Discovery and Characterization of AP Endonuclease 1 Small Molecule Inhibitors: Impact on DNA Repair and Cancer Cell Viability

By

Hongmei Wu

B.S. Biological Sciences, University of California, Irvine, 2023

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Graduate Program of Biotechnology at Brown University

PROVIDENCE, RHODE ISLAND

May 2026

AUTHORIZATION TO LEND AND REPRODUCE THE THESIS

As the sole author of this thesis, I authorize Brown University to lend it to other institutions for the purpose of scholarly research.

Date 4/30/2026 Hongmei Wu

Hongmei Wu, Author

I further authorize Brown University to reproduce this thesis by photocopying or other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Date 4/30/2026 Hongmei Wu

Hongmei Wu, Author

This thesis by Hongmei Wu is accepted in its present form by the Graduate Program in Biotechnology as satisfying the thesis requirements for the degree of Master of Science

Date 4.30.26 Signature: _____

Dr. Robert Sobol, Advisor

Date 4/30/2026 Signature: _____

Dr. Diana Horrigan, Reader

Date 4/30/2026 Signature: _____

Dr. Wynand Roos, Reader

Approved by the Graduate Council

Date _____ Signature: _____

Dr. David Lindstrom, Dean of the Graduate School

Acknowledgement

I extend my deepest gratitude to my supervisor, Dr. Robert W. Sobol, for his unwavering support and guidance throughout my research journey. His deep commitment to academic excellence and meticulous attention to detail have significantly shaped this dissertation. I am also thankful to the other members of my thesis committee, Dr. Wynand Roos and Dr. Diana Horrigan, for their constructive feedback, guidance, and essential suggestions that enhanced the quality of my work.

My appreciation also goes to the staff and students in Dr. Sobol lab: Dan Ivanov who worked together with me on DRMB assay optimization and generated dose response curve data of E3330, Karhie Langham who provided cell viability data for E3330 and PARP inhibitors combinatory treatments, and other current and previous lab members including Charlotte R. Pearson, Emelia Margo, Nicole Marion Villarroel, Matt Wing, and Dr. Rasha Al-Rahahleh, who participated in the study or provided technical support. Their collective effort and support have been a cornerstone of my research experience.

Additionally, I would like to thank the collaborators of this project, including Dr. Karen Almeida from Rhode Island College who developed and optimized the DRMB assay, Dr. Yuan Liu from Florida International University who supplied purified APE1 protein, Dr. Debanus Das from Xpose Therapeutics Inc. who provided new APE1 inhibitor candidate compounds, and Dr. Adel Nefzi from Florida International University who provided a new multiplex compound library.

Finally, my sincere thanks go to my family, friends, peers, and partner for their continuous encouragement throughout my research experience. The completion of my thesis is inseparable from their support physically, mentally, and spiritually.

Table of Contents

Table of Figures

Table of Tables

Abstract of Discovery and characterization of AP Endonuclease 1 small molecule inhibitors: impact on DNA repair and cancer cell viability, by Hongmei Wu, ScM, Brown University, May 2026

Abstract

Apurinic/Apyrimidinic Endonuclease 1 (APE1) is a multifunctional protein that plays critical roles in base excision repair (BER), redox regulation of transcription factors, and RNA processing. APE1 is frequently overexpressed in diverse cancer types, where it contributes to tumor progression, therapeutic resistance, and poor clinical outcomes by enhancing DNA repair capacity and activating oncogenic signaling pathways. Accordingly, small-molecule APE1 inhibitors have emerged as promising agents to resensitize cancer cells to DNA-damaging chemotherapies, including temozolomide (TMZ). Despite this potential, the mechanisms of action of many reported APE1 inhibitors and their direct effects on DNA repair remain incompletely understood.

In this study, we systematically characterized five commercially available small-molecule APE1 inhibitors and performed compound screening to identify novel candidates using a newly developed DNA Repair Molecular Beacon (DRMB) assay. Using this approach, we demonstrate that E3330, previously characterized as a selective inhibitor of APE1 redox (Ref-1) activity, also potently inhibits APE1 AP endonuclease function. Furthermore, we show that E3330 disrupts BER by suppressing Poly (ADP-ribose) polymerase (PARP) activation and enhances sensitivity to alkylating agents. Together, these findings reveal an unanticipated dual mechanism of E3330 and underscore the utility of the DRMB assay for identifying and mechanistically characterizing APE1 inhibitors with therapeutic potential.

Chapter 1. Background

1.1 Introduction

DNA integrity plays a fundamental role in the regulation of cell metabolism and activities. Environmental factors, endogenous metabolic by-products, and numerous exogenous agents can result in a wide range of DNA damage types, including base lesions, apurinic/apyrimidinic (AP) sites, DNA single-strand breaks, and DNA double-strand breaks. If left unrepaired, these lesions can block replication and transcription or can lead to mutagenesis and genomic instability, which are hallmarks of cancer and other diseases.

AP Endonuclease 1 (APE1), encoded by the APEX1 gene, is a class II endonuclease that targets apurinic/apyrimidinic (AP) sites of double-stranded DNA. As the major AP-endonuclease in human cells, APE1 accounts for more than 95 % of cellular AP-endonuclease activity and initiates base excision repair (BER) by hydrolyzing the phosphodiester backbone 5′ to the AP site, generating a suitable substrate for DNA polymerases and ligases to complete subsequent DNA repair processes (Hickson *et al*., 2001; Demple & Sung, 1991).

1.2 APE1 and DNA Repair

As one of the cellular DNA repair mechanisms to restore DNA integrity, the BER pathway is responsible for the detection and repair of thousands of damaged bases per cell, per day. A critical step in BER is the processing of AP sites, which arise following spontaneous base loss, oxidation damage by reactive oxygen species (ROS), or the action of DNA glycosylases on damaged bases. Accumulation of AP sites destabilizes the DNA backbone because these lesions lack a templating

base, impairing normal base pairing and polymerase progression. During replication or transcription, unrepaired AP sites can stall DNA polymerases or lead to strand cleavage, generating single-strand breaks that may convert into highly cytotoxic double-strand breaks if encountered by replication forks. Consequently, persistent AP sites disrupt genome integrity and cell viability, underscoring the essential role of base excision repair in preventing their accumulation (Wallace, 2014; Caldecott, 2008).

APE1 recognizes these AP sites and specifically cleaves the DNA phosphodiester backbone 5′ to the lesion, generating a single-strand break with a 3′-hydroxyl (3′-OH) terminus and a 5′-deoxyribose phosphate (5′-dRP) end. This incision is essential for subsequent DNA synthesis and ligation steps that complete the repair. A recent study shows that APE1 is critical to the DNA gap tailoring and DNA synthesis by coordinating the recruitment of X-ray repair cross-complementing protein 1 (XRCC1), DNA Polymerase β (pol-β), and ligase 3 (Lig3) to complete BER repair (Almeida *et al*., 2024). Without efficient AP endonuclease activity, AP sites accumulate, leading to stalled replication forks, double-strand breaks, or cytotoxicity (Hickson *et al*., 2001).

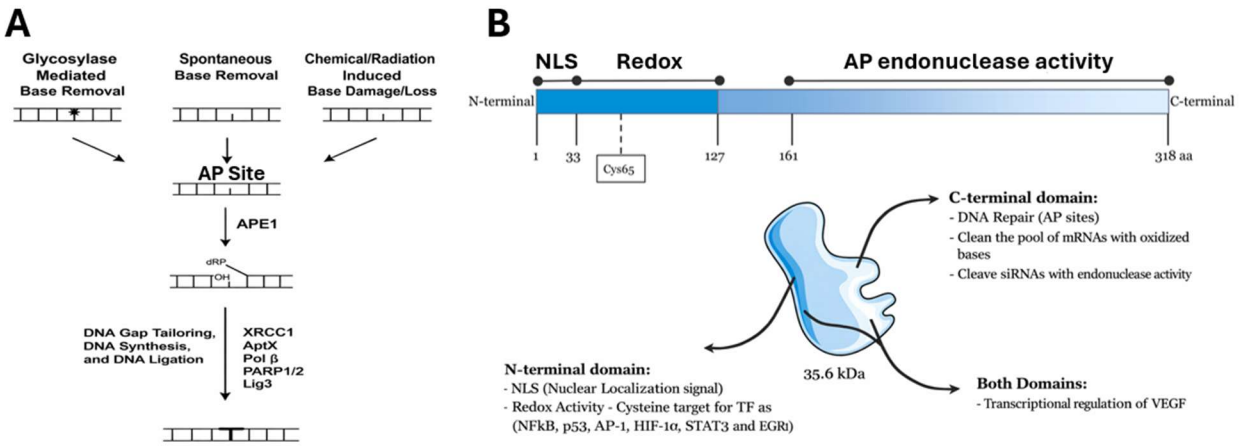

Figure 1: Functions of APE1. (A) Mechanism of APE1 activity in Base Excision Repair (Almeida *et al*., 2024). **(B)** Illustration of APE1 domains and functions. (Oliveira *et al.*, 2022)

The expression of functional APE1 is essential for embryonic development. Homozygous APEX1 gene knockout in mice results in embryonic lethality (Xanthoudakis *et al*., 1996). Depletion of APE1 protein in somatic cells was found to induce DNA damage accumulation and senescence phenotypes through activation of stress response and telomere dysfunction (Li *et al.*, 2018). These observations underscore APE1's key role in maintaining genome integrity and cellular homeostasis.

In addition to BER, recent studies suggest that APE1 participates in broader DNA damage response networks, including interactions with ATR/Chk1 checkpoint signaling via processing of single-strand breaks and potential influence on non-homologous end joining (NHEJ) of double-strand breaks under oxidative stress (Zhang *et al*., 2023). At the same time, APE1 is also found to have exonuclease activity and can cleave mismatched bases at the 3' end of the DNA strand (Liu *et al.*, 2021). Such multifunctionality indicates that APE1's endonuclease activity has implications beyond classic BER, contributing to global DNA repair network modulation.

1.3 APE1's Role in Redox Function and Transcriptional Regulation

Beyond its canonical role in BER, APE1 also exhibits multifunctional activities, including redox regulation of transcription factors (Ref-1) and modulating RNA processing and gene expression. Structurally, APE1 has two separate domains that control redox activity and AP endonuclease activity, respectively. The C-terminal domain controls DNA repair function and RNA processing, and the N-terminal domain controls redox regulation. APE1 was originally identified as a redox factor (Ref-1) (Xanthoudakis *et al*., 1992). The Cys65 side chain in the redox domain can bind numerous redox-sensitive transcription factors, including AP-1, NF-κB, p53, HIF-1α, and STAT3, which subsequently regulate gene expression programs involved in proliferation, survival,

angiogenesis, and stress response. This dual functionality positions APE1 as an integrator of DNA damage signaling and transcriptional control (Oliveira *et al*., 2022).

1.4 APE1 Expression and Cancer Progression

APE1 overexpression has been broadly observed in human cancers. Elevated APE1 mRNA and protein levels are reported in various malignant cancers, including pancreatic, prostate, cervical, ovarian, lung, colon, and other malignancies, often correlating with increased tumor growth, migration, therapeutic resistance, and poor clinical prognosis (Malfatti *et al*., 2023). Significantly lower overall survival rate is observed in APE-1-positive ovarian and gastric cancers as compared to APE-1-negative controls (Al-Attar *et al.*, 2010). Therefore, APE1 expression level may be considered as a biomarker for tumor progression.

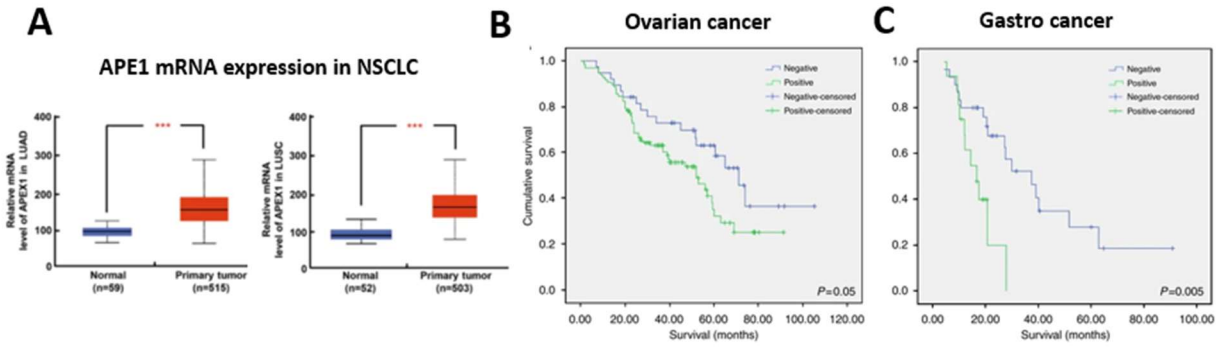

Figure 2: APE1 Expression and Cancer Progression. (A): APE1 mRNA expression in LUAD and LUSC subtypes of NSCLC (Malfatti *et a*l., 2023). (**B) & (C):** Overall survival rate in APE1 positive vs. APE1 negative phenotype in ovarian and gastric cancer (Al-Attar *et al.*, 2010).

The mechanisms behind APE1's role in cancer progression and drug resistance are complicated and not well known. Overexpression of APE1 may facilitate repair of DNA damage in rapidly proliferating cancer cells, contributing to resistance to chemotherapeutic agents and radiotherapy.

Recent studies showed that reducing APE1 activity with small-molecule APE1 inhibitors resensitizes cancer cells to DNA-damaging agents (Long *et al*., 2021).

Additionally, APE1 can also support oncogenic transcriptional programs via redox-mediated modulation of key factors, such as activation of cancer-promoting transcription factors including NF-κB, HIF-1α, STAT3, and AP-1 (Shah *et al*., 2017).

Moreover, APE1 has been implicated in non-canonical regulation of gene expression beyond redox modulation. A recent study found that APE1 binds G-quadruplex DNA structures, such as those in the KRAS promoter, promoting stable structure formation required for efficient transcription in Pancreatic ductal adenocarcinoma (PDAC) (Pramanik *et al*., 2022).

Both the AP endonuclease activities and redox regulation activities have been targets in drug development, particularly for cancer therapeutics that aim to sensitize tumor cells to genotoxic therapies by disrupting DNA repair or transcriptional support of survival pathways.

1.5 Additional APE1 Cellular Functions and Broader Implications

Beyond DNA repair and redox regulation, APE1 has emerging roles in RNA metabolism and quality control, interactions with nucleolar components such as nucleophosmin (NPM1), and involvement in RNA processing (Li *et al*., 2022). Its subcellular localization, dynamic regulation, and interaction networks extend its effect into mitochondrial DNA repair, stress responses, and immune regulation. This suggests APE1's broader implications beyond cancer and in other chronic conditions, including cardiovascular and inflammatory diseases (Yuan *et al.*, 2025).

1.6 Development of APE1 Inhibitors

Over the past 2 decades, several APE1 inhibitors have been discovered and tested for potential use in cancer therapeutics. Some APE1 inhibitors, including APE1 inhibitor compound 3 (cat# 262017), lucanthone (cat# 262015), and CRT 0044876, were found to inhibit the DNA repair function of APE1. Other APE1 inhibitors, such as APX2009 and APX3330, were found to inhibit the redox function of APE1. In this section, we review and summarize the properties and effects of the above 5 commercial APE1 inhibitors based on a literature review.

1.6.1. APE1 Inhibitor Compound 3 (Cat #262017)

Mechanism & Activity: Compound 3 is a small molecule identified in focused screens for inhibitors of APE1's endonuclease activity, likely binding the active site and acting competitively to block AP site cleavage and DNA binding. In colon cancer models, Compound 3 has been shown to inhibit APE1 endonuclease activity, leading to accumulation of unrepaired AP sites, enhanced p53 activation, metabolic disruption, and increased apoptotic cell death when combined with genotoxic stress (Codrich *et al*.,2019). Meanwhile, inhibition of exonuclease activities and redox activities by compound 3 has not been reported in primary literature (Xue & Demple,2022).

Development Status & Limitations: Compound 3 remains largely in bench research only. Despite its direct inhibition of APE1 endonuclease activity and ability to sensitize tumor cells to DNA-damaging agents, compound 3 is found to have considerable potential off-target effects, and the characterization of its selectivity remains incomplete, especially in complex biological systems. Some recent studies suggest that compound 3 has off-target cytotoxicity at high doses in APE-1-deficient cells (Xue &Demple, 2022).

1.6.2. Lucanthone (Cat #262015)

Mechanism & Activity: Lucanthone (1-[2-diethylaminoethylamino]-4-methylthioxanthen-9-one) was originally developed as an anti-schistosomal agent and recently repurposed in research as an inhibitor of APE1's DNA repair activity. Early studies demonstrate that lucanthone inhibits APE1's AP endonuclease function *in vitro* and in cell extracts and enhances cell killing by alkylating agents such as methyl methane sulfonate (MMS) and temozolomide (TMZ), likely through the accumulation of unrepaired AP sites (Luo & Kelley, 2004). Biochemical assays and molecular docking studies show evidence of direct binding of Lucanthone with the hydrophobic pocket of APE1, which enhances the degradation of APE1. The IC_{50} value of Lucanthone is estimated to be 5μM in a DNA incision assay with depurinated plasmid DNA, and around 7.2μM in cell-based assays (Naidu *et al.*, 2011). No significant inhibition of APE1 redox activity or exonuclease activities has been observed for Lucanthone. Additionally, Lucanthone also acts as a potent inhibitor of autophagy and induces lysosomal membrane permeabilization (LMP), potentially enhancing the effectiveness of radiation and other cancer therapies (Carew *et al.*, 2011).

Development Status: In 2016, Lucanthone entered Phase II clinical trials as an adjunct to whole-brain radiation therapy (WBRT) for patients with brain metastases from non-small cell lung cancer (NCT02014545) and adjunct treatment to temozolomide and radiation therapy for glioblastoma multiforme (NCT01587144). However, both clinical studies have been terminated or withdrawn due to safety concerns and limited efficacy. Though Lucanthone shows potent inhibition of APE1 in biochemical and cell-based assays according to prior studies, its specificity as a DNA repair inhibitor still needs further characterization. Its lack of redox inhibition may also limit its impact on transcription-driven survival pathways.

1.6.3. CRT0044876

Mechanism & Activity: CRT0044876 (7-Nitroindole-2-carboxylic acid) is one of the first direct small molecule inhibitors identified for APE1. This indole-carboxylic acid analog binds to the active site of APE1 and inhibits the AP endonuclease, associated 3′-phosphodiesterase, and 3′-phosphatase activities with IC50 in the low micromolar range (~3 μM) (Madhusudan *et al*., 2005). No significant inhibition of APE1 redox activity is found in CRT0044876.

Development Status: CRT0044876 has been extensively used as a research tool of APE1's repair functions, featuring its relative specificity for the APE1 active site and strong potency in biochemical assays. However, it has limited druglike properties, only modest cell potency, and potential off-target activities in cellular contexts. Currently, CRT0044876 has not been advanced to the clinical stage.

1.6.4. E3330 / APX3330

Mechanism & Activity: APX3330 (also known as E3330) is a quinone-derived small molecule characterized as a selective inhibitor of APE1's redox regulatory function. Unlike repair inhibitors that target the active site, APX3330 prevents APE1's redox activation of transcription factors such as NF-κB, HIF-1α, AP-1, and STAT3 by promoting disulfide bond formation in key cysteine residues (Cys 65) associated with redox activity, thereby blocking transcription factor binding and downstream gene expression (Long *et al.*, 2021). E3330 was found to have a moderate potency in redox activity inhibition of APE1, with an IC_{50} of approximately 55μM in NF-κB transactivation and 7μM in AP-1 DNA binding (Zhang et al., 2014). In recent studies with NSCLC, E3330 greatly

enhanced cancer cell cytotoxicity and impaired migration and invasion, resensitizing cancer cells to treatments including Cisplatin (Manguinhas *et al.*, 2020).

APE1's endonuclease activity was found to be not significantly inhibited by E3330 according to literature reports. It was also previously confirmed that E3330 does not sensitize cancer cell lines to alkylating DNA-damaging agents (Luo *et al*., 2005). However, one recent study shows a significant conformational change of the APE1 protein endonuclease domain based on NMR structural analysis, suggesting a potential effect on APE1's DNA repair function at high concentration above 100μM (Manvilla *et al.*, 2011). The mechanism behind E3330's potential interaction with the APE1 DNA repair domain is unknown.

Development Status: E3330 is currently the most clinically advanced APE1 inhibitor that has completed a Phase I trial in advanced solid tumors (NCT03375086). It showed manageable safety and target engagement signals with some disease stabilization. More analogues of E3330 are being developed to enhance its potency and bioavailability.

1.6.5. APX2009

Mechanism & Activity: APX2009 is a second-generation analog of APX3330 with improved physicochemical properties, including a smaller molecular weight (without the long alkyl chain and carboxylate group found in APX3330), lower lipophilicity, increased oral bioavailability, and 4-10 times enhanced potency for redox inhibition as compared to E3330 (Gampala *et al.*, 2024). APX2009 is found to specifically inhibit the redox function of APE1, resulting in reduced transcription factor activation and oncogenic signaling in multiple cancer cell lines, including

pancreatic and breast cancer (Siqueira *et al*., 2024, Gampala *et al.*, 2024). However, no inhibition of AP endonuclease activity or exonuclease activities by APX2009 was observed.

Development Status: APX2009 remains a promising preclinical lead compound for cancers dependent on APE1-mediated transcriptional networks. However, its effects on APE1 are primarily restricted to modulating transcriptional rather than direct DNA repair inhibition. More mechanistic studies are needed to fully characterize APX2009 and its interactions with APE1.

1.6.6 Summary of current APE1 inhibitors

According to previous literature, the above 5 APE1 inhibitors (compound 3, Lucanthone, CRT0044876, E3330, and APX2009) all have moderate to strong IC_{50} values, most of which are below 10μM. However, current commercial APE1 inhibitors have limited inhibition to either the endonuclease activity or redox activities, which do not cover the full spectrum of APE1 functions. Moreover, some recent studies had contradictory results showing non-significant binding to AP sites by APE1 active site inhibitors (compound 3, Lucanthone, and CRT004487) (Xue & Demple, 2022), and some of them were found to have off-target effects and cytotoxicity.

Currently, only E3330 is advanced to the clinical stage as an APE1 redox function inhibitor, but prior studies with E3330 show minimal or no effect on APE1's DNA repair function at low dosage despite its potential interaction with the APE1 endonuclease function domain.

More characterization is needed to study how current APE1 inhibitors affect DNA repair and cancer cell survival. Meanwhile, the discovery of new small molecule APE1 inhibitors with stronger potency to DNA repair inhibition and better safety profile is critical to the development of novel cancer therapeutics that overcome drug resistance by disrupting DNA repair mechanisms.

Inhibitor	Primary Target	IC_{50} (APE1)	Structure & Binding Mode	Other Affected APE1 Activities	Development / Notes
Compound 3 (262017)	DNA repair active site	Not widely published (estimated low µM)	Small molecule competitive binding to the APE1 repair active site	Exonuclease activity inhibition likely but not reported	Research tool; limited published data and potential off target effects
Lucanthone (262015)	DNA repair active site	~5 µM (repair inhibition)	Planar polyaromatic ring structure that binds in the APE1 repair active site	N/A	Research tool; endonuclease inhibitor; narrow specificity
CRT0044876	DNA repair active site	~3.0 µM (AP endo) & ~5 µM (3′-PG diesterase)	7-nitro-1H-indole-2-carboxylic acid binds in the APE1 DNA repair active site	Inhibited exonuclease activity in bacterial exonuclease III model	Research tool; direct endonuclease inhibitor limited in vivo use
E3330 (APX3330)	Redox domain (Ref-1)	~50 µM (redox IC_{50}) ~6.5 µM (redox EMSA)	Quinone-derivative that binds APE1 redox region, but binding may overlap the DNA repair site and affect conformational dynamics	Minimal change to endonuclease activity at low µM concentrations	Clinically advanced: completed Phase I solid tumor trial with target engagement data
APX2009	Redox domain (Ref-1)	~0.45 µM (redox EMSA)	Structural analog of APX3330 with modifications improving potencies	minimal direct effect in endonuclease activity	Preclinical; second-generation redox inhibitor with improved potency

Table 1: Summary of properties and features of 5 commercial APE1 inhibitors

Chapter 2. Methods and Experimental Design

2.1 Introduction of Experimental Design

The goals of this study fall into two aspects. Firstly, we aim to identify and characterize small molecule inhibitors targeting the apurinic/apyrimidinic (AP) endonuclease function of APE1. Secondly, we want to study the downstream effects of APE1 inhibitors in the DNA repair process. To screen compounds for AP endonuclease activities, we designed a novel DNA Repair Molecular Beacon (DRMB) assay that can detect AP endonuclease activity with cell lysate or purified APE1 protein in real time (Almeida *et al.*, 2024). Selected compound hits from the DRMB assay are characterized and validated for their effects in DNA repair via several other biochemical and cell-based assays (Figure 3). The LivePAR assay is used to quantify APE1 inhibitors' effect in downstream PARP activation in BER following DNA damage by visualizing poly(ADP-ribose) (PAR) foci formation in cells engineered with an EGFP-fused protein construct that binds to PAR. An immunoblot specific to PAR is used as a secondary assay to validate PAR formation after treatment with APE1 inhibitors and DNA-damaging agents. γ-H2AX immunostaining is conducted to visualize potential DNA double-strand break (DSB) resulting from oxidative stress and lack of APE1 activities. Finally, a cell survival assay is used to study the cytotoxicity of APE1 inhibitors and their potential effects in sensitizing cancer cells to DNA alkylating agents. The killing effects of APE1 inhibitors are also studied in cancer cell lines with XRCC1 knockout or BRCA1 knockout to respectively study APE1's effect in BER protein recruitment, and synthetic lethality of APE1 inhibition and deficiency in homologous recombination (HR).

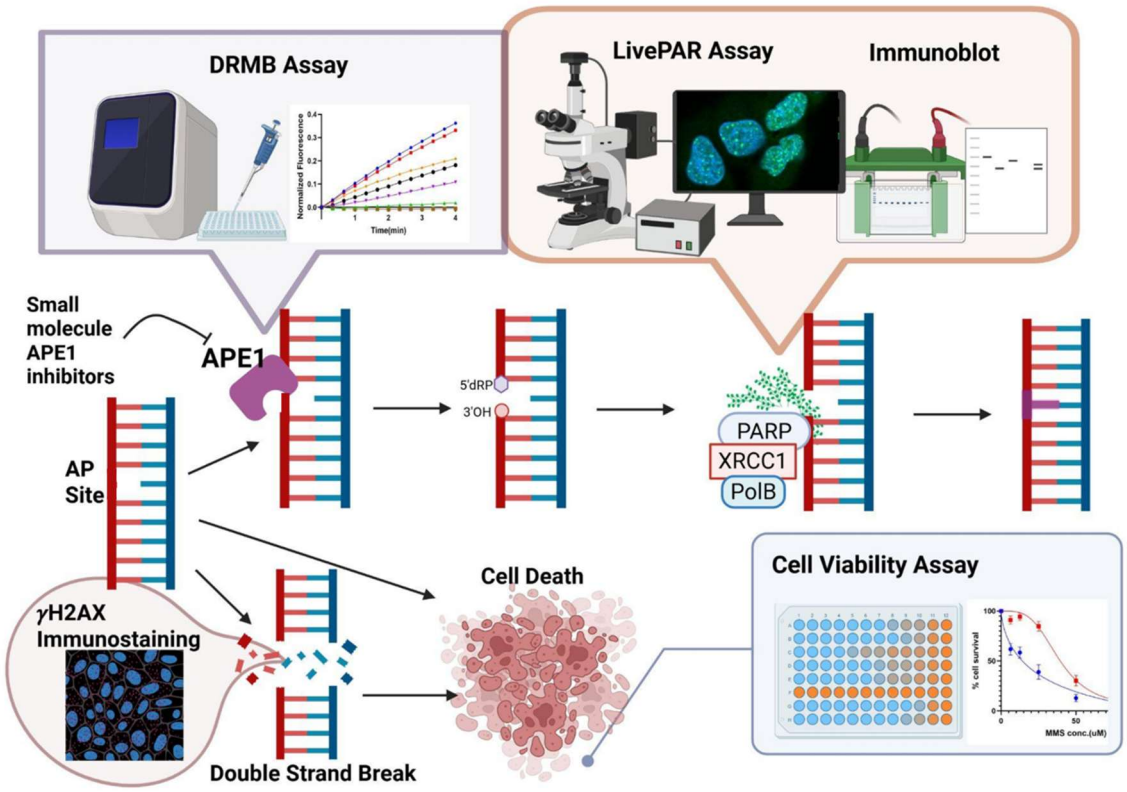

Figure 3: Illustration of Experiment Design (created with BioRender)

2.2 DNA Repair Molecular Beacon (DRMB) Assay

2.2.1 DRMB Assay Design and Optimization

To better measure AP endonuclease activity in real time, our lab developed a DNA Repair Molecular Beacon Assay. The reporter beacon in this assay is composed of a stem-loop oligonucleotide structure where the 5' end is conjugated to a FAM fluorophore and the 3' end is conjugated to a Dabcyl quencher. Under normal conditions, once hybridized, the fluorophore is quenched by the Dabcyl, and no fluorescence is detected. The THF5 beacon is engineered with a tetrahydrofuran (THF) lesion 5 base pairs from the 5' end that mimics an AP site and serves as substrate for APE1 (Figure 4C; Almeida *et al*., 2024). Upon the start of the reaction, APE1

hydrolyzes the THF lesion site and releases the fluorophore, causing an increase in fluorescence (Figure 4A; Li *et al.*, 2018). As a control, the control beacon CON5 does not have a lesion and does not react to APE1 (Figure 4C). Fluorescence is measured over time through the BioRad StepOne PCR instrument, and the fluorescence readout is positively related to AP endonuclease activity.

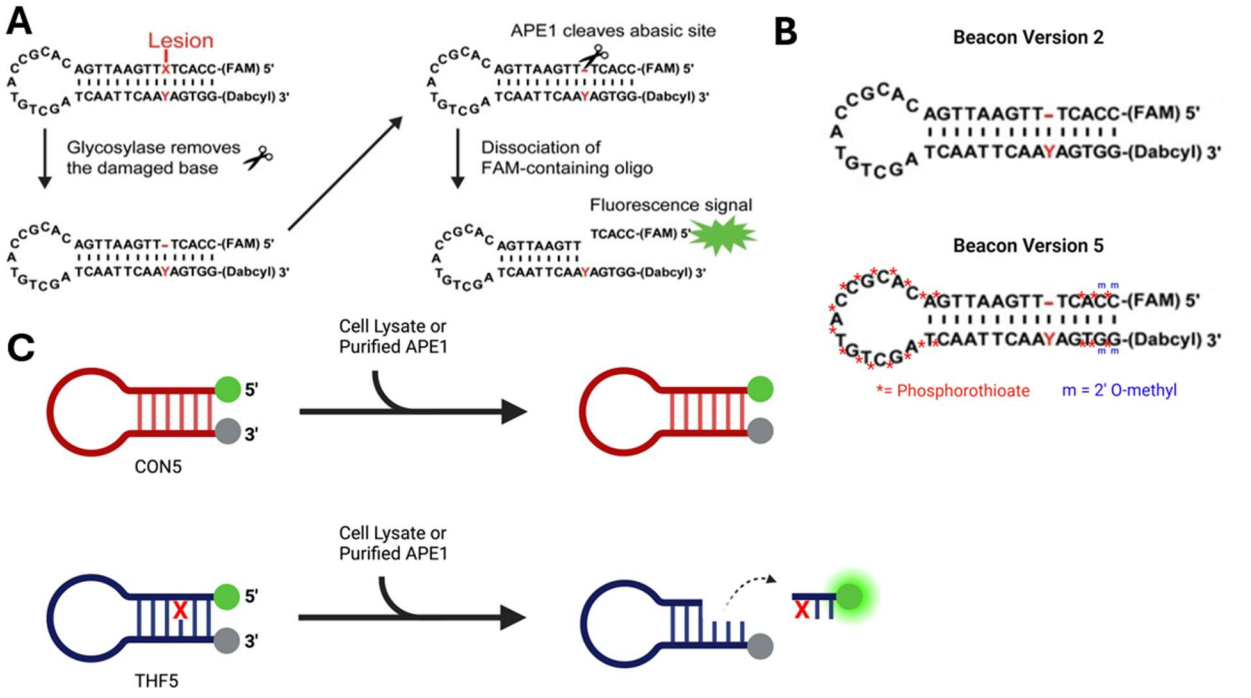

Figure 4: **Design of DRMB Assay**. **(A)** DNA Repair Molecular Beacon (DRMB) assay design and mechanism (Li *et al*., 2018). **(B)** Comparison of beacon design in version 2 and version 5 DRMB assay (Li *et al*.,2018; Almeida *et al.*, 2024) **(C)** Illustration of experiment design to evaluate AP endonuclease activity in version 5 DRMB assay (Almeida *et al.*, 2024)

The optimization of APE1-specific DRMB assays has undergone multiple versions and revisions. As compared to the previous version (Version 2) (Li *et al*., 2018; Svilar *et al*., 2012), the newest version (Version 5) includes a modified DNA backbone by adding phosphorothioate and 2' methoxy groups (Figure 4B), which stabilizes the hairpin structure as well as fluorophore and quencher conjugation in the beacons. These modifications prevent undesired exonuclease activities and allow the addition of 6mM Mg^{2+} in the buffer to enhance sensitivity to APE1 (Almeida *et al*.,

2024). Both sensitivity and specificity of this assay are significantly improved after these modifications.

2.2.2 APE1 Inhibitor Screening Methods using the DRMB Assay

Cell lysate preparation: The Retinal Pigment Epithelial (RPE-1) cell line (Amelia Technologies) and an RPE-1/APE1-KO (knock out) cell line (developed in the Sobol Lab) are cultured in DMEM/F12 media with 10%FBS and 1% penicillin/streptomycin and harvested at 80% confluence. Cells are detached with 0.25% Trypsin-EDTA solution (Gibco, cat# 25200-056) and centrifuged at 500g, 4°C for 5 minutes, forming about 50ul packed cell pellets containing 1-3 x 10^7 cells. The NucBuster Protein Extraction kit (Novagen, cat# 71183-3) is used to extract nucleic protein per the manufacturer's instructions. The cell pellet is resuspended in 150 µL NucBuster Reagent 1 per 50ul cell and centrifuged at 16,000g, 4°C for 5 minutes after mixing and 5 min incubation on ice. After removing the supernatant, the cell pellet is resuspended in 75 µL NucBuster Reagent 2 supplemented with 1 µL 100x protease inhibitor cocktail and 1 µL 100mM DTT (provided in kit) per 50 µL cell pellet volume, fully mixed and centrifuged at 16,000g, 4°C for 5 minutes. Supernatant is then collected and stored in aliquots at -80°C.

Buffer Preparation: Basic Common BER Reaction (CBR) buffer is prepared using the following formula: 25mM HEPES-KOH (Fisher BioReagents, cat# BP299-100), 150mM KCl (RICCA, cat# 5899-32), 0.5mM EDTA (Fisher BioReagents, cat# BP2482-500), and 1% glycerol (Fisher BioReagents, cat# BP299-1) in ddH2O adjusted to pH 7.8. Basic Common BER Reaction w/ magnesium (CBR-M) buffer is prepared by adding 6mM $MgCl_2$ (Honeywell Fluka, cat# 63020-1L) to the CBR buffer.

Beacon Preparation: HPLC-purified molecular beacons were purchased from IDT Technologies and dissolved to 200nM in CBR buffer. Molecular Beacon sequences are as follows:

CON5: /56-FAM/∗mC∗mC ACT ATT GAA TTG ∗A∗C∗A ∗C∗G∗C ∗C∗A∗T∗G∗T∗C ∗G∗A∗T∗CAA TTC AAT AGT∗ mG∗mG∗/3Dab/ where ∗ is a phosphorothioate linkage.

THF5: /56-FAM/∗mC∗mC∗ ACT /idSp/TT GAA TTG ∗A∗C∗A ∗C∗G∗C ∗C∗A∗T∗G∗T∗C ∗G∗A∗T∗CAA TTC AAT AGT∗ mG∗mG∗/3Dab/ where ∗ is a phosphorothioate linkage.

Beacons are annealed in a 100°C water bath for 15 minutes, then cooled overnight, and stored at -20°C in sterile black tubes. When properly annealed, the 6-FAM fluorophore is positioned near the Dabcyl quencher such that no fluorescence is emitted before the endonuclease reaction.

DRMB Assay: Purified human APE1 protein (kindly provided by Dr. Yuan Liu's lab, FIU, 200pM final concentration) or RPE-1 cell lysates (500nM final concentration) are diluted in CBR buffer supplemented with 12mM DTT (Thermo Scientific, cat# J1539703) and 2.5% EDTA-free Complete mini protease inhibitor cocktail (Millipore Sigma, cat# 11836170001). In a 96-well PCR plate (Applied Biosystems, cat# 4346906), each well is loaded with 15µl CBR-M, 2.5µl 200pM purified APE1 protein or 20ng/µl RPE 1 cell lysate, and 2.5µl DMSO (Fisher BioReagents, cat#BP231-100) or potential APE1 inhibitor compounds or CBR buffer. Commercial APE1 inhibitors used in this project include E3330 (Sigma Aldrich, cat# E8534-5MG), APX2009 (Sigma Aldrich, cat# SML 1887-5MG), CRT0044876 (Sigma Aldrich, cat# C0496-10MG), APE1 Inhibitor III (EMD Millipore Corp., cat#262017-10MG), and DNA Base Excision Repair Pathway Inhibitor (EMD Milliport Corp., cat# 262015-25MG), diluted to 10mM stock in DMSO and further diluted in CBR buffer to desired concentration. Compounds are mixed and incubated with purified APE1 protein or RPE1 cell lysate in quadruplicate for 30min on ice. After incubation, 5µl of 200nM THF5 or CON5 beacon is carefully added and allowed to hang on the wall of each well

without touching the mixture. Final beacon concentrations of the reagents in the reaction mixture are 40nM for beacons, 20 pM for purified APE1, and 2ng/ul for RPE1 cell lysates. To ensure the same reaction start time for all wells, the PCR plate is gently centrifuged or tapped, allowing beacon droplets to fall into the mixture before loading onto the StepOne PCR instrument. The AP endonuclease activity is very rapid and is completed within 10 minutes once started. It is therefore critical to start capturing fluorescence signals immediately after the reaction starts.

Experimental setting on the StepOne PCR instrument contains the following 6 consistent-temperature stages:

Stage #1–180 cycles, 20 s, 37 °C

Stage #2–15 cycles, 20 s, 60 °C

Stage #3–15 cycles, 20 s, 65 °C

Stage #4–15 cycles, 20 s, 70 °C

Stage #5–15 cycles, 20 s, 75 °C

Stage #6–15 cycles, 20 s, 80 °C

Data Analysis: The raw fluorescence readout (multicomponent data) of each well with a total of 255 cycles is acquired from the Applied Biosciences StepOne Real-Time PCR instrument and exported as an Excel file. The background adjusted value of each cycle is calculated as follows: adjusted background value = each experiment well readout - average of "beacon only" control well readouts - average of "APE1 protein/cell lysate only" control well readouts + average of "CBR buffer only" control well readouts. The baseline adjusted value is calculated by subtracting the adjusted background value of each cycle from the adjusted background value of cycle 1. The maximum fluorescence (Fmax) is defined as the maximum fluorescence readout among the averages of individual stages #2 through #6. The normalized fluorescence (Fnorm) is calculated

by averaging the baseline-adjusted value divided by Fmax of the respective wells. Data of the first 12 cycles (4 minutes) is then plotted as normalized fluorescence vs. reaction time (minutes). The slope of the graph is positively correlated with AP endonuclease activity.

2.2.3 APE1 Exonuclease Activity Measurement in DRMB Assay

APE1 exonuclease activity is measured by using CON2 beacon (sequence: /56-FAM/CCA CTA TTG AAT TGA CAC GCC ATG TCG ATC AAT TCA ATA GTG G/3Dabcyl), which is analogous to CON5 beacon but without phosphorothioate linkage and 2' methoxy groups. This unstabilized backbone structure allows us to measure exonuclease activity by APE1 at high enzyme concentration, where APE1 cuts the unstabilized 3' end of the beacon sequence, removes the Dabcyl quencher at the 3' end, and exposes the 5' FAM fluorophore, thereby increasing the fluorescence detected. Since the CON2 beacon does not have a THF lesion that serves as a substrate for the endonuclease activity of APE1, the fluorescence detected using the CON2 beacon is restricted to the exonuclease activity of APE1 only. In the modified protocol to test APE1 exonuclease activity, purified APE1 protein is used at 20nM final concentration, and CON2 beacon is used at 40nM final concentration, while all other steps remain the same as the general DRMB running procedure.

2.3 LivePAR Assay

2.3.1 Introduction of LivePAR assay

The Live PAR assay is a cell-based assay to quantify PARP1 activation. Poly(ADP-ribose) polymerase 1 (PARP1) is an enzyme that forms PAR [poly(ADP-ribose)] and facilitates DNA repair, especially for single-strand breaks (SSBs). In BER, single-strand breaks created by APE1

endonuclease activity trigger PARP1 binding and activation, inducing PARylation, initiating the recruitment of other DNA repair scaffold proteins such as XRCC1 to DNA damage sites.

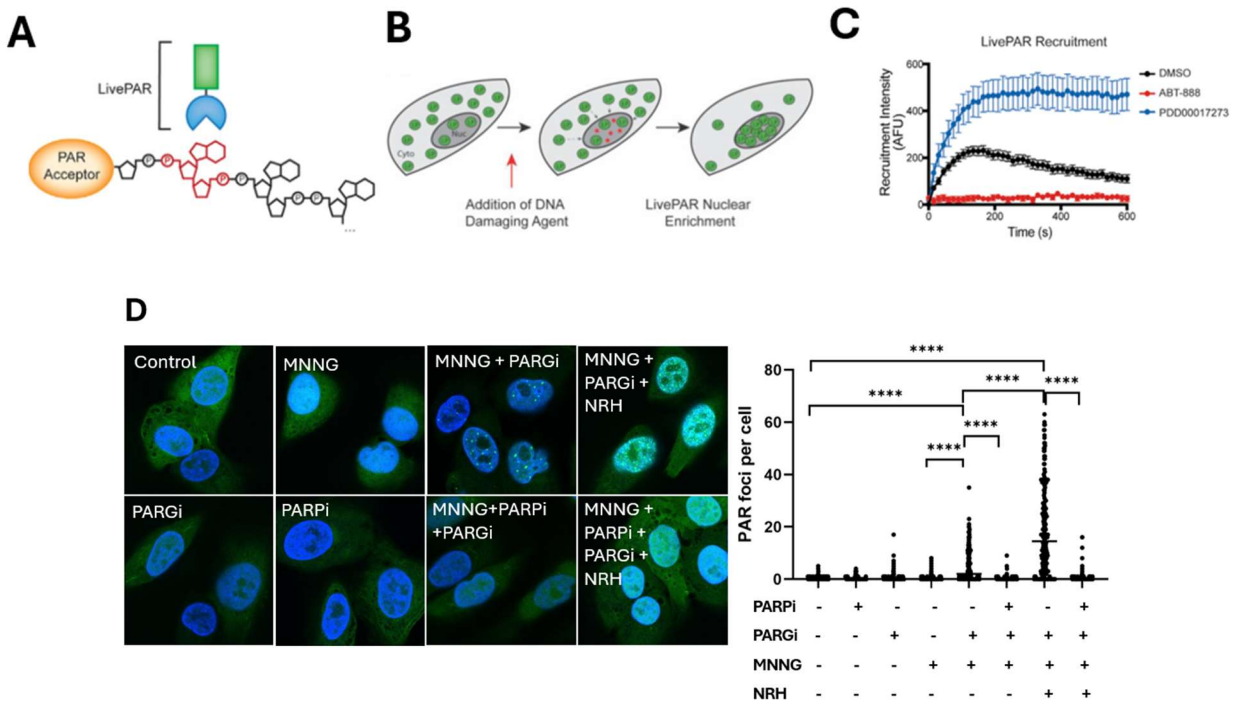

Figure 5: Design of LivePAR Assay. (A) Design of LivePAR construct (Koczor *et al.*, 2021). **(B)** Illustration of LivePAR nucleus localization following DNA damage (Koczor *et al.*, 2022). **(C)** Real-time LivePAR recruitment quantification following laser-induced micro-irradiation and treatment with PARP inhibitor ABT-888, PARG inhibitor PDD00017273, or DMSO control (Koczor *et al.*, 2021) **(D)** Confocal microscopic analysis (n=3) of PAR foci formation following treatment with PARP inhibitor ABT-888, PARG inhibitor PDD00017273, MNNG, and NRH (**** denotes $p<0.0001$, Kolmogorov-Smirnov test)

In the assay, U2OS/LivePAR cells are engineered by expression of a fusion protein, EGFP fused to a poly(ADP-ribose) (PAR)-binding motif that binds to the iso-ADP-ribose moiety of PAR. This is used to detect PAR foci in live cells when evaluated by confocal fluorescent microscopy (Figure 5A). N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) is an alkylating agent that induces DNA damage and initiates DNA repair. The small molecule inhibitor PDD00017273 inhibits Poly(ADP-ribose) glycohydrolase (PARG), the enzyme that breaks down the PAR complex, increasing PAR foci formation (Figure 5C). Dihydronicotinamide riboside (NRH) acts as a potent NAD^+ precursor that increases intracellular NAD^+ levels (Chini *et al.*, 2022), which subsequently drives elevated

activation of poly(ADP-ribose) polymerase 1 (PARP1) and increases PAR synthesis. In the LivePAR assay, combination treatment of MNNG, PARG inhibitor PDD00017273, and NRH has shown the greatest amount of PAR foci (Figure 5D), which serves as a positive control for the testing of APE1 inhibitor compounds. Meanwhile, PARP inhibitor ABT-888 (Veliparib) has shown almost complete inhibition of PAR foci formation, making it a negative control in the LivePAR assay (Figure 5D).

2.3.2 LivePAR Assay Procedure

20*20mm coverslips (Fisher Scientific, cat# 22-170371) are pre-soaked for 20min in diethyl ether (Fisher Scientific, cat# 60-29-7) and washed with 100% ethanol, 70% ethanol, and ddH_2O, respectively, followed by another 20 min incubation in 1N HCl and 3 washes in ddH_2O. U2OS LivePAR cells are seeded at a density of 2.24 x 10^5 cells per 60mm petri dish with 2 coverslips and allowed for 24hr growth. Cells were pre-treated for 30 min at 37°C with APE1 inhibitors at the desired concentration or DMSO and then treated with a mixture of 10μM PARG inhibitor PDD00017273 (Tocris, cat#5952) and 100μM NRH (Dr. Marie Migaud lab), with 10μM PARP inhibitor ABT-888 (Veliparib, BPS Bioscience, cat# 27101) also added in one dish as a negative control. After another 30 min incubation at 37°C, cells are treated with 10 μM MNNG (Molport, cat#N493990) and incubated at 37°C for 90 min.

Before fixing cells, the medium is removed after incubation and washed 3 times with PBS. Cells are prefixed in 4% formaldehyde (Thermo Scientific, cat# J61899.AP) in PBS, followed by 3x wash with PBS. Then, cells are fixed by adding 3 ml of cold 7:3 methanol (Fisher Bioreagents, cat#A452SK-4): acetone (Sigma Aldrich, cat# 650501) solution and incubating at -20°C for 9 min, followed by 3 washes with PBS to remove the fixative and rehydrate the cells. Coverslips with

cells are mounted onto glass slides with 15 µL Vectashield (H-1000) anti-fade medium (Fisher Scientific, cat# NC1695563) with corners sealed by clear nail enamel (Al-Rahahleh *et al*., 2025). Slides are imaged at 60X magnification, 488nm (green) and 405nm (blue) channels with the Nikon confocal microscope imaging system. The number of PAR foci within the nucleus is analyzed using ImageJ software.

2.4 γ-H2AX Immunostaining

2.4.1 Introduction of γ-H2AX Assay

DNA double-strand breaks (DSBs) and blocked replication forks activate DNA damage response (DDR) kinases, which then phosphorylate various downstream DNA repair pathways. γ-H2AX foci, a biomarker for DNA double-strand breaks, are microscopic structures formed in the cell nucleus through the phosphorylation of histone H2AX at serine 139 by DDR kinases (Krumm, 2016). By immunostaining of cells with a phosphorylated H2AX antibody, DNA double-strand breaks can be quantified and visualized microscopically as γ-H2AX foci surrounding lesion sites.

2.4.2. γ-H2AX Immunostaining Procedure

Coverslips are prepared in the same procedure as in the LivePAR assay prior to the experiment. U2OS cells are seeded at 2*10^5 cells per 60mm petri dish with 2 coverslips and allowed for 24hr growth. Cells are then treated with potential APE1 inhibitors for 24 hrs before fixation in the same way as in the LivePAR assay. After fixation, cells are incubated in the blocking solution (5%BSA in 0.25% Triton-X100/PBS) for 1 hour at room temperature. Then, the blocking solution is removed, and 100 µL of anti-phospho-H2AX rabbit monoclonal antibody (Cell Signaling, cat #9718, 1:500 dilution in 0.25% Triton X100/PBS) is added dropwise onto each coverslip and incubated at 4°C and in a hydrated container overnight (Khan *et al.*, 2025). On the next day, cells

are washed 2x with PBS and 1x with 0.25% Triton X100/PBS before incubation in 100µl Alexa Fluor 568 goat anti-rabbit secondary antibody (Invitrogen; REF: A11011; 1:500 dilution) for 2-3 hours at room temperature in the dark. Cells are washed again with 2x with PBS and 1x with 0.25% Triton X100/PBS before mounting to glass slides in Vectashield (H-1000) as indicated in LivePAR assay procedure. Finally, slides are imaged at 60X magnification, 568nm (red) and 405nm (blue) channels with the Nikon confocal microscope imaging system. The number of γ-H2AX foci within the nucleus is analyzed using ImageJ software.

2.5 Cell Viability Assay

The cell viability assay is designed to test the drug-killing effect and cytotoxicity of APE1 inhibitor candidates by measuring the number of live and dead cells following treatments. Cells are seeded at 8000 cells/ml, 100µl media/well in 96-well cell culture plates (Costar, cat#3596) and are cultured overnight before treatment with 100µl/well APE1 inhibitor candidates at various concentrations via serial dilution, with each treatment condition in quadruplicate. When studying combined killing effects with DNA-damaging agents such as MMS, APE1 inhibitors are added 30 minutes before other compounds. Cells are grown for 5 days post-treatment. Cells are stained by adding 50µl/well of PBS supplemented with 0.5% Hoechst dye (Thermo Fisher Scientific, cat # 6224910) and 1.5% propidium iodide (Sigma, cat#P4170) and incubated at 37°C in the dark for 15 minutes. The plate is then loaded into the Celigo image cytometer and imaged in blue 377/477nm illumination (live channel) and red 531/629 nm illumination (dead channel). Cell count data for each well is analyzed as the number of live cells minus the number of dead cells, normalized to the average cell count of control wells. The analyzed data is graphed as % cell survival vs. treatment dose.

2.6 Cell Culture

RPE-1 and RPE-1/APE1-KO cell lines are cultured in DMEM/F12 media (DMEM/F12 with L-glutamine, 10% heat inactivated FBS, 1% penicillin-streptomycin [Invitrogen #15140-122]). The U2OS/LivePAR cell line, RPE-1/p53-KO and RPE1/p53-KO/BRCA1-KO cell lines are cultured in DMEM media (DMEM [CellGrow #10-013-CV, 10% heat inactivated FBS, 1% L-glutamine, 1% penicillin/streptomycin). ES-2 and ES-2/XRCC1-KO cells are cultured in RPMI media (RPMI 1640 [CellGrow #MT10-040-CV], 10%FBS, 1% penicillin/streptomycin, 1% L-glutamine). LN428 and LN428/XRCC1-KO cells are cultured in MEMalpha media (MEM alpha [Invitrogen #12571-063], 10% heat inactivated FBS, 1% antibiotic/antimycotic [Invitrogen # 15240-062], 1% L-glutamine, 0.05mg/ml gentamicin). All cell lines are passaged or seeded for experiments at 80% confluence with media change every 3-5 days.

2.7 Immunoblot

Cell lysate preparation: To make working sample lysis buffer solution, 50µl β-mercaptoethanol (Sigma, cat#M3148) is added per 1ml 2X clear Laemmli sample lysis buffer (2% SDS [Thermo Fisher, cat#24730020], 20% glycerol [LabChem, cat# LC148502], 62.5mM Tris-HCl [pH 6.8, Bio-Rad, cat# 161-0799] in MilliQ sterile H_2O). 1-2 million cells are cultured in 100mm dishes and washed twice with cold PBS before harvesting. 250µl working sample lysis buffer solution is added evenly in drops to each dish after removal of PBS. Cells are then scraped using a sterile cell scraper, and cell/lysis buffer mixtures are collected in 1.5ml Eppendorf tubes. Collected samples are boiled for 5-10 minutes on 99°C heat blocks before immunoblotting or storage at -80°C.

SDS-Page: All samples are adjusted to 20 µg per well by diluting with the 2X clear Laemmli sample lysis buffer. 4X Laemmli sample buffer (Bio-Rad, cat#1610747) is added at the appropriate volume. Samples are then heated on a 99°C heat block for 2 minutes before loading on the NuPAGE 4-12%Bis-Tris gels (Thermo Fisher Scientific, cat# NP0321BOX). 1X MOPS gel running buffer is prepared by diluting 20x MOPS buffer stock (InVitrogen, cat# NP0001-02). 20µl of each processed sample and 10µl pre-stained color protein standard markers (10-250kDa, New England Biolabs; cat# P7719S) are loaded and the gels are electrophoresed at 100V for 1.5 hours.

Transfer and Blocking: Transfer buffer is prepared by mixing 100 ml Trans-Blot Turbo 5X transfer buffer (Bio-Rad; cat#10026938) with 100ml pure ethanol and 300ml milli-Q water. Proteins separated by SDS-PAGE are transferred to the Transblot Turbo Mini-size Nitrocellulose membrane (Bio-Rad, cat# 1704270) using the Bio-Rad Trans-Blot Turbo Transfer system for 18 minutes. After the transfer, nitrocellulose membranes are washed with water and stained with Ponceau S stain (Fluka Analytical, cat# 81462) to check if proteins are successfully transferred. The membranes are washed again with TBST (5% 20xTBS (VWR Life Science, cat# J640-4L) with 0.5% Tween (BioRad, cat#1610781) diluted in MilliQ sterile H_2O) before blocking with 5% non-fat dry milk (Bio-Rad, cat#1706404) in TBST for 1 hour.

Primary and Secondary Antibodies: After blocking, the membranes are properly cut based on the molecular weight of the proteins and incubated in selected primary antibodies that are diluted in blocking solution (Table 2) at 4°C overnight on a rocker, followed by three 10-minute washes with TBST. The membrane is then incubated for 2 hours in HRP-conjugated secondary antibodies at room temperature, followed by three 10-minute washes with TBST.

Type	Antibody Target/Name	Manufacturer	Cat #	Dilution ratio	Source
Primary Ab	APE1	Thermo Fisher Scientific	MA1-440	1:1000	mouse
	PolB	Sigma Aldrich	SAB4502236	1:500	rabbit
	XRCC1	Cell Signaling Technology	76998S	1:1000	rabbit
	PCNA	Abcam	ab92552	1:1000	rabbit
	H3	Cell Signaling Technology	4499S	1:5000	rabbit
	PAR	Roland Inc.	Clone 10H	1:1000	mouse
Secondary Ab	Goat anti-rabbit (H+L) HRP conjugated Ab	Bio-Rad	1706515	1:2000	goat
	Goat anti-mouse (H+L) HRP conjugated Ab	Bio-Rad	1706516	1:2000	goat

Table 2. List of antibodies used in immunoblot.

Imaging and Data Analysis: Membranes are incubated for 5 minutes in a 1:1 mixture of 1ml Bio-Rad Clarity Western ECL Peroxide solution + Clarity Western ECL Luminol/Enhancer (cat#1705061) before imaging using Chemi-doc software. For quantification of protein expression, the band density in captured images is analyzed in ImageJ with targeted protein band density data normalized to that of the H3 protein.

Chapter 3. Results

3.1 Discovery and Characterization of APE 1 Inhibitors via DRMB Assay

3.1.1 Validation of DRMB Assay

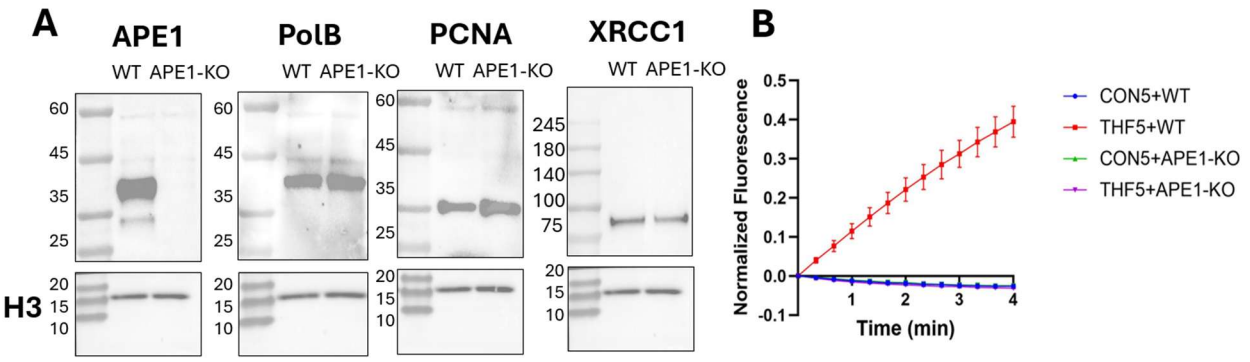

Figure 6: Validation of DRMB Assay. (A) Immunoblot validation of RPE1 WT and RPE1/APE1-KO cell line. **(B)** Validation of AP endonuclease activity capture in DRMB Assay

Our lab has previously knocked out the APEX 1 gene in the RPE-1 cell line using the CRISPR/Cas9 system, creating RPE-1/APE1-KO cells. Immunoblots are used to verify protein expression of APE1, PolB, PCNA, and XRCC1 in RPE-1 WT and RPE-1/APE1-KO cells. The immunoblot result (Figure 6A) verifies successful knockout of APE1 in RPE-1/APE1-KO cells without significant change in the expression of DNA polymerase B (PolB), X-ray repair cross-complementing protein 1 (XRCC1), or Proliferating Cell Nuclear Antigen (PCNA). To validate the functionality and specificity of the DNA Repair Molecular Beacon (DRMB) Assay in detecting AP endonuclease activity, cell lysate from RPE-1 and RPE-1/APE1-KO cells was used. The THF5 beacon is used as a substrate for APE1 and a reporter for AP endonuclease activity, while the CON5 beacon is used as a control reporter. According to the DRMB assay normalized fluorescence data (Figure 6B), a linear increase in normalized fluorescence is seen in the RPE-1 WT cell lysate with the addition of the THF5 beacon, while analysis of the RPE-1/APE1-KO cell lysate and the

CON5 beacon does not change the fluorescence detected. This indicates that the presence of the RPE1 protein lysates can hydrolyze the APE1-specific THF lesion (mimics of AP site), which validates the specificity of the DRMB assay.

3.1.2 APE1 inhibitor Screening in DRMB Assay

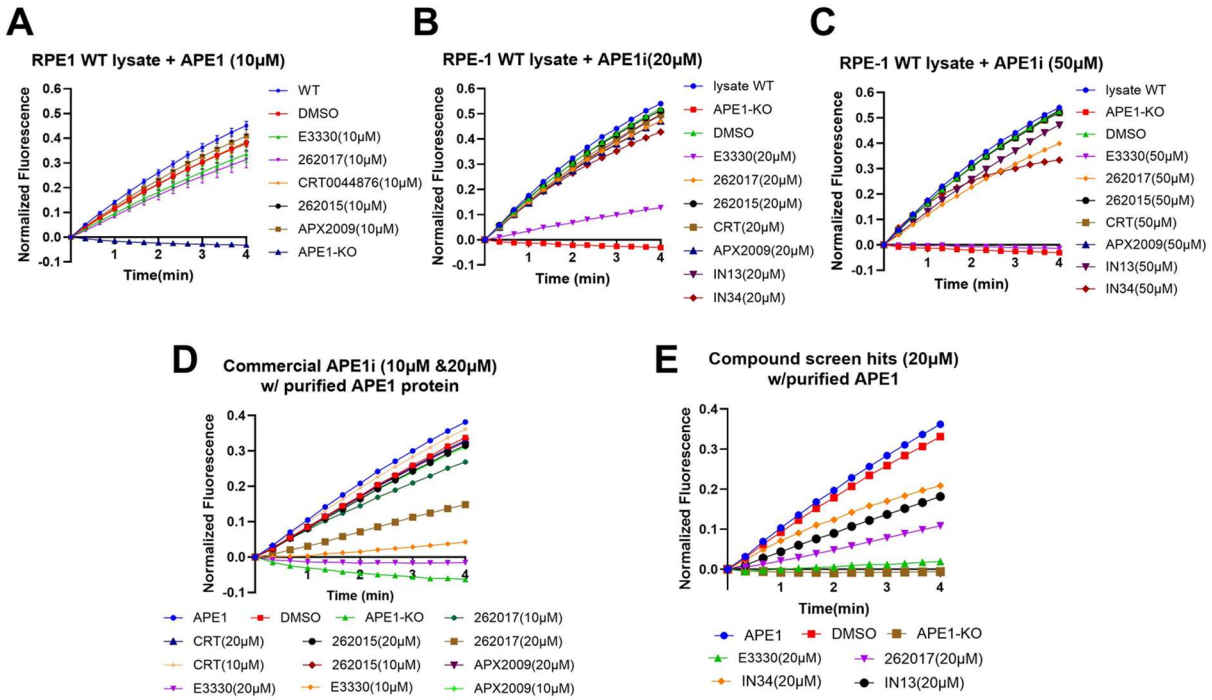

Figure 7: APE1 Inhibitor Screening in DRMB Assay. (A)-(C): Normalized fluorescence over time as measured in the DRMB assay (version 5) with RPE-1 wildtype cell lysate and THF5 beacon, preincubated 30 minutes at 4°C with 10μM, 20μM, or 50μM potential APE1 inhibitors. **(D):** Normalized fluorescence over time in DRMB assay (version 5) with purified APE1 protein and THF5 beacon, preincubated 30 minutes at 4°C with 10μM or 20μM individual commercial APE1 inhibitors, including E3330, CRT0044876, Lucanthone (262015), compound 3 (262017), and APX2009. **(E):** Characterization of compound screening hits in the DRMB assay (version 5) with purified APE1 protein. XPTx-0506 (IN13) and XPTx-0524 (IN34) are selected among 34 unknown candidate compounds from our collaborator, Xpose Therapeutics.

To test the AP endonuclease inhibition effect of both commercial APE1 inhibitors and unknown compounds, RPE-1 WT cell lysate is preincubated for 30 minutes with 10μM, 20μM, and 50μM

of the selected commercial APE1 inhibitors and unknown compounds on ice before the addition of the THF5 beacon. Surprisingly, our analysis using the DRMB assay shows no significant reduction in fluorescence over time for the commercial APE1 inhibitors Lucanthone (262015) or CRT0044876 at 10μM (Figure 7A), which demonstrates a minimal inhibition of AP endonuclease activity by CRT0044876 and weak inhibition of AP endonuclease activity by Lucanthone (262015). Next, we increased the dose of the compounds to 20μM and 50μM, respectively, and found no significant increase in AP endonuclease activity inhibition for Lucanthone (262015) or for CRT0044876. Compound 3 (262017) shows weak inhibition of AP endonuclease activity at 10μM, 20μM, and 50μM (Figure 7A-7C). This contradicts previous literature, potent inhibition of APE1 activity by Lucanthone (262015), by compound 3 (262017), and by CRT0044876 at doses below 10μM (Naidu *et al*, 2011; Madhusudan *et al*, 2005). Interestingly, we found that E3330 shows strong inhibition of AP endonuclease activity in the DRMB (version 5) assay at 20μM and 50μM, which contradicts prior literature suggesting no inhibition of AP endonuclease activity by E3330 at doses below 100μM (Luo *et al*, 2005). However, APX2009, an analog of E3330, does not show inhibition of AP endonuclease activity in our DRMB assay with RPE-1 WT cell lysate, which is consistent with prior studies. Considering potential off-target interaction by APE1 commercial inhibitors in cell lysates (Xue & Demple,2022), we also tested all 5 commercial APE1 inhibitors in our DRMB (version 5) assay with 20 pM purified APE1 protein, kindly provided by Dr. Yuan Liu (Florida International University). Compound 3 (262017) and E3330 showed more potent inhibition of AP endonuclease activity at 20μM with purified APE1 protein than in the RPE-1 WT cell lysate (Figure 7B & 7D), which may be due to potential off-target interactions of compound 3 (262017) and of E3330 with other proteins in RPE-1 cell lysates. Lucanthone (262015), CRT0044876, and APX2009 continue to show no significant change of normalized

fluorescence with purified APE1 protein, which confirms no significant AP endonuclease inhibition by Lucanthone (262015), by CRT0044876, or by APX2009. Additionally, we screened 34 unknown candidate compounds from our collaborator Xpose Therapeutics in the DRMB (version 5) assay with both the RPE1 WT cell lysate and with purified APE1 protein. Among the 34 candidate compounds, XPTx-0506 (IN13) and XPTx-0524 (IN34) are hits that show a potency similar to the commercial inhibitor compound 3 (262017) (Figure 7B, 7C, and 7E).

3.1.3 Characterization of E3330's inhibition of AP Endonuclease Activity

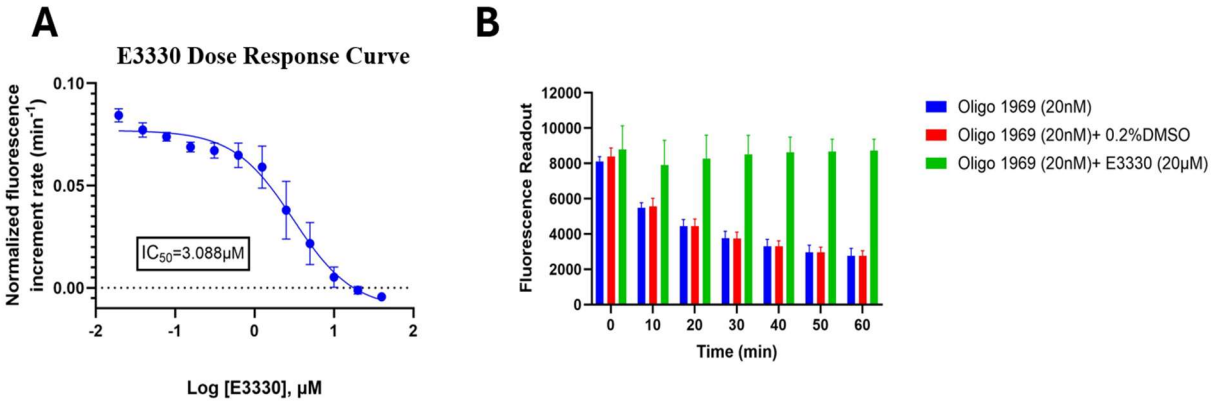

Figure 8: Inhibition of AP Endonuclease Activity by E3330. (A) Dose response curve of E3330 (n=3). AP endonuclease activity quantified based on the slope of normalized fluorescence in the first 4 minutes of the reaction at 37°C, data provided by Dan Ivanov from the Sobol lab. **(B)** The effect of E3330 on fluorescence signals of oligo 1969, an analogous beacon of CON5 but without the Dabcyl quencher.

To further characterize E3330's inhibition of AP endonuclease activity, we performed dose-response experiments on E3330 (0.02μM-40μM) using the DRMB assay with 20 pM purified APE1 protein and 40nM THF5 beacon. AP endonuclease activity is quantified based on the rate of normalized fluorescence per minute in the first 4 minutes of the reaction at 37°C, with a lower fluorescence rate — a smaller slope of linear regression fit in the normalized fluorescence vs. time

plot — indicating stronger inhibition. The dose response curve of E3330 (Figure 8A) is generated using a 4-parameter nonlinear regression fit. The average IC_{50} of E3330 is 3.088μM on AP endonuclease activity inhibition at 37°C reaction temperature. This confirms a potent inhibition of AP endonuclease activity by E3330.

Due to the contradictory results of E3330 from previous literature, we were concerned that there is a likelihood that the reduction of fluorescence in the DRMB assay may be caused by some intrinsic fluorescence quenching effect of E3330. To test this hypothesis, we designed oligo 1969 (sequence:/56-FAM/*mC*mC*A CTA TTG AAT TG*A* C*A*C* G*C*C* A*T*G* T*C*G* A*T*C AAT TCA ATA GT*mG* mG), an analogous beacon of CON5 but without the 3'Dabcyl quencher, and monitored the fluorescence level over time with or without 20μM E3330. The result (Figure 8B) shows that instead of quenching the fluorescence signal, E3330 stabilizes the fluorescence signal by preventing the degradation of the fluorescence signal over time at 37°C, which rejects the hypothesis and proves that the reduction in fluorescence rate in the prior DRMB experiments is most likely caused by E3330 inhibition of AP endonuclease activity.

3.1.4 APE1 inhibitors' effect on exonuclease activities of APE1

Since APE1 also has intrinsic exonuclease activity that cleaves the 3' end of the DNA strand (Liu *et al*, 2021), we performed experiments to test if selected compounds E3330, 262017, inhibitor 13, and inhibitor 34 also display inhibition of the exonuclease activity of APE1. This is achieved by using the CON2 beacon that has the same sequence as the CON5 beacon but without the phosphorothioate linkage stabilization, which makes the CON2 beacon subject to exonuclease cleavage of the 3'Dabcyl quencher, resulting in an increase in fluorescent readout. In this experimental design, the slope of normalized fluorescence over time is indicative of exonuclease

activity of APE1. With 20nM purified APE1 and 40nM CON2 beacon used in the DRMB assay, we found that E3330 also has a potent dose-dependent inhibition of exonuclease activity of APE1 starting at 10μM (Figure 9A), which was not shown in the previous literature. Meanwhile, compound 3 (262017) shows no inhibition of exonuclease activity, which supports previous literature (Xue & Demple,2022). The candidate compound XPTx-0524 (IN34) also shows minimal inhibition of exonuclease activity (Figure 9D) while XPTx-0506 (IN13) shows significant inhibition of exonuclease activity only at a concentration of 50μM (Figure 9C).

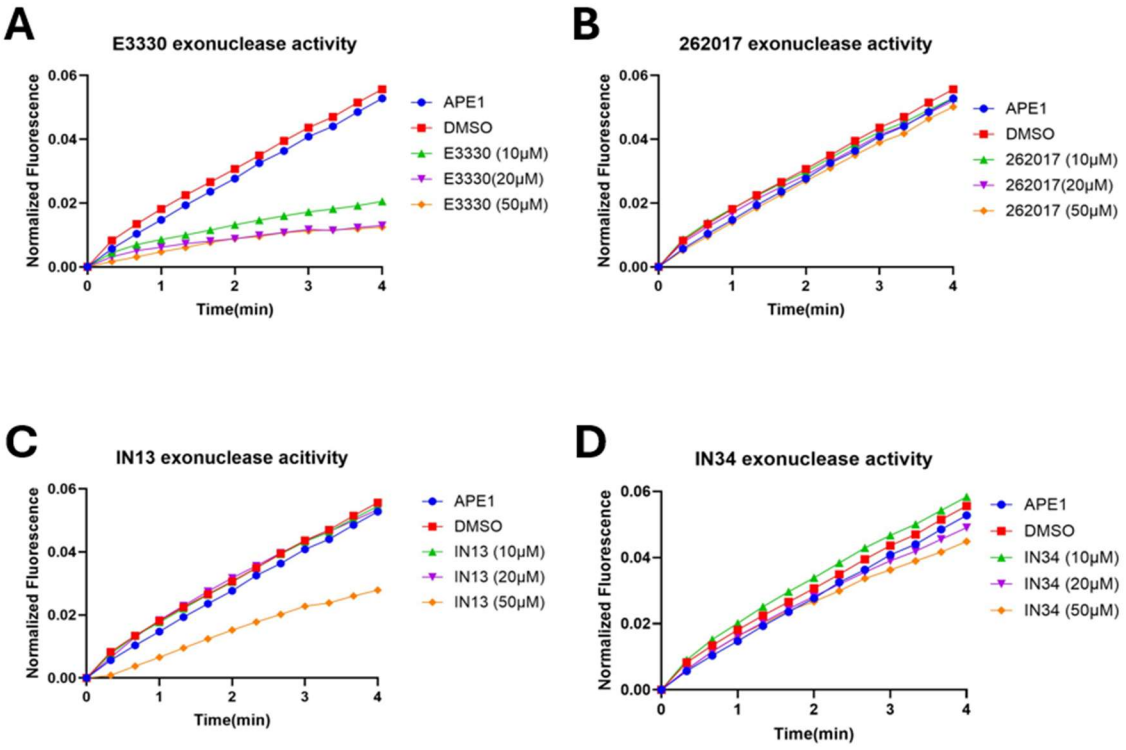

Figure 9: Effect of selected APE1 inhibitors on the exonuclease activity of APE1. (A)-(D): Normalized fluorescence over time in the DRMB assay (version 5) with RPE-1 wildtype cell lysate and CON2 beacon, preincubated 30 minutes at 4°C with 10μM, 20μM, or 50μM of **(A)** E3330, **(B)** compound 3 (262017), **(C)** Inhibitor 13, and **(D)** Inhibitor 34.

3.2 APE1 Inhibitors' Impact on MNNG-Induced PARP Activation

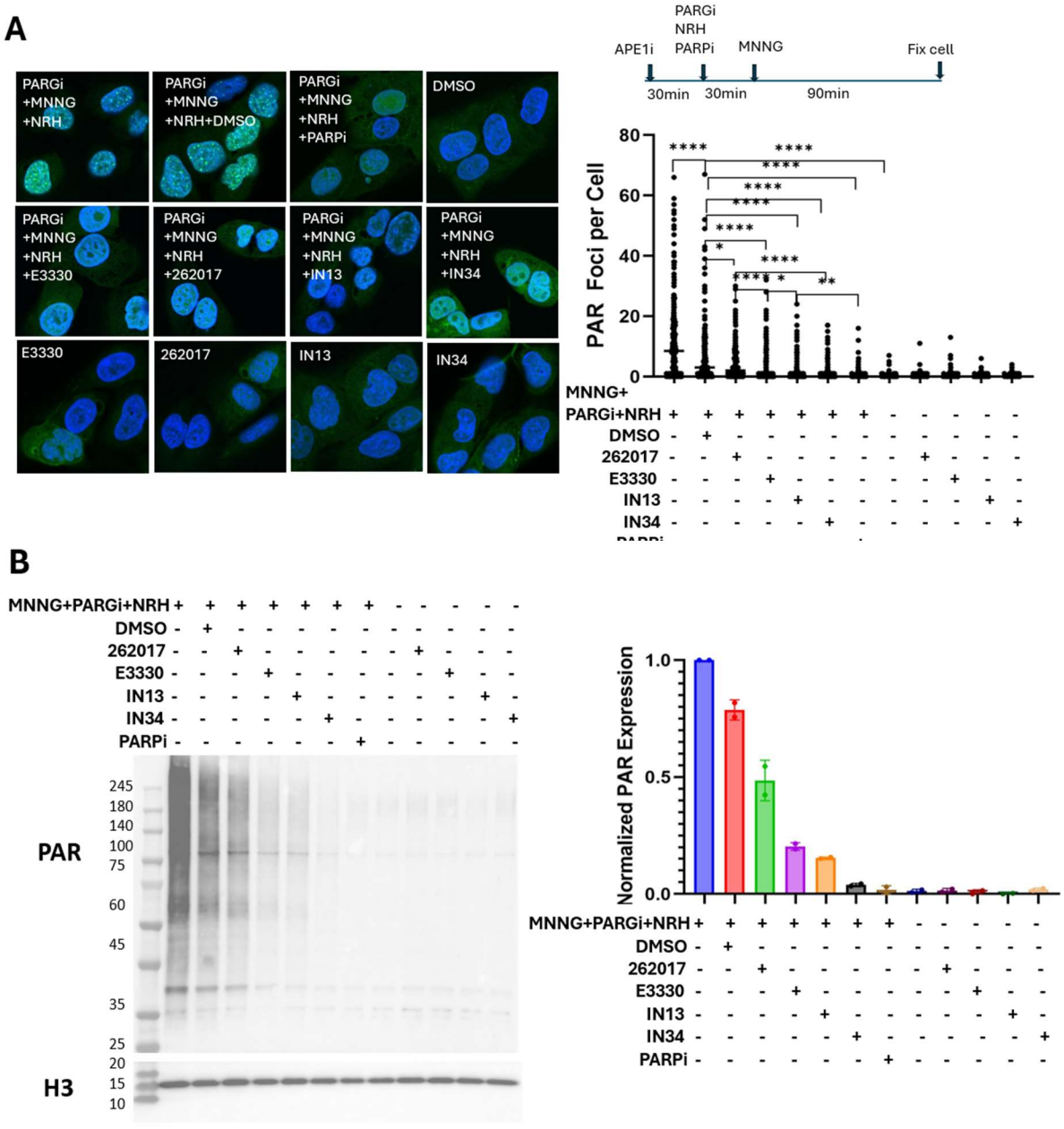

Figure 10: PAR expression in U2OS LivePAR cells treated with APE 1 inhibitors. (A) Number of PAR foci per U2OS LivePAR cell treated with 10µM MNNG, 10µM PARG inhibitor PDD00017273 (PARGi), 100µM NRH, with or without 20µM APE1 inhibitor candidates E3330, compound 3 (262017), XPTx-0506 (IN13), XPTx-0524 (IN34), or PARP inhibitor ABT-888 (PARPi) (**** denotes $p<0.0001$, ** denotes $p<0.01$, * denotes $p<0.05$, Kolmogorov-Smirnov test). **(B)** Validation of PAR expression via immunoblot by harvesting U2OS/LivePAR cell lysate just before fixation.

To study if APE1 inhibitors affect the downstream activation of PARP1/PARP2 in base excision repair, we evaluated the formation of poly(ADP-ribose) (PAR) foci via confocal microscopy using U2OS/LivePAR cells. In the LivePAR assay, cells were preincubated for 30 minutes with 20μM APE1 inhibitor candidates E3330, compound 3 (262017), XPTx-0506 (IN13), XPTx-0524 (IN34), or 0.2% DMSO control, before the addition of 100μM NRH and 10μM of the PARG inhibitor PDD00017273, with or without 10μM PARP inhibitor ABT-888 for another 30-minute incubation. After that, cells are incubated in 10μM MNNG for 90 minutes to induce DNA damage before being fixed for microscopic analysis or harvested for immunoblot analysis. According to the confocal microscopy analysis, there is a significant decrease in the number of PAR foci per cell in cells pretreated with 20μM of any of the 4 APE1 inhibitor candidates when compared to the DMSO control, with XPTx-0524 (IN34) showing the strongest inhibition of PAR foci formation (Figure 10A). We also validated PAR expression by immunoblot, and the immunoblot quantification data are consistent with the confocal microscope quantification of PAR foci (Figure 10B). Note that the reduction in PAR levels by APE1 inhibitors is only observed in the presence of MNNG, as none of the 4 APE1 inhibitor candidates tested alone change PAR levels without MNNG treatment (Figure 10A & 10B). This indicates that the inhibition of PARP1/PARP2 activation is due to disrupted DNA base excision repair by APE1 inhibitors following DNA damage, rather than the APE1 inhibitor candidates alone impacting PARP1/PARP2. Interestingly, the decrease of PAR foci is only observed when cells are preincubated with APE1 inhibitors before the addition of the PARG inhibitor + NRH, and no significant change is observed when APE1 inhibitors are added together (at the same time) with the PARG inhibitor + NRH. In conclusion, the results of our experiments using the LivePAR assay confirm the regulatory role of APE1 in the activation of PARP1/PARP2 following DNA damage (Ortega *et al*., 2025; Almeida & Sobol, 2007) and proves

that APE1 inhibitor candidates E3330, compound 3 (262017), XPTx-0506 (IN13), and XPTx-0524 (IN34) significantly inhibit the downstream PARP1/PARP2 activation in base excision repair.

3.3 APE1 Inhibitor Induction of DNA Double-Strand Breaks

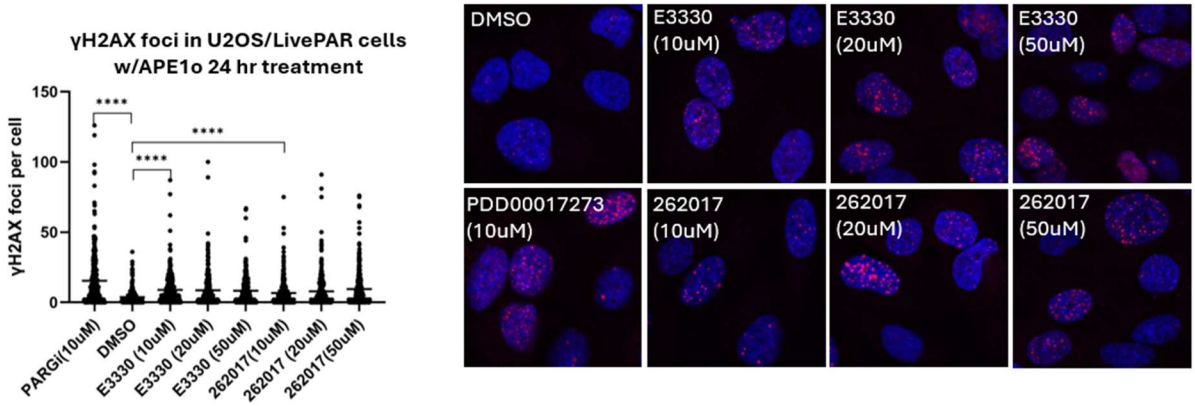

Figure 11: Confocal microscopy analysis of γ-H2AX foci in U2OS/LivePAR cells treated for 24 hrs with APE 1 inhibitors (n=3, **** denotes p<0.0001, Kolmogorov-Smirnov test).

Prior studies have shown mixed conclusions on the role of APE1 in the formation of DNA double-strand breaks (DSB). According to an earlier study, APE1 inhibition results in the accumulation of AP sites that can block replication fork progression and generate DNA single-strand breaks (SSBs) that eventually progress to toxic DSBs and G2/M cell cycle arrest (Sultana *et al*, 2012). A recent study also suggests that APE1 deficiency leads to DSB accumulation at a later phase following oxidative stress (after 24 hr) via increased ubiquitylation of Artemis, an important endonuclease in non-homologous end joining (NHEJ) and the repair of radiation-induced DSBs, resulting in more DSBs (Zhang *et al*, 2023). To study APE1 inhibitor effects on the formation of DNA double-strand breaks, we performed γ-H2AX immunostaining on U2OS/LivePAR cells following 24 hours of treatment with 10 μM, 20μM, or 50μM of APE1 inhibitors E3330 or compound 3 (262017). Our result shows a significantly increased number of γ-H2AX foci per cell

with 24-hour E3330 or compound 3 (262017) treatment starting at a 10μM dose (Figure 11). This confirms that APE1 inhibitors can increase DNA double-strand breaks, though the specific mechanism still needs to be determined.

3.4 APE1 Inhibitors' Effect on Cancer Cell Survival

3.4.1 Cytotoxicity of APE1 inhibitor Candidates

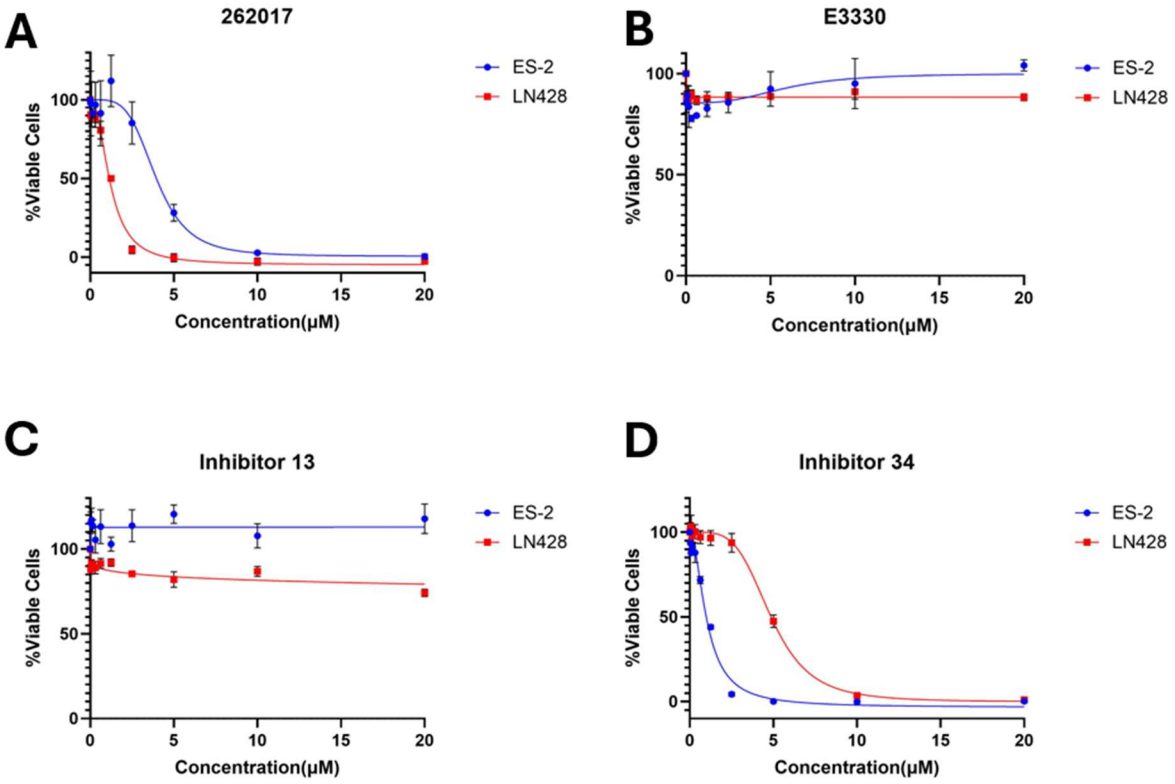

Figure 12: Cell killing curve (n=3) of APE1 inhibitor candidates: **(A)** compound 3 (262017), **(B)** E3330, **(C)** XPTx-0506 (IN13), and **(D)** XPTx-0524 (IN34) following 5-day incubation.

Cytotoxicity of 4 APE1 inhibitors [E3330, compound 3 (262017), XPTx-0506 (inhibitor 13), and XPTx-0524 (inhibitor 34)] was tested in the human ovarian clear cell carcinoma cell line ES-2 and the human glioblastoma-derived cell line LN428 by measuring % viable cells following 5-day treatment at 0-20μM compound. According to the cell killing curve data, compound 3 (262017),

and XPTx-0524 (inhibitor 34) show potent cell killing effects at doses below 5µM, indicating strong cytotoxicity (Figures 12A & 12D). The potent cell-killing effect of compound 3 (262017) shown in this experiment supports recent studies that suggest potential cytotoxicity and off-target effects with compound 3 (262017) (Xue & Dimple, 2022). However, E3330 and XPTx-0506 (inhibitor 13) do not show cytotoxic activity at doses up to 20µM (Figures 12B & 12C).

3.4.2 E3330 Synergy with DNA Alkylating Agent and DDR inhibitors

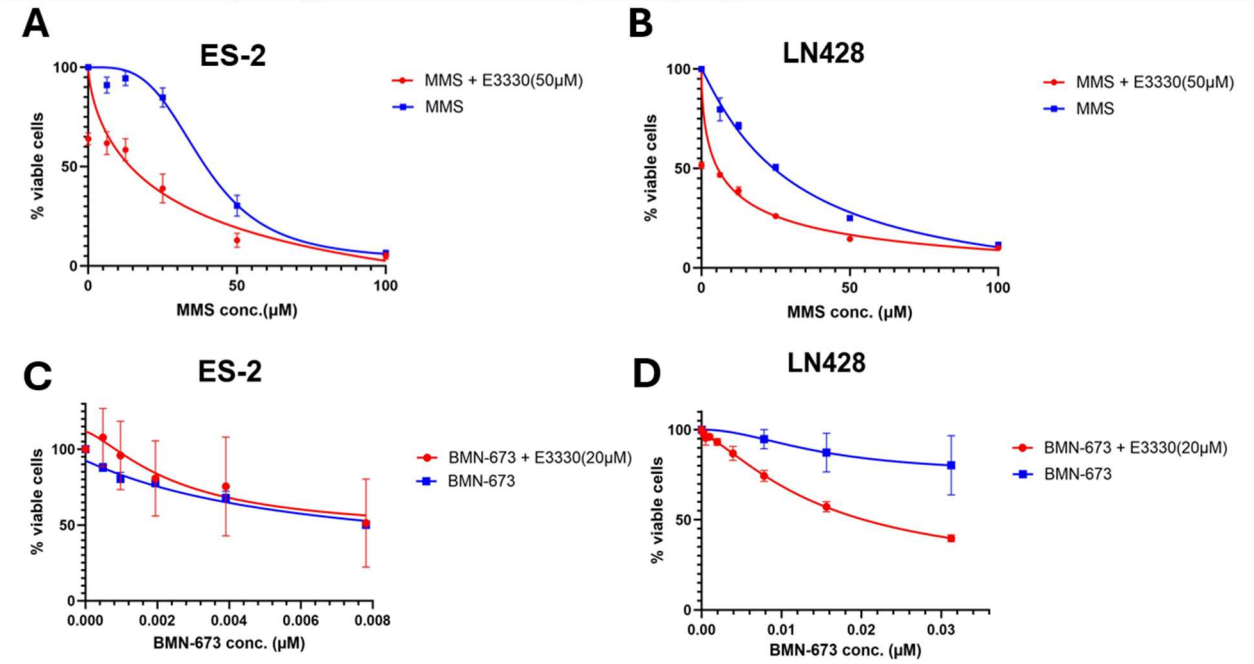

Figure 13: E3330 Synergy with DNA Alkylating Agent and DDR inhibitors. (A)-(B): Cell killing curve (n=3) of methyl methanesulfonate (MMS) with or without 50µM E3330 in **(A)** ES-2 cell line and **(B)** LN428 cell line. **(C)-(D):** Cell killing curve (n=3) of PARP inhibitor BMN-673 with or without 50µM E3330 in **(C)** ES-2 cell line and **(D)** LN428 cell line, data provided by Karhie Langham from the Sobol lab.

APE1 plays an important role in the development of drug resistance to chemotherapies using DNA alkylating agents, and inactive variants of the APE1 protein are shown to resensitize cancer cells to alkylating agents, including temozolomide (TMZ) (McNeill *et al*, 2009). Prior literature has also shown that the APE1 small molecule inhibitor Lucanthone enhances cancer cell killing by alkylating agents such as methyl methanesulfonate (MMS) and temozolomide (Luo & Kelley,

2004). Previous studies also showed that E3330 and its analogues can sensitize cancer cells to DNA alkylating agents through inhibition of the APE1 redox function but not via DNA repair pathways (Luo *et al*, 2005). Based on our testing of E3330 in the DRMB assay, in the LivePAR assay, and when evaluating γ-H2AX staining that confirmed its inhibition of AP endonuclease activity and downstream BER processing, we propose that E3330 will also enhance cancer cell killing by DNA alkylating agents through the disrupted DNA repair process following DNA damage. We analyzed the percent cell viability of ES-2 cells and LN428 cells after 5-day methyl methanesulfonate (MMS) treatment with or without 50μM E3330. Our result shows a significantly reduced cell viability in the presence of 50μM E3330 as compared to MMS only control in both the ES-2 and LN428 cell lines (Figure 13A & 13B). This suggests that E3330, at 50μM, can sensitize cancer cells to DNA alkylating agents, including MMS, potentially through inhibition of BER. Interestingly, our experiments only show a significant reduction in cell viability at 50μM E3330 but not at 20μM E3330 when coupled with MMS treatments, and E3330 alone is also found to kill 10-30% of ES-2 and LN428 cells at 50μM after a 5-day treatment. More synergistic analysis is needed to determine if the increased cancer cell killing is due to additional killing effects by E3330 or synergy with MMS.

Additionally, synergistic effects among DNA repair response (DDR) inhibitors remain our areas of interest for potential combination cancer therapies targeting the DNA repair process. To study if E3330 enhances cancer cell killing by other DDR inhibitors, our team performed cell survival assay experiments on various small molecule DDR inhibitors, including PARP inhibitors (BMN-673, ABT-888, AZD5305), the PARG inhibitor PDD00017273, the USP1 inhibitor ML323, and the ATR inhibitor AZD6738 in the presence or absence of 20μM E3330 using ES-2 and LN428 cells. No enhancement in cell killing is found in all the DDR inhibitor compounds tested when

combined with 20μM E3330, except the PARP1/PARP2 inhibitor BMN-673 (Talazoparib, MedChemExpress, cat# HY-16106). BMN-673 is a potent PARP inhibitor (PARP1 IC_{50} = 0.57 nM) that exhibits selective anti-tumor cytotoxicity (Shen *et al*, 2013) and has recently completed phase 3 clinical trials for BRCA-mutated breast and ovarian cancer (NCT01945775). Interestingly, in our experiments, E3330 significantly enhances cell killing of BMN-673 in the LN428 cell line but not in the ES-2 cell line (Figure 13C & 13D). The synergistic effect between E3330 and BMN-673 can potentially be explained with a synthetic lethal effect targeting DNA base excision repair (BER) by APE1 and single-strand break repair (SSBR) by PARP1/PARP2 where inhibition of the two parallel pathways lead to accumulation of toxic DNA damage, replication fork collapse, excessive DNA double-strand breaks (DSBs), which enhance cell death (Sultana *et al*, 2012).

3.4.3 E3330's Killing Effect in XRCC1 and BRCA1 deficient cells

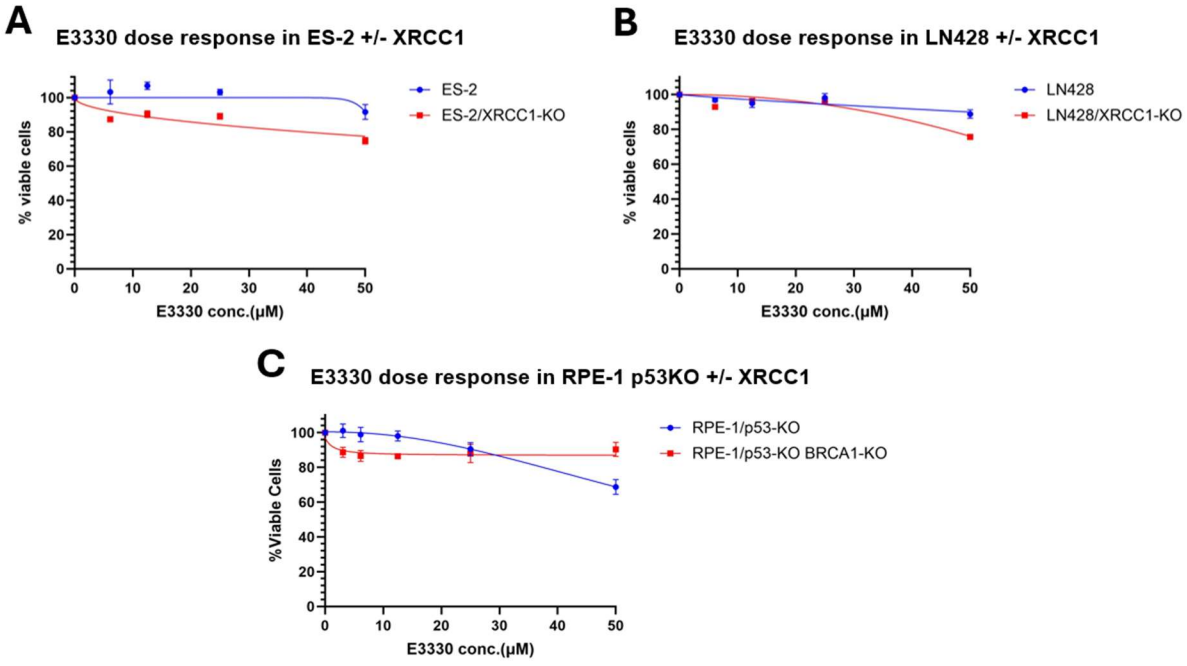

Figure 14: E3330's Killing Effect in XRCC1 and BRCA1-deficient cells. Cell killing curve (n=3) of E3330 in **(A)** ES-2 vs. ES-2 XRCC1 KO cell line, **(B)** LN428 vs. LN428 XRCC1 KO cell line, and **(C)** RPE-1 p53 KO vs. RPE-1 p53 KO BRCA1 KO cell line.

APE1 is suggested to compensate for DNA repair deficiency in X-ray repair cross-complementing protein 1 (XRCC1) or Breast Cancer Type 1 Susceptibility Protein (BRCA1) deficient cells (Sossou *et al*., 2005; Sultana *et al*., 2012). XRCC1 acts as a scaffold protein that facilitates repair of DNA single-strand breaks (SSBs) in the BER pathway. An earlier study found that overexpression of APE1 can compensate for the impaired capability of XRCC1-deficient cells to repair SSBs induced by oxidative DNA damage, both *in vivo* and in whole-cell extracts (Sossou *et al*., 2005). We hypothesize that cell viability will thereby decrease in XRCC1-deficient cells treated with APE1 inhibitors. In our cell survival assay, we found that cell viability following 5-day (6.25μM-50μM) E3330 treatment is reduced by 10-20% in our ES-2/XRCC1-KO cell line as compared to the ES-2 wildtype cell line (Figure 14A). Lower cell viability is observed in the LN428 cell line only at 50μM E3330 treatment (Figure 14B). This indicates that XRCC1-deficient cells are more sensitive to killing by the APE1 inhibitor E3330.

BRCA1 and BRCA2 play a crucial role in the repair of DNA double-strand breaks through homologous recombination (HR) (Prakash *et al*., 2015). Previous studies have also found synthetic lethality between APE1 inhibition and BRCA2 deficiency, where treatment with small molecule APE1 inhibitors resulted in reduced cell viability in BRCA2-deficient cells, though E3330 did not show enhanced cytotoxicity in BRCA2-deficient cells (Sultana *et al*, 2012). The specific mechanisms behind this synthetic lethality still remain unclear, but it is likely due to the accumulation of toxic DNA DSBs and AP sites in the absence of both BER by APE1 inhibition and homologous recombination repair by BRCA2. To find out if E3330, now identified as an APE1 DNA repair function inhibitor, plays a role in the synthetic lethality between APE1 and BRCA1 deficiency, we measured the cell survival rate in RPE-1/p53-KO and RPE-1/p53-KO/BRCA1-KO

cells following 5-day incubation with E3330 at various concentrations. We find slightly decreased cell viability in RPE-1/p53-KO/BRCA1-KO cells compared to the RPE-1/p53-KO control cell line at E3330 treatment concentrations below 20μM (Figure 14C). However, E3330 treatment at 50μM shows higher cell viability in the RPE-1/p53-KO/BRCA1-KO cells as compared to the RPE-1/p53-KO control cell line. Therefore, no clear conclusions can be drawn regarding whether BRCA1-deficient cells are more sensitive to killing by E3330 based on our experiments. This is likely due to an insufficient cell growth period in our experiment for the RPE-1/p53-KO/BRCA1-KO cells to show significant effects, or the p53 knockout alleviating the sensitivity of BRCA1-deficient cells to APE1 inhibitors (Peng *et al,* 2025).

Chapter 4. Discussion and Future Perspectives

4.1 Discussion

APE1 is a central regulator of genomic stability through its essential role in base excision repair (BER) and a key modulator of transcriptional programs via its redox (Ref-1) function. Its overexpression in multiple malignancies and association with therapeutic resistance have made APE1 an attractive target for anticancer drug development (Tell *et al.*, 2009; Kelley *et al*., 2014). While numerous small-molecule inhibitors of APE1 have been reported, a persistent challenge in the field has been the incomplete characterization and understanding of their mechanisms of action—particularly whether they target the enzyme's DNA repair activity, redox function, or both. In this study, we systematically evaluated several commercially available APE1 inhibitors using a newly developed DNA Repair Molecular Beacon (DRMB) assay and identified an unexpected role for E3330 as a potent inhibitor of the endonuclease activity of APE1. These findings provide new mechanistic insights into APE1 inhibition and highlight the complexity of targeting multifunctional DNA repair proteins.

4.1.1 E3330 as a Dual-Function APE1 Inhibitor

E3330 (APX3330) has been extensively characterized in prior literature as a selective inhibitor of APE1's redox activity with minimal effects on its endonuclease function (Fishel *et al*., 2010; Shah *et al*., 2017). In contrast, our results demonstrate that E3330 potently inhibits APE1 AP endonuclease activity, with an IC_{50} of approximately 3.1 μM in our DRMB assay with purified protein, and robust inhibition is also observed in cell lysates at 20–50 μM. This finding challenges

the prevailing view that E3330 acts exclusively through redox inhibition and suggests a previously underappreciated dual mechanism of action.

Several factors may explain this discrepancy with prior reports. First, differences in assay design are likely critical. Traditional biochemical assays often rely on gel-based or endpoint measurements of AP site cleavage, whereas the DRMB assay provides a real-time, fluorescence-based readout of DNA repair activity that may be more sensitive to partial inhibition or transient enzyme–substrate interactions. Second, previous studies may have used conditions that favored detection of redox effects over catalytic inhibition, such as transcription factor binding assays or cellular reporter systems. Third, structural studies have suggested that E3330 may bind APE1 in a manner that induces conformational changes affecting multiple functional domains, rather than exclusively targeting the redox-active cysteine residues (Georgiadis *et al*., 2008; Kelley *et al.*, 2014). Thus, it is possible that E3330 can interfere with the endonuclease activity of APE1 indirectly through allosteric modulation.

Importantly, our observation that E3330 also inhibits APE1 exonuclease activity in a dose-dependent manner further supports a broader impact on the enzyme's catalytic functions. Together, these findings position E3330 as a dual-function inhibitor that can simultaneously disrupt both DNA repair and redox signaling pathways. This dual activity may enhance its therapeutic potential, as it can target multiple cancer-promoting mechanisms mediated by APE1 and its impact on DNA repair process.

4.1.2 Limited Endonuclease Inhibition by Other APE1 Inhibitors

In contrast to E3330, other tested inhibitors, including lucanthone, CRT0044876, and APX2009, did not exhibit detectable inhibition of APE1 endonuclease activity in our assays, despite prior

reports suggesting such effects (Barzilay *et al*., 1995; Madhusudan *et al*., 2005). Compound 3 (cat# 262017) showed only weak to moderate inhibition, while two novel candidates (XPTx-0506 and XPTx-0524) demonstrated moderate activity comparable to Compound 3.

These discrepancies may again reflect differences in assay sensitivity, enzyme source (purified protein versus cell lysate), or compound stability and bioavailability. Notably, both Compound 3 and E3330 exhibited stronger inhibition when tested using purified APE1 as compared to cell lysates, which suggests that cellular factors such as protein binding partners, post-translational modifications, or intracellular metabolism may attenuate inhibitor activity in more complex biological contexts. For example, APE1 is known to interact with multiple BER proteins, including XRCC1 and PARP1, which may influence its accessibility or conformation (Caldecott, 2008). These interactions could reduce the effective binding of inhibitors to the active site.

The lack of endonuclease inhibition by APX2009 is consistent with its design as a second-generation redox inhibitor, reinforcing the notion that redox-targeting compounds may not directly impair BER. However, the failure to detect inhibition by CRT0044876 and lucanthone—both widely cited as APE1 repair inhibitors—raises important questions about their specificity and mechanism of action. It is possible that their reported effects on DNA repair arise from indirect mechanisms or off-target activities, underscoring the need for more rigorous characterization of APE1 inhibitors.

4.1.3 APE1 Inhibition Promotes DNA DSBs and Impairs PARylation

A key functional consequence of APE1 inhibition observed in this study is the increase of DNA double-strand breaks (DSBs), which is indicated by the increased γ-H2AX foci following treatment with E3330 or Compound 3. This is consistent with the role of APE1 in processing AP

sites and preventing their conversion into strand breaks during replication or transcription (Wallace, 2014). The increase in DSBs may arise through several mechanisms. First, unrepaired AP sites can block DNA polymerases, leading to replication fork stalling and collapse (Hoitsma *et al*., 2023), which in turn generates DSBs. Second, accumulation of single-strand breaks (SSBs) due to impaired BER can be converted into DSBs during S phase (Kuzminov, 2001; Sultana *et al*, 2012). Third, inhibition of APE1 may disrupt coordination between BER and other repair pathways related to DSBs such as non-homologous end joining (NHEJ), exacerbating genomic instability (Zhang *et al*., 2023).

In parallel, our LivePAR assay revealed a significant reduction in PARylation following treatment with APE1 inhibitors, indicating impaired activation of PARP1 during BER. PARP1 is rapidly activated in response to base damage and SSBs and facilitates recruitment of repair proteins through poly(ADP-ribose) (PAR) chain formation (Ray Chaudhuri & Nussenzweig, 2017). The observed decrease in PAR foci suggests that APE1 activity is required for efficient PARP activation, likely by generating the DNA intermediates that serve as PARP1 substrates (Almeida & Sobol., 2007). Alternatively, inhibition of APE1 may disrupt protein–protein interactions within the BER complex that are necessary for PARP1 recruitment or activation.

These findings highlight the central role of APE1 in coordinating BER and suggest that its inhibition not only blocks repair but also perturbs downstream signaling events, including PARylation. This disruption of the DNA damage response may contribute to the cytotoxic effects of APE1 inhibitors and their ability to sensitize cancer cells to genotoxic stress.

4.1.4 Synthetic Lethality and Therapeutic Implications

The concept of synthetic lethality—where simultaneous disruption of two pathways leads to cell death while inhibition of either alone is tolerated—has become a cornerstone of targeted cancer therapy (Lord & Ashworth, 2017). Our findings provide several lines of evidence supporting the potential for synthetic lethal interactions involving APE1 inhibition.

First, E3330 significantly enhances the cytotoxicity of the DNA alkylating agent MMS, consistent with previous studies showing that BER inhibition sensitizes cells to alkylating damage (Madhusudan *et al*., 2005). Alkylating agents generate lesions that are primarily repaired by BER, and inhibition of APE1 prevents the completion of this pathway, leading to accumulation of toxic intermediates.

Second, we observed selective enhancement of cytotoxicity when E3330 was combined with the PARP inhibitor BMN-673 (talazoparib), but not with other DNA damage response (DDR) inhibitors. This finding is particularly intriguing, as PARP inhibitors are known to trap PARP1 on DNA and induce replication stress, especially in cells with defective homologous recombination (Murai *et al*., 2012). The combination of APE1 inhibition and PARP trapping may synergistically increase DNA damage by both preventing repair and stabilizing toxic DNA–protein complexes. The lack of synergy with other PARP inhibitors or DDR agents may reflect differences in potency, trapping efficiency, or cellular context.

Third, increased sensitivity to E3330 in XRCC1-deficient cells further supports a synthetic lethal interaction. XRCC1 is a scaffold protein that coordinates BER by interacting with APE1, PARP1, and DNA ligase III (Caldecott, 2008). Loss of XRCC1 compromises BER efficiency, making cells more reliant on residual repair capacity. In this context, inhibition of APE1 further reduces repair

capacity, leading to increased cell death. This finding is consistent with previous reports linking XRCC1 deficiency to hypersensitivity to DNA damage and BER inhibitors (Sossou *et al*., 2005).

In contrast, our results with BRCA1-deficient cells were inconclusive, likely due to experimental limitations such as insufficient growth time or compensatory effects of p53 loss. Nevertheless, given the established role of BRCA1 in homologous recombination (Prakash *et al.*, 2015), it is plausible that APE1 inhibition could also exhibit synthetic lethality in HR-deficient contexts, particularly in combination with agents that induce replication stress.

4.2 Future Perspectives

4.2.1 Future Experiments

The findings of this study open several avenues for future research. First, the identification of E3330 as a dual-function inhibitor underscores the need for detailed structural and biochemical studies to elucidate its binding mode. High-resolution crystallography or cryo-EM studies of APE1 in complex with E3330 could reveal whether the compound binds directly to the active site, an allosteric site, or induces conformational changes that affect multiple functional domains. Such insights would inform rational design of next-generation inhibitors with improved specificity and potency.

Second, the DRMB assay demonstrated robust performance in detecting APE1 activity and inhibitor effects, highlighting its potential for high-throughput screening. Expanding this approach to screen large chemical libraries could identify novel inhibitors with diverse chemical scaffolds. To do this, our lab is currently screening a 37-million compound multiplex scaffold library kindly

provided by Dr. Adel Nefzi from Florida International University. Through the first round of screening using the version 5 DRMB assay with purified APE1 protein, we have observed some potential sub-scaffold libraries of interest. Using this approach, we can potentially narrow down specific molecular structures of potential APE1 inhibitors following multiple rounds of screenings. Hits from such screens could be further optimized through medicinal chemistry to enhance selectivity, cellular activity, and pharmacokinetic properties.

Third, further investigation of the cellular consequences of APE1 inhibition is warranted. For example, genome-wide analyses of DNA damage, replication stress, and transcriptional changes could provide a more comprehensive understanding of how APE1 inhibitors affect cellular physiology. Additionally, studies in more physiologically relevant models, such as patient-derived xenografts or organoids, would be important to evaluate therapeutic potential.

4.2.2 Significance of Study

This study provides important insights into the mechanisms of APE1 inhibition and its potential as a therapeutic target in cancer. The discovery that E3330 inhibits both redox and endonuclease activities challenges the traditional view of APE1 inhibitors as functionally selective and highlights the need to consider the full spectrum of APE1 functions when evaluating inhibitor effects. The use of the DRMB assay enabled sensitive detection of APE1 activity and revealed discrepancies with prior literature, emphasizing the importance of assay selection in mechanistic studies.

From a therapeutic perspective, APE1 inhibitors represent a promising strategy to enhance the efficacy of DNA-damaging agents and exploit vulnerabilities in DNA repair pathways. The observed interactions with alkylating agents, PARP inhibitors, and BER deficiencies suggest

multiple areas of interest for combination therapy. However, the development of clinically effective APE1 inhibitors will require careful balancing of potency, specificity, and toxicity, given the essential role of APE1 in normal cells.

4.2.3 Conclusions

Our findings advance the current understanding of APE1 biology and its pharmacological inhibition by identifying E3330 as a dual-function inhibitor with previously unrecognized effects on APE1-mediated DNA repair. In addition, we establish the DNA Repair Molecular Beacon (DRMB) assay as a sensitive and robust platform for the discovery and mechanistic characterization of APE1 inhibitors. Functionally, we demonstrate that APE1 inhibition suppresses the base excision repair (BER) pathway, as evidenced by reduced PARP activation and increased accumulation of DNA double-strand breaks. Furthermore, the enhanced cytotoxic effects observed with combined treatment of E3330 and the alkylating agent MMS, as well as with the PARP inhibitor BMN-673 and XRCC1 deficiency, support a potential synthetic lethal interaction between APE1 inhibition and disruptions in complementary DNA damage response pathways. Collectively, these results highlight the therapeutic potential of targeting APE1, particularly in combination with DNA-damaging agents or DNA repair inhibitors. Continued characterization and discovery of APE1 small molecule inhibitors and their integration into rational combination strategies may provide a promising avenue for improving cancer treatment outcomes.

References:

Al-Attar, A., Gossage, L., Fareed, K. *et al.* Human apurinic/apyrimidinic endonuclease (APE1) is a prognostic factor in ovarian, gastro-oesophageal and pancreatico-biliary cancers. *Br J Cancer* **102**, 704–709 (2010). https://doi.org/10.1038/sj.bjc.6605541

Al-Rahahleh RQ, Roos WP, Saville KM, *et al*. (2025). Overexpression of the WWE domain of RNF146 modulates poly-(ADP)-ribose dynamics at sites of DNA damage. *DNA Repair* (Amst). 2025;150:103845. doi:10.1016/j.dnarep.2025.103845

Almeida, K. H., Andrews, M. E., & Sobol, R. W. (2024). AP endonuclease 1: Biological updates and advances in activity analysis. *Methods in enzymology*, 705, 347–376. https://doi.org/10.1016/bs.mie.2024.07.011

Almeida, K. H., & Sobol, R. W. (2007). A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. *DNA repair*, 6(6), 695–711. https://doi.org/10.1016/j.dnarep.2007.01.009

Barzilay, G., Mol, C. D., Robson, C. N., Walker, L. J., Cunningham, R. P., Tainer, J. A., & Hickson, I. D. (1995). Identification of critical active-site residues in the multifunctional human DNA repair enzyme HAP1 (APE1). *Nature Structural Biology, 2*(7), 561–568. https://doi.org/10.1038/nsb0795-561

Caldecott, K. W. (2008). Single-strand break repair and genetic disease. *Nature Reviews Genetics, 9*(8), 619–631. https://doi.org/10.1038/nrg2380

Carew, Jennifer S. *et al*.(2011). Lucanthone Is a Novel Inhibitor of Autophagy That Induces Cathepsin D-mediated Apoptosis. *Journal of Biological Chemistry,* Volume 286, Issue 8, 6602 - 6613

Chini CCS, Peclat TR, Gomez LS, *et al.* (2022). Dihydronicotinamide Riboside Is a Potent NAD+ Precursor Promoting a Pro-Inflammatory Phenotype in Macrophages. *Front Immunol*. 2022;13:840246. Published 2022 Feb 25. doi:10.3389/fimmu.2022.840246

Codrich, M. *et al*.(2019). Inhibition of APE1-endonuclease activity affects cell metabolism in colon cancer cells via a p53-dependent pathway, *DNA Repair,* Volume 82, 2019, 102675, ISSN 1568-7864, https://doi.org/10.1016/j.dnarep.2019.102675.

Fishel, M. L., Kelley, M. R., & Ivanov, A. V. (2010). APE1/Ref-1 redox function and its

inhibition in cancer therapeutics. *Antioxidants & Redox Signaling, 12*(3), 301–315. https://doi.org/10.1089/ars.2009.2823

Gampala, S., Moon, H. R., Wireman, R., *et al*. (2024). New Ref-1/APE1 targeted inhibitors demonstrating improved potency for clinical applications in multiple cancer types. *Pharmacological research*, 201, 107092. https://doi.org/10.1016/j.phrs.2024.107092

Georgiadis, M. M., Luo, M., Gaur, R. K., Delaplane, S., Li, X., & Kelley, M. R. (2008).

Evolution of the redox function in mammalian apurinic/apyrimidinic endonuclease. *Biochemistry, 47*(45), 12099–12110. https://doi.org/10.1021/bi801432y

Hickson, I.D., Gorman, M.A., Freemont, P.S. (2001). Structure and Functions of the Major

Human AP Endonuclease HAP1/Ref-1. In: Nickoloff, J.A., Hoekstra, M.F. (eds) DNA Damage and Repair. *Contemporary Cancer Research.* Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-59259-095-7_4

Hoitsma NM, Norris J, Khoang TH, *et al*.(2023). Mechanistic insight into AP-endonuclease 1

cleavage of abasic sites at stalled replication fork mimics. *Nucleic Acids Res*. 2023;51(13):6738-6753. doi:10.1093/nar/gkad481

Li, J., Zhao,H., McMahon, A., Yan, S. (2022). APE1 assembles biomolecular condensates to promote the ATR–Chk1 DNA damage response in nucleolus, *Nucleic Acids Research*, Volume 50, Issue 18, 14 October 2022, Pages 10503–10525, https://doi.org/10.1093/nar/gkac853

Khan MM, Roos WP, Pearson CR, *et al*. (2025). DNA polymerase beta expression in head & neck cancer modulates the poly(ADP-ribose)-mediated replication checkpoint. *DNA Repair* (Amst). 150:103853. doi:10.1016/j.dnarep.2025.103853

Koczor, C. A., Haider, A. J., Saville, K. M., Li, J., Andrews, J. F., Beiser, A. V., & Sobol, R. W. (2022). Live Cell Detection of Poly(ADP-Ribose) for Use in Genetic and Genotoxic Compound Screens. *Cancers,* 14(15), 3676. https://doi.org/10.3390/cancers14153676

Koczor, C. A., Saville, K. M., Andrews, J. F., Clark, J., Fang, Q., Li, J., Al-Rahahleh, R. Q., Ibrahim, M., McClellan, S., Makarov, M. V., Migaud, M. E., & Sobol, R. W. (2021). Temporal dynamics of base excision/single-strand break repair protein complex assembly/disassembly are modulated by the PARP/NAD+/SIRT6 axis. *Cell reports*, 37(5), 109917. https://doi.org/10.1016/j.celrep.2021.109917

Kelley, M. R., Logsdon, D., & Fishel, M. L. (2014). Targeting DNA repair pathways for cancer

treatment: What's new? *Future Oncology, 10*(7), 1215–1237. https://doi.org/10.2217/fon.13.245

Krumm, A., Barckhausen, C., Kücük, P., Tomaszowski, K. H., Loquai, C., Fahrer, J., Krämer, O.

H., Kaina, B., & Roos, W. P. (2016). Enhanced Histone Deacetylase Activity in Malignant Melanoma Provokes RAD51 and FANCD2-Triggered Drug Resistance. *Cancer research*, 76(10), 3067–3077. https://doi.org/10.1158/0008-5472.CAN-15-2680

Kuzminov A. Single-strand interruptions in replicating chromosomes cause double-strand breaks.(2001). *Proc Natl Acad Sci* U S A. 2001;98(15):8241-8246. doi:10.1073/pnas.131009198

Li, J., Svilar, D., McClellan, S., Kim, J., Ahn, E. E., Vens, C., Wilson, D. M., & Sobol, R. W. (2018). DNA Repair Molecular Beacon assay: a platform for real-time functional analysis of cellular DNA repair capacity. *Oncotarget*, 9(60), 31719–31743. https://doi.org/10.18632/oncotarget.25859

Li, M., Yang, X., Lu, X., Dai, N., Zhang, S., Cheng, Y., Zhang, L., Yang, Y., Liu, Y., Yang, Z., Wang, D., & Wilson, D. M., 3rd (2018). APE1 deficiency promotes cellular senescence and premature aging features. *Nucleic acids research*, 46(11), 5664–5677. https://doi.org/10.1093/nar/gky326

Liu, TC., Lin, CT., Chang, KC. *et al.* APE1 distinguishes DNA substrates in exonucleolytic cleavage by induced space-filling. *Nat Commun* **12**, 601 (2021). https://doi.org/10.1038/s41467-020-20853-2

Long, K., Gu, L., Li, L. *et al*.(2021).Small-molecule inhibition of APE1 induces apoptosis, pyroptosis, and necroptosis in non-small cell lung cancer. *Cell Death Dis* **12**, 503 (2021). https://doi.org/10.1038/s41419-021-03804-7

Lord, C. J., & Ashworth, A. (2017). PARP inhibitors: Synthetic lethality in the clinic. *Science, 355*(6330), 1152–1158. https://doi.org/10.1126/science.aam7344

Luo, M., & Kelley, M. R. (2004). Inhibition of the human apurinic/apyrimidinic endonuclease (APE1) repair activity and sensitization of breast cancer cells to DNA alkylating agents with lucanthone. *Anticancer research*, *24*(4), 2127–2134.

Luo, M., Nyland, R.L., Richard L.F. *et al*.(2005). Specific inhibition of the redox function of the dual DNA repair/redox protein Ape1 using small molecules results in tumor cell killing. *Cancer Res* 1 May 2005; 65 (9_Supplement): 1180.

Madhusudan, S., Smart, F., Shrimpton, P., Parsons, J. L., Gardiner, L., Houlbrook, S., Talbot, D. C., Hammonds, T., Freemont, P. A., Sternberg, M. J., Dianov, G. L., & Hickson, I. D. (2005). Isolation of a small molecule inhibitor of DNA base excision repair. *Nucleic Acids Research, 33*(15), 4711–4724. https://doi.org/10.1093/nar/gki781

Malfatti, M. C., Bellina, A., Antoniali, G., & Tell, G. (2023). Revisiting Two Decades of

Research Focused on Targeting APE1 for Cancer Therapy: The Pros and Cons. *Cells*, *12*(14), 1895. https://doi.org/10.3390/cells12141895

Manguinhas, R., Fernandes, A. S., Costa, J. G., Saraiva, N., Camões, S. P., Gil, N., Rosell, R., Castro, M., Miranda, J. P., & Oliveira, N. G. (2020). Impact of the APE1 Redox Function Inhibitor E3330 in Non-small Cell Lung Cancer Cells Exposed to Cisplatin: Increased Cytotoxicity and Impairment of Cell Migration and Invasion. *Antioxidants* (Basel, Switzerland), 9(6), 550. https://doi.org/10.3390/antiox9060550

Manvilla, B. A., Wauchope, O., Seley-Radtke, K. L., & Drohat, A. C. (2011). NMR studies reveal an unexpected binding site for a redox inhibitor of AP endonuclease 1. *Biochemistry,* 50(48), 10540–10549. https://doi.org/10.1021/bi201071g

McNeill, D. R., Lam, W., DeWeese, T. L., Cheng, Y. C., & Wilson, D. M., 3rd (2009). Impairment of APE1 function enhances cellular sensitivity to clinically relevant alkylators and antimetabolites. Molecular cancer research : MCR, 7(6), 897–906. https://doi.org/10.1158/1541-7786.MCR-08-0519

Murai, J., Huang, S. Y. N., Das, B. B., Renaud, A., Zhang, Y., Doroshow, J. H., Ji, J., Takeda,

S., & Pommier, Y. (2012). Trapping of PARP1 and PARP2 by clinical PARP inhibitors. *Cancer Research, 72*(21), 5588–5599. https://doi.org/10.1158/0008-5472.CAN-12-2753

Naidu, M. D., Agarwal, R., Pena, L. A., Cunha, L., Mezei, M., Shen, M., Wilson, D. M., 3rd, Liu, Y., Sanchez, Z., Chaudhary, P., Wilson, S. H., & Waring, M. J. (2011). Lucanthone and its derivative hycanthone inhibit apurinic endonuclease-1 (APE1) by direct protein binding. *PloS one*, *6*(9), e23679. https://doi.org/10.1371/journal.pone.0023679

Oliveira, T. T., Coutinho, L. G., de Oliveira, L. O. A., Timoteo, A. R. S., Farias, G. C., & Agnez-Lima, L. F. (2022). APE1/Ref-1 Role in Inflammation and Immune Response. *Frontiers in immunology*, 13, 793096. https://doi.org/10.3389/fimmu.2022.793096

Ortega, P. *et al.* ,Mechanism of DNA replication fork breakage and PARP1 hyperactivation during replication catastrophe.*Sci. Adv*.11,eadu0437(2025).DOI:10.1126/sciadv.adu0437

Peng M, Lee S, Nair HG, MacGilvary N, Cong K, Kraemer M, Li R, McConnell J, Baer C, Deng B, Zhu L, Cantor SB. (2025).RAD51 is chromatin enriched and targetable in BRCA1-deficient cells. *Mol Cell*. 2025 Sep 18;85(18):3373-3387.e6. doi: 10.1016/j.molcel.2025.08.017. Epub 2025 Sep 10. PMID: 40934926; PMCID: PMC12502998.

Prakash R, Zhang Y, Feng W, Jasin M.(2015). Homologous recombination and human health:

the roles of BRCA1, BRCA2, and associated proteins. Cold Spring Harb *Perspect Biol*. 7(4):a016600. Published 2015 Apr 1. doi:10.1101/cshperspect.a016600

Pramanik S. *et al*. (2022). The human AP-endonuclease 1 (APE1) is a DNA G-quadruplex

structure binding protein and regulates *KRAS* expression in pancreatic ductal adenocarcinoma cells, *Nucleic Acids Research*, Volume 50, Issue 6, 8 April 2022, Pages 3394–3412, https://doi.org/10.1093/nar/gkac172

Ray Chaudhuri, A., & Nussenzweig, A. (2017). The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. *Nature Reviews Molecular Cell Biology, 18*(10), 610–621. https://doi.org/10.1038/nrm.2017.53

Shah, F., Logsdon, D., Messmann, R.A. *et al.*(2017). Exploiting the Ref-1-APE1 node in cancer signaling and other diseases: from bench to clinic. *npj Precision Onc* **1**, 19 (2017). https://doi.org/10.1038/s41698-017-0023-0

Shen, Y., Rehman, F. L., Feng, Y., Boshuizen, J., Bajrami, I., Elliott, R., Wang, B., Lord, C. J., Post, L. E., & Ashworth, A. (2013). BMN 673, a novel and highly potent PARP1/2 inhibitor for the treatment of human cancers with DNA repair deficiency. Clinical cancer research : an official journal of the American Association for Cancer Research, 19(18), 5003–5015. https://doi.org/10.1158/1078-0432.CCR-13-1391

Siqueira, P., Rodrigues, M., Amorim, , Rodrigues, J., Oliveira, M., Fonseca, A., Pires, B., & Mencalha, A. (2024). The inhibitor of the redox activity of APE1/REF-1, APX2009, reduces the malignant phenotype of breast cancer cells. *Braz. J. Med. Biol. Res*., 57, e13250.

Sossou, M., Flohr-Beckhaus, C., Schulz, I., Daboussi, F., Epe, B., & Radicella, J. P. (2005). APE1 overexpression in XRCC1-deficient cells complements the defective repair of oxidative single strand breaks but increases genomic instability. *Nucleic acids research,* 33(1), 298–306. https://doi.org/10.1093/nar/gki173

Sultana, R., McNeill, D. R., Abbotts, R., Mohammed, M. Z., Zdzienicka, M. Z., Qutob, H., Seedhouse, C., Laughton, C. A., Fischer, P. M., Patel, P. M., Wilson, D. M., 3rd, & Madhusudan, S. (2012). Synthetic lethal targeting of DNA double-strand break repair deficient cells by human apurinic/apyrimidinic endonuclease inhibitors. *International journal of cancer,* 131(10), 2433–2444. https://doi.org/10.1002/ijc.27512

Svilar D, Vens C, Sobol RW (2012). Quantitative, real-time analysis of base excision repair activity in cell lysates utilizing lesion-specific molecular beacons. *J Vis Exp*. (66):e4168. Published 2012 Aug 6. doi:10.3791/4168

Tell, G., Quadrifoglio, F., Tiribelli, C., & Kelley, M. R. (2009). The many functions of APE1/Ref-1: Not only a DNA repair enzyme. *Antioxidants & Redox Signaling, 11*(3), 601–620. https://doi.org/10.1089/ars.2008.2194

Thakur, S., Sarkar, B., Cholia, R. *et al.*(2014). APE1/Ref-1 as an emerging therapeutic target for

various human diseases: phytochemical modulation of its functions. *Exp Mol Med* **46**, e106. https://doi.org/10.1038/emm.2014.42

Wallace, S. S. (2014). Base excision repair: A critical player in many games. *DNA Repair, 19*, 14–26. https://doi.org/10.1016/j.dnarep.2014.03.030

Xanthoudakis S, Curran T.(1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J.* 1992;11(2):653-665. doi:10.1002/j.1460-2075.1992.tb05097.x

Xanthoudakis S, Smeyne RJ, Wallace JD, Curran T.(1996). The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proceedings of the National Academy of Science. 1996;93:8919–8923. doi: 10.1073/pnas.93.17.8919

Xue, Z., & Demple, B. (2022). Knockout and Inhibition of Ape1: Roles of Ape1 in Base Excision DNA Repair and Modulation of Gene Expression. *Antioxidants* (Basel, Switzerland), 11(9), 1817. https://doi.org/10.3390/antiox11091817

Yuan, H. H., Yin, H., Marincas, M., Xie, L. L., Bu, L. L., Guo, M. H., & Zheng, X. L. (2025). From DNA Repair to Redox Signaling: The Multifaceted Role of APEX1 (Apurinic/Apyrimidinic Endonuclease 1) in Cardiovascular Health and Disease. *International journal of molecular sciences,* 26(7), 3034. https://doi.org/10.3390/ijms26073034

Zhang, J., Luo, M., Marasco, D., Logsdon, D., LaFavers, K. A., Chen, Q., Reed, A., Kelley, M. R., Gross, M. L., & Georgiadis, M. M. (2013). Inhibition of apurinic/apyrimidinic endonuclease I's redox activity revisited. *Biochemistry,* 52(17), 2955–2966. https://doi.org/10.1021/bi400179m

Zhang, Q., Yang, L., Gao, H., Kuang, X., Xiao, H., Yang, C., Cheng, Y., Zhang, L., Guo, X., Zhong, Y., & Li, M. (2023). APE1 promotes non-homologous end joining by initiating DNA double-strand break formation and decreasing ubiquitination of artemis following oxidative genotoxic stress. *Journal of translational medicine,* 21(1), 183. https://doi.org/10.1186/s12967-023-04022-9