signal for proliferative, mitogenic, and pro-survival signals among a host of transcriptional factors and phosphorylation cascades. The data obtained with SPC and CB-64D under conditions favoring cell death suggests that the sigma-2 receptor may play a pro-survival or proliferative role under similar conditions. The unexpected result of the SK-N-SH neuroblastoma cells becoming quiescent negated our ability to test survival under serum starvation conditions. The observation of the cells becoming quiescent corresponded with previous observations of the lack of responsiveness to sigma-2 ligands in our calcium assay and cytotoxicity. We found that
cell density was pivotal in obtaining a response in our calcium assay, in which a cell number of 15-30K cells per well in the 96-well plate format, was found to be optimal. This particular cell number corresponded to 60-70% confluency, which we had previously observed to be optimal in acquiring the most potent EC$_{50}$ with CB-64D. Based on these observations, it appears that the sigma-2 receptor is down-regulated as the cells increase in confluency, which is a result of the decreased rate of proliferation. Our findings that SPC or CB-64D did not stimulate doubling time or DNA-synthesis, along with the proliferative effects observed in the survival experiment suggest that the time frame observed for induction of DNA-synthesis or doubling time were too early in the signaling cascade.

The ubiquitous nature of calcium as an intracellular second messenger and its broad range of effects are greatly augmented by the vast number of modulatory proteins, which still leave the possibility of the sigma-2 receptor acting as a differentiating factor via calcium signaling. We must also take under consideration the fact that exogenously added SPC will be subject to the very intricate sphingolipid metabolism demonstrated in the literature and therefore have different effect than intracellularly produced SPC. Said metabolism has been shown to complicate the characterization of SPC and its ability to elicit a calcium signal, which is partly why many of the effects attributed to SPC have also been attributed to sphingosine-1-phosphate (S1P). In addition, levels of freely available SPC within circulation have been shown to be very low, which might provide additional clues regarding the differences in SPC's metabolism and targets.

In spite of the progress allowed by CB-64D and CB-184, there are limitations to how much we can accomplish without the elucidation of an endogenous ligand or the cloning of the receptor. The synthesis of the putative sigma-2 receptor antagonists AC927 (Figure 18), SN79 (Figure 19) UMB408 (Figure 27), UMB414 (Figure 27) and
MV1036A (Figure 33), have provided us with a significant toolset for the further characterization of the receptor. No single putative antagonist has yet showed the ability to block all of the effects attributed to the sigma-2 receptor in this system. AC927 showed the ability to block the CB-184-induced production of SPC. AC927 also completely blocked the CB-64D-induced calcium release and had no effect in the CB-64D-induced cytotoxicity. The putative sigma-2 antagonist SN79 behaved much like a partial agonist, in that it showed the ability to attenuate the CB-64D-induced calcium signal, while still producing a small calcium signal of its own. Additionally, SN79 showed a dose-dependent augmentation of the cytotoxic effect of CB-64D, which provided an unexpected, yet significant insight into the signaling mechanism of the sigma-2 receptor. While SN79 does not show any cytotoxic effect of its own, its ability to augment CB-64D’s cytotoxicity is the first such effect observed in the sigma-2 receptor. Currently, there is no evidence in the literature for multiple sigma-2 receptor subtypes from radioligand binding assays. However, data recently obtained in the Bowen lab shows that a [3H]DTG (O-di-tolyl guanidine) binding site remains after the classical sigma-2 receptors are blocked by an isothiocyanate derivative of SN79, under conditions were sigma-1 receptors are masked. This novel sigma-like binding site could possibly represent a sigma-2 subtype (A. Comeau, unpublished data). That site is under further investigation.

Using this data, we formulated a hypothetical model explaining the observed effects of SN79 and AC927 on the basis of the proposed existence of multiple sigma-2 receptor subtypes (Figure 37). This hypothesis is primarily based on the biphasic calcium signal elicited by CB-64D and the CB-184-dependent production of SPC and ceramide. Both the transient calcium signal and the production of SPC are attributed to non-cytotoxic signals, while ceramide and the prolonged calcium signal are generally
cytotoxic. These observations, along with the lack of any known mammalian enzyme with the ability to modulate both the production of ceramide and SPC add to the hypothesis of a bifurcating pathway via multiple sigma-2 receptor subtypes. Adding to this hypothesis, characterization of the putative sigma-2 ligand MV1036A resulted in the first ligand able to block the CB-64D-induced cytotoxic effect, while having no cytotoxic effect of its own. MV1036A produced a calcium signal whose recovery kinetics more closely resembled that of a full agonist. Based on its lack of cytotoxicity, it appears that MV1036A may be an agonist at the proposed sigma-2 receptor subtype responsible for the non-cytotoxic effects.

The observed effects of the putative sigma-2 receptor antagonists against the induced effects of CB-64D strengthen our hypothetical model (Figure 37) and provide a clearer picture of the signaling mechanism of the sigma-2 receptor. In this model, MV1036A antagonizes the actions of the subtype of the sigma-2 receptor responsible for the cytotoxic effects, while acting as an agonist at the non-cytotoxic side and producing a robust, transient calcium signal. SN79 and AC927 bind to the site responsible for the non-cytotoxic effects, which results in the release of a calcium signal and no cytotoxic effect. This observed signaling profile classifies SN79 and AC927 as partial agonists at the proposed sigma-2 subtype responsible for the non-cytotoxic effects. Additionally, UMB408 and UMB414 act as weak agonists at the cytotoxic site, which results in their cytotoxic effect, while being unable to produce a calcium signal or antagonize the effects of CB-64D. In this model, both CB-64D and CB-184 bind to both sigma-2 receptor sites and are able to produce both cytotoxic and non-cytotoxic effects. AC927 and SN79 would bind to and block the site responsible for the non-cytotoxic effects, which would explain AC927’s ability to block the production of SPC and calcium, as well as SN79’s ability to block the calcium signal elicited by CB-64D. This hypothetical model also
provides an explanation for the augmentation of CB-64D’s cytotoxic signal, since we hypothesize that CB-64D binds to, and activates both subtypes. In this model, SN79 mutes the non-cytotoxic signal, which allows for the cytotoxic signal to be more pronounced.

Although the tools for characterizing the sigma-2 receptor are extremely limited, continued synthesis of ligands targeted towards the receptor will continue to provide small clues towards the receptor’s endogenous function and value as a viable clinical target. The lack of an endogenous ligand and cloning of the receptor have slowed, and hindered efforts to extensively, and definitively elucidate the physiological role, intracellular function and signaling mechanism of the receptor. The identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site provides a significant step forward in the characterization of the sigma-2 receptor (Xu et al., 2012). Given the availability of biological tools for the cloned PGRMC1, it is now possible to directly address the implication of the sigma-2 receptor in the various effects reported to date, as well as the possibility of multiple receptor subtypes. In this study, we have provided evidence for a hypothetical signaling model of the sigma-2 receptor, based on data acquired through an exhaustive characterization of synthetic ligands and biological observations. Although much work remains to be done, the potential of the sigma-2 receptor as a clinical target, as well as its biological function make the sigma-2 receptor a very promising biological system. The range of tissues in which the sigma-2 receptor has been found, along with the cellular functions observed, suggest that the sigma-2 receptor’s role extends far beyond its clinical implications.
References


41. Michalak M, Robert Parker JM, Opas M. Ca2+ signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium. 2002 Nov-Dec;32(5-6):269-78.


