INHIBITION OF APE1'S DNA REPAIR ACTIVITY AS A TARGET IN CANCER: IDENTIFICATION OF NOVEL SMALL MOLECULES THAT HAVE TRANSLATIONAL POTENTIAL FOR MOLECULARLY TARGETED CANCER THERAPY

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DEDICATION

I dedicate my thesis to three of the most important people in my life: My wonderful parents, Ajit and Ranjana Bapat and my amazing husband, Dhruv Bhate. Their unconditional love, encouragement and support have been my rock in my pursuit of this PhD.

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ABSTRACT

Aditi Ajit Bapat

INHIBITION OF APE1'S DNA REPAIR ACTIVITY AS A TARGET IN CANCER: IDENTIFICATION OF NOVEL SMALL MOLECULES THAT HAVE TRANSLATIONAL POTENTIAL FOR MOLECULARLY TARGETED CANCER THERAPY

The DNA Base Excision Repair (BER) pathway repairs DNA damaged by endogenous and exogenous agents including chemotherapeutic agents. Removal of the damaged base by a DNA glycosylase creates an apurinic / apyrimidinic (AP) site. AP endonuclease1 (Ape1), a critical component in this pathway, hydrolyzes the phosphodiester backbone 5' to the AP site to facilitate repair. Additionally, Ape1 also functions as a redox factor, known as Ref-1, to reduce and activate key transcription factors such as AP-1 (Fos/Jun), p53, HIF-1α and others. Elevated Ape1 levels in cancers are indicators of poor prognosis and chemotherapeutic resistance, and removal of Ape1 via methodology such as siRNA sensitizes cancer cell lines to chemotherapeutic agents. However, since Ape1 is a multifunctional protein, removing it from cells not only inhibits its DNA repair activity but also impairs its other functions. Our hypothesis is that a small molecule inhibitor of the DNA repair activity of Ape1 will help elucidate the importance (role) of its repair function in cancer progression as wells as tumor drug response and will also give us a pharmacological tool to enhance cancer cells' sensitivity to chemotherapy. In order to discover an inhibitor of Ape1's DNA repair function, a fluorescence-based high throughput screening (HTS) assay was used to screen a library of drug-like

compounds. Four distinct compounds (AR01, 02, 03 and 06) that inhibited Ape1's DNA repair activity were identified. All four compounds inhibited the DNA repair activity of purified Ape1 protein and also inhibited Ape1's activity in cellular extracts. Based on these and other *in vitro* studies, AR03 was utilized in cell culture-based assays to test our hypothesis that inhibition of the DNA repair activity of Ape1 would sensitize cancer cells to chemotherapeutic agents. The SF767 glioblastoma cell line was used in our assays as the chemotherapeutic agents used to treat gliobastomas induce lesions repaired by the BER pathway. AR03 is cytotoxic to SF767 glioblastoma cancer cells as a single agent and enhances the cytotoxicity of alkylating agents, which is consistent with Ape1's inability to process the AP sites generated. I have identified a compound, which inhibits Ape1's DNA repair activity and may have the potential in improving chemotherapeutic efficacy of selected chemotherapeutic agents as well as to help us understand better the role of Ape1's repair function as opposed to its other functions in the cell.

Mark R. Kelley Ph.D., Chair

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ABBREVIATIONS

5' dRP	5' deoxyribose Phosphate
5' dRPase	5' deoxyribose Phosphatase
6-FAM	Fluorescein
8-OxoG	8-oxo-7,8-dihydroguanine
Aag	3meA DNA Glycosylase
AP1	Activator Protein 1
AP sites	Apurinic / Apyrimidinic sites
Ape1	Apurinic / Apyrimidinic endonuclease 1
AR	Ape Repair Inhibitor
ARP	Aldehyde Reactive Probe
Bcl 2	B-cell CLL / lymphoma 2
BER	Base Excision Repair
BM	Bone marrow
bp	Base pair
^{o}C	Degree centigrade
C	Cysteine
CGCF	Chemical Genomics Core Facility
clogP	Octanol-water partition coefficient
δn	Standard deviation of the negative reaction
δp	Standard deviation of the positive reaction
D	Aspartic Acid

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA Pol β	DNA Polymerase β
DMEM	Dulbecco's Minimal Essential Medium
DSB	Double-strand breaks
DTT	Dithithreitol
E	Glutamic Acid
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra-acetic acid
EMS	Ethyl methane sulphonate
ES cells	Embryonic Stem cells
EtOH	Ethanol
FBS	Fetal Bovine Serum
Fen-1	Flap Endonuclease-1
FID	Fluorescent Intercalator Displacement
Gzm A	Granzyme A
H	Histidine
HCl	Hydrogen chloride
HEPES	N-2-hydroxythylpoperazine-N'-2-ethanesulfonic acid
HEX	Hexachloro phosporamide
Hif 1-α	Hypoxia-inducible factor 1-alpha
H_2O_2	Hydrogen Peroxide
HOS	Human osteosarcoma

HR	Homologous Recombination
HRP	Horse Radish Peroxidase
HTS	High-Throughput Screen
IC_{50}	Inhibition Concentration 50%
IgG	Immunoglobin G
IR	Ionizing Radiation
K	Lysine
KCl	Potassium Chloride
L	Liter
LP-BER	Long patch-BER
LOPAC	Library of pharmacologically active compounds
M	Molar
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
µn	Average of the negative reaction
µp	Average of the positive reaction
ml	Milliliter
mM	Millimolar
MMR	Mismatch Repair
MMS	Methyl methane sulfonate
MPG	N-methyl purine DNA glycosylase

MTT	Tetrazole 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MX	Methoxyamine
N	Asparagine
NaCl	Sodium Chloride
NCA or CRT0044876	7-nitroindole, 2-carboxylic acid
NCI Diversity Set Library	National Cancer Institute Diversity Set Library
NEIL	Nei Endonuclease VIII like
NER	Nucleotide Excision Repair
ng	Nanogram
NHEJ	Non-Homologous End Joining
NIR	Nucleotide Incision Repair
NK	Natural Killer
nm	Nanometer
nM	Nanomolar
NO	Nitric Oxide
NTH	Endonuclease three like
OGG1	8-oxoguanine DNA glycosylase
PARP	Poly (ADP ribose) polymerase 1
PBS	Phosphate buffered saline
PCNA	Proliferating Nuclear Cell Antigen
PEF	Primary Embryonic Fibroblasts
PTH	Para Thyroid Hormone

Q	Dabcyl
Rac 1	Ras-related C3 botulinum toxin substrate 1
Ref-1	Redox effector factor-1
RF-C	Replication Factor-C
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT	Room Temperature
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulphate
siRNA	Small-interfering RNA
SP-BER	Short patch-BER
SSB	Single-strand breaks
TBE	Tris-borate EDTA buffer
TBS	Tris buffered saline buffer
TBST	Tris buffered saline with Tween 20 buffer
TE	Tris-EDTA buffer
TEN	Tris-EDTA-Sodium chloride buffer
THF	Tetrahydrofuran
TMZ	Temozolomide
U	Units
V	Volts
XRCC1	X-ray Cross Complementing factor 1
Y	Tyrosine

CHAPTER I

INTRODUCTION

The ability of cancer cells to recognize and repair chemotherapy-induced damage is an important factor in resistance to chemotherapy (131). Therefore, inhibiting DNA damage repair pathways and using inhibitors against specific proteins of these pathways is an excellent strategy to develop targeted therapies for cancer treatment (14, 42, 83, 131, 133). Apurinic / apyrimidinic endonuclease 1 (Ape1) is a an essential protein functioning in the Base Excision Repair (BER) pathway, which repairs damage caused by endogenous as well as exogenous agents including chemotherapeutic agents (32, 47, 56). Ape1 is unique such that it is the only cellular protein that can process the apurinic / apyrimidinic sites (AP sites) generated as a result of the action of the DNA glycosylases, which initiate BER and there is no backup for this critically important repair function of Ape1 in the cells. Given Ape1's importance in normal cellular functioning, altered or elevated levels of Ape1 have been observed in a variety of cancers including breast cancer, gliomas, sarcomas (osteosarcomas, rhabdomyosarcomas), ovarian and multiple myeloma among others (47, 103, 108, 155, 162, 173, 195). These high levels of Ape1 have not only been speculated to be a cause of resistance to chemotherapy but have also been linked to tumor promotion, progression and poor prognosis associated with shorter relapse-free survival and poor outcome from chemotherapy (108). Furthermore, Ape1 also functions as redox regulatory protein (also known as Ref-1 (1, 205-207)) where it activates transcription factors by reducing cysteine residues on their DNA binding subunits to alter gene transcription, in addition to which it interacts with several proteins

from different signaling pathways (206). There is a vast amount of data showing that down-regulating or inhibiting Ape1 in cancer cells using RNA interference and DNA antisense oligonucleotide techniques can sensitize them to laboratory and clinical chemotherapeutic agents (17, 18, 103, 115, 162, 173, 194, 197). However, reduction of Ape1 protein levels using RNA interference or antisense DNA technology not only prevents its ability to repair DNA but also disrupts key protein – protein interactions within the BER pathway as well as its redox signaling. Therefore, development of good and selective inhibitors of the repair function of Ape1 would provide us with useful tools in order to improve the efficiency of chemotherapeutic regimens.

Hypothesis

The hypothesis was that since Ape1 is involved in the repair of DNA damaged by chemotherapeutic agents, identification of a small molecule inhibitor of the DNA repair activity of Ape1 protein using a high-throughput screening assay will help us elucidate the importance (role) of its repair function in cancer progression as well as tumor drug response while maintaining its other functions and interactions intact. Such an inhibitor of Ape1's DNA repair activity will also give us a pharmacological tool to enhance cancer cells' sensitivity to chemotherapy.

Specific Aims of the Project

Specific Aim 1:

To identify and characterize novel inhibitors of Ape1's DNA repair activity using a High-Throughput Screening (HTS) assay. A library of 60,000 compounds will be

screened to identify small molecule inhibitors of the DNA repair function of Ape1 using a modified fluorescence based assay as described by Madhusudan *et al* (132). The compounds shortlisted after two rounds of screening will be validated using another gel – based AP endonuclease assay to determine inhibition of Ape1's DNA repair activity and IC_{50} values will be calculated using the aforementioned HTS assay.

Specific Aim 2:

To determine the selectivity of the potential repair inhibitors to inhibit Ape1's DNA repair activity. For the compounds identified to be selective for Ape1, their ability to inhibit a structurally different but related *Escherichia coli* endonuclease IV (63, 141, 199) will be assayed as well as their ability to inhibit Ape1 in a cellular environment.

Specific Aim 3:

To determine the efficacy of these inhibitors in the SF767 glioblastoma human cancer cell line and to test their ability to enhance cytotoxicity of laboratory and clinical chemotherapeutic agents. The survival of SF767 human glioblastoma cells will be monitored after treatment with the compounds singly as will the ability of these compounds to enhance the cytotoxicity of laboratory and clinical chemotherapeutic agents (Methyl methane sulfonate (MMS) and Temozolomide (TMZ)) known to induce DNA damage repaired by the BER pathway (32, 56). The Aldehyde Reactive Probe (ARP) assay will be used to assay for the persistence AP sites as a result of inhibition of Ape1 by these compounds (109, 143).

Thus, dissecting out the two functions of Ape1 and exploring them individually will allow us to delve further into delineating the importance of these functions. Since Ape1 has been known to be involved in resistance to chemotherapy, developing unique inhibitors of Ape1's repair function will help us increase the efficiency of the current chemotherapy and radiation regimens.

CHAPTER II

REVIEW OF RELATED LITERATURE

Importance of DNA Repair Pathways and Cancer

DNA repair pathways protect the genome from damage caused by endogenous and exogenous DNA damaging agents including chemotherapeutic agents and radiation damage (32, 56, 57, 117, 118), and the persistence of unrepaired DNA damage results in cell cycle arrest, apoptosis and accumulation of mutations (61, 127). To protect cellular DNA, several DNA repair pathways such as the Base Excision Repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR), Homologous Recombination (HR) and Non-homologous End Joining (NHEJ) exist in the cell to ensure efficient repair of a variety of damage (32, 56). The importance of multiple DNA repair pathways is highlighted by several cancer predisposing syndromes, which harbor germline mutations in DNA repair genes. Currently, surgeries to resect the tumor and chemotherapy and radiation therapy are the mainstream treatment options available to treat cancers. Many chemotherapeutic drugs act by damaging DNA, which leads to an accumulation of damage resulting in impaired cell signaling and ultimately causing cell death (79). Normal cells are proficient in all forms of DNA repair; however, deficiency of a particular DNA repair pathway in cancer cells can lead to elevated levels of other DNA repair pathway proteins leading to efficient repair of DNA damage and reducing the efficacy of cancer therapy. Cancer cells deficient in the proteins of the HR pathway for instance may be unable to efficiently repair damage through this pathway and may look to compensate for this deficiency by completing repair through alternative pathways such

as the NHEJ or BER pathway (21, 51, 104, 116). The ability of cancer cells to identify and repair such DNA damage undermines the efficacy of these agents, and acquired or intrinsic cellular resistance to these clinical DNA-damaging agents is governed by the enhanced or elevated levels of DNA repair proteins (14, 42, 83, 131). Although it may sound paradoxical to inhibit DNA repair pathway proteins since cancer promotion and deregulated cellular growth is aided by deficient DNA repair pathways, a fine balance exists between induction of DNA damage and its efficient repair, which is often responsible for resistance to chemotherapy (14, 133). Thus, inhibiting specific proteins from DNA repair pathways in cancer cells would provide us with a selective way to sensitize cancer cells to chemotherapeutic agents (131, 133). Additionally, combining DNA repair inhibition with other current chemotherapy regimens (21) and thus developing targeted therapies are generating robust interest.

The DNA Base Excision Repair (BER) Pathway

The BER pathway recognizes and repairs single base lesions caused by endogenous and exogenous agents including radiation and chemotherapy-induced damage (56, 57, 117, 118). Such lesions include N-alkylated purines (N^3-methyladenine, N^7-methylguanine and N^3-methylguanine), 8-oxo-7,8-dihydroguanine (8-OxoG), thymine glycols, 5-OH and 6-OH dihydrothymine, uracil glycol, 5-hydroxycytosine and urea residues in addition to a number of additional adducts (4, 32, 47). Repair of the damaged base is initiated by a DNA glycosylase (Figure 1), which specifically recognizes and excises the damaged base. Different DNA glycosylases recognize specific and different types of base damage (38, 168). Glycosylases are of two types: monofunctional and

bifunctional glycosylases. Monofunctional glycosylases (eg: N-methyl purine DNA glycosylase, MPG) excise the damaged base to generate an apurinic / apyrimidinic (AP) site. In contrast, bifunctional glycosylases in addition to exhibiting glycosylase activity also have an AP lyase function (26, 36). Bifunctional glycosylases such as 8-oxoguanine DNA glycosylase (OGG1), Nei endonuclease VII like, NEIL1, NEIL2 and NTH not only excise the damaged base but also nick the phosphodiester backbone 3' to the AP site (32, 47). Removal of the damaged base by a DNA glycosylase creates an AP site, and AP sites are also generated by spontaneous base loss in the genome (38, 47, 127, 161, 199).

The second critical component of the pathway is the multifunctional protein apurinic / apyrimidinic endonuclease (Ape1). Following hydrolysis by a DNA glycosylase, Ape1 processes the AP site by making an incision in the phosphodiester backbone immediately 5' to the AP site. This incision creates 3'OH and 5'deoxy Ribose Phosphate (5'dRP) termini (201). At this stage, repair can proceed via one of two pathways. The short-patch BER (SP-BER) pathway repairs regular AP sites. In the short-patch pathway, DNA polymerase β (Pol β) removes the 5'dRP moiety via its deoxy Ribose Phosphatase (5'dRPase) activity and uses the 3'OH terminus to insert the correct base. Subsequently, DNA ligase III / XRCC1 (X-ray cross-species complimenting 1) seals the nick and repair is completed (40, 57, 161, 175) (Figure 1). The long-patch BER (LP-BER) pathway preferentially repairs modified (oxidized, reduced) AP sites (60, 106, 161). In this minor BER pathway, a flap of 3-8 nucleotides surrounding the AP site is displaced. The correct nucleotides are inserted by DNA polymerase β, δ or ε along with proliferating cell nuclear antigen (PCNA) and replication factor - C (RF-C). Following resynthesis, flap endonuclease 1 (FEN1) removes the displaced strand and then the nick

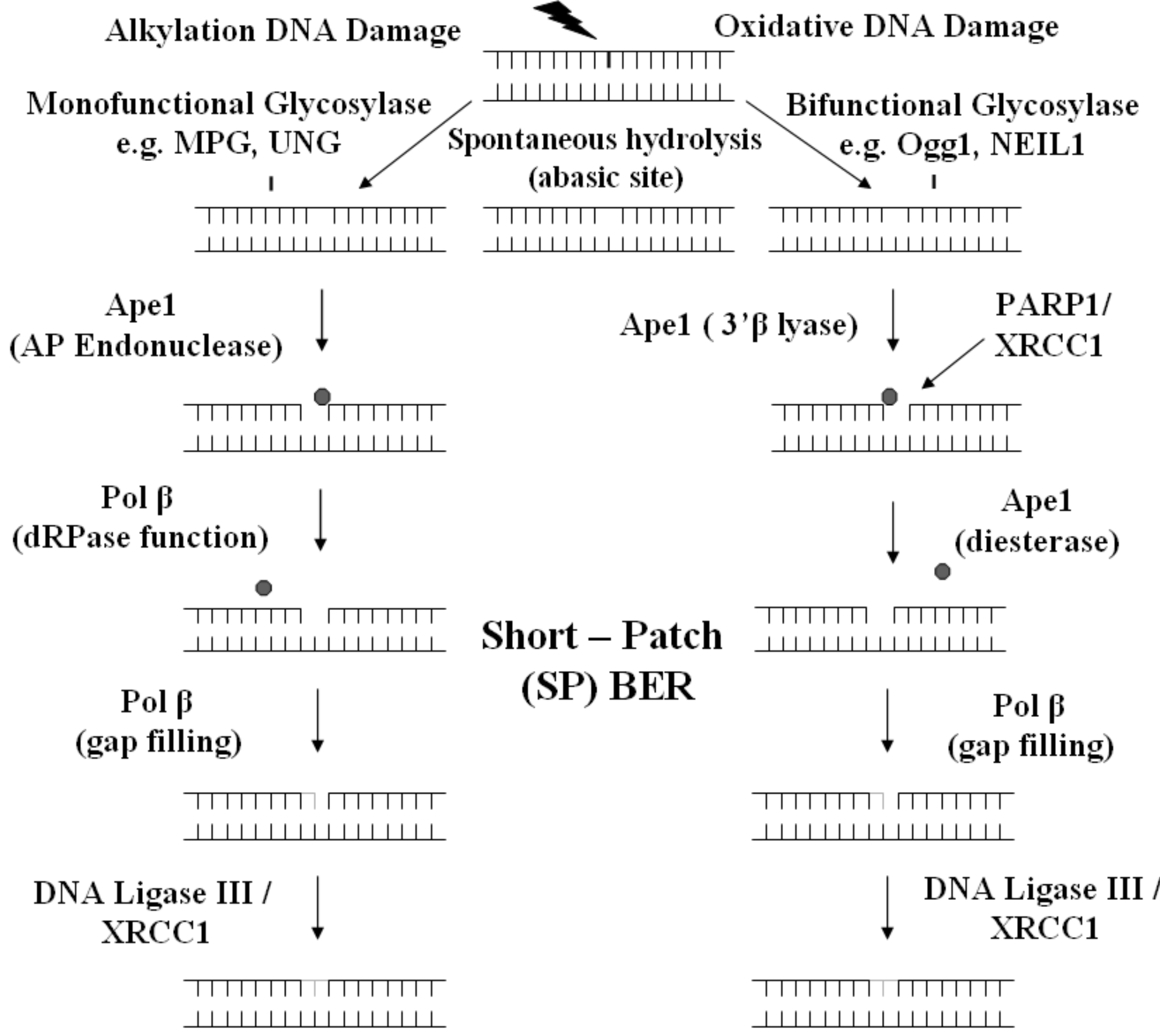


Figure 1: The Short-Patch DNA Base Excision Repair (BER) Pathway
A schematic representation of the BER pathway, AP sites genereated by the action of DNA glycosylases or by spontaneous hydrolysis are processed by the Short-Patch BER pathway.

is sealed by DNA Ligase I or DNA Ligase III / XRCC1 (47, 106) (Figure 2). Oxidative DNA lesions can also be excised by the recently identified Neil glycosylases NEIL1 and NEIL2, which show homology to the *E. coli* endonuclease VIII (8, 74-76, 96, 183) and the subsequent AP sites generated are processed by Ape1 to complete repair.

AP Endonucleases and the Ape1 Protein

Based on the method of incision, AP Endonucleases can be classified into two classes:

Class I AP Endonucleases

Class I AP endonucleases are also known as AP Lyases (or β-lyases) as they process the AP sites by the β-elimination reaction, which involves the removal of a hydrogen atom from the 2' position and cleave the phosphodiester backbone 3' to the AP site generating a 5' phosphate and a 3' α,β-unsaturated aldehyde end (7). This AP lyase activity is usually associated with complex DNA glycosylases, which are responsible for repairing oxidatively damaged DNA (36). The *E. coli* endonuclease III and endonuclease VIII (73) and the human homologue NTH1 (80, 81, 90) belong to this class of endonucleases.

Class II AP Endonucleases

Class II AP endonucleases are the major class of endonucleases and are also known as hydrolytic endonucleases as they hydrolyze the phosphodiester backbone 5' to the AP site. Based on homology, Class II AP Endonucleases can be further classified into two families, the exonuclease III (xth) and the endonuclease IV (nfo) family. The

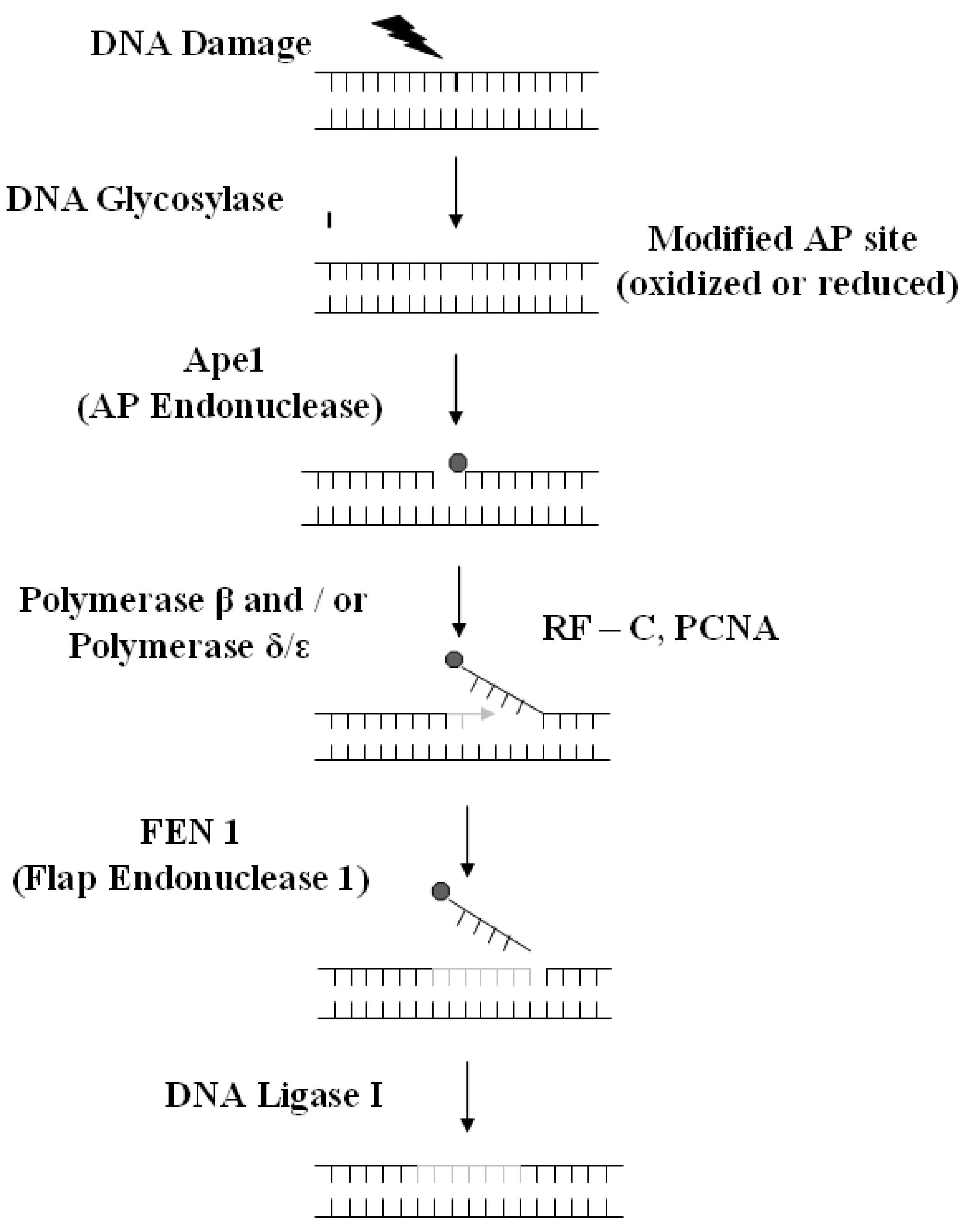


Figure 2: The Long-Patch BER Pathway
In this minor BER pathway, modified or oxidized AP sites are repaired by the insertion of a patch of 3-8 nucleotides to complete the repair of the damaged base.

Exonuclease III family consists of human Ape1 in addition to enzymes from various phyla, and these enzymes possess a strong AP endonuclease activity (38, 47, 156, 168, 170, 203). Although, Ape1 also possesses a much weaker (almost 200-fold weaker) 3'-repair diesterase activity (26) than the AP endonuclease activity, it is important in the removal of 3' blocking lesions such as phosphoglycolate moieties in order to complete repair (25, 26, 47, 54, 150).

The endonuclease IV family of enzymes is the second major family of Class II AP endonucleases which include the *E. coli* endonuclease IV (Figure 3) and Apn1 from *Saccharomyces cerevisiae* (yeast), which is responsible for 90% of AP endonuclease activity in *S. cerevisiae* (yeast) (47, 100, 154, 193). Apn1 can repair both alkylation and oxidative damage including oxidized AP sites and unlike Ape1, Apn1 has a higher 3'-repair diesterase activity (55). Although the enzymes from both families share the AP endonuclease function, they do not share sequence or structural similarity (38, 141).

The Structure of the Ape1 protein

The Ape1 protein is a ~37kDa, 318 amino acid protein, which is encoded by a 2.6kb gene on chromosome 14, q11.2-12 (47, 72, 163). The Ape1 protein is a multifunctional protein and with two main activities: the redox activity and the AP endonuclease or DNA repair activity (207). The first 36 amino acids at the N-terminal part of the protein comprise the nuclear localization sequence (NLS) (47). The redox activity resides in the N-terminal part of the protein and C65 has been shown to be the critical residue required for redox activity (47).

The AP endonuclease activity or the DNA repair function resides in the C-terminal portion of the protein. Similar to exonuclease III and DNase I, Ape1 is a globular protein and forms a four-layered α / β sandwich. This α/β sandwich is made up of two β-sheets, each of which is comprised of six strands and each β-sheet is surrounded by α helices (67, 207) (Figure 3). There is a single active site for the repair function and the H309 residue has been shown to be critical for catalysis as site-directed mutagenesis studies have shown that an H309N mutant protein has a 2000-fold decrease in activity. Additionally, H309 interacts with D283 to form the active–site nucleophile, which is responsible for bond cleavage. D283 in turn interacts with D308 to maintain the conformation of the active site and to align H309 accurately in the active site (10, 47, 128). Mg^{2+} is a critical requirement for the activity of Ape1, and E96 and K98 play an important role in positioning Mg^{2+} in the active site (10, 95). Y171 is another residue critical for catalysis, and mutating Y171 drastically reduced activity of Ape1 as did mutating the D210 residue. D210 has been speculated to play a role as a proton donor (46, 140), and N212 has been shown to be responsible for substrate recognition (47, 167).

Functions of Ape1

The AP Endonuclease Activity of Ape1

Ape1 is responsible for 95% of the endonuclease activity in the cell and is a critical part of both the SP and LP-BER pathways (38, 168). Ape1 processes AP sites by hydrolyzing the phosphodiester backbone 5’ to the AP site to generate a 3’OH and a 5’dRP terminus. Subsequently, the 5’dRP moiety is removed by the dRPase function of Ape1 or DNA Pol β and repair is completed via the SP-BER or LP-BER pathways

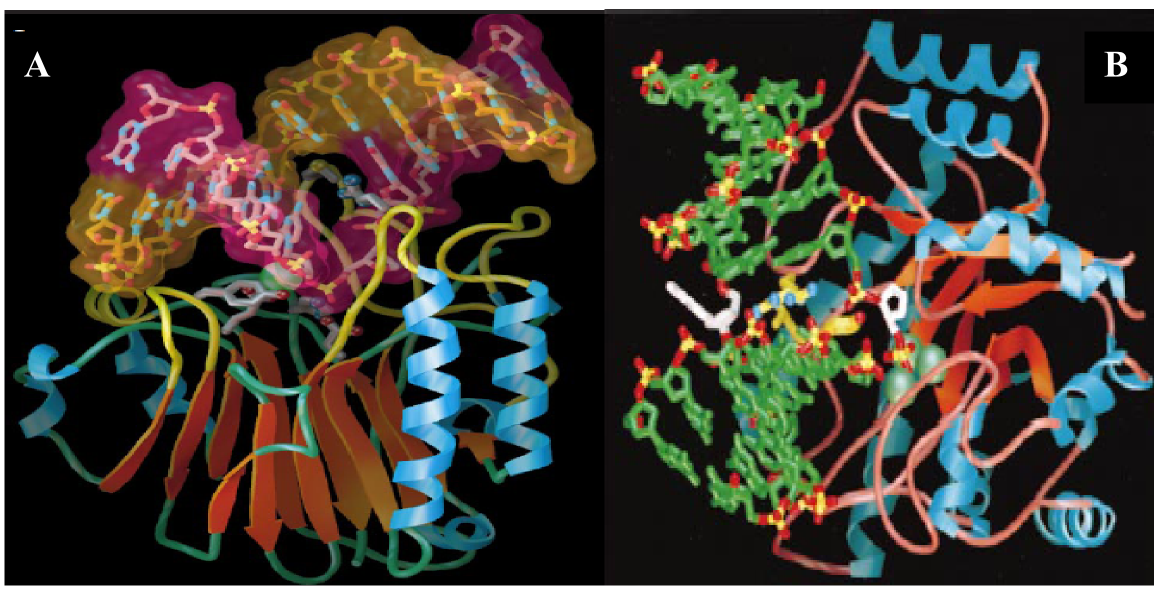


Figure 3: Structures of the human Ape1 and the *Escherichia coli* endonuclease IV proteins
The human Ape1 (140) **(A)** and the *E. coli* endonuclease IV proteins (89) **(B)** function similar to each other, but their structures are distinct from each other.

(47, 161). Ape1 is essential to complete the repair of AP sites, which are generated by spontaneous base hydrolysis in the cell (142) and by the action of different DNA glycosylases on a variety of DNA lesions, including oxidative DNA lesions, which can also be excised by the recently identified NEIL glycosylases (8, 74-76, 183). These AP sites if they are left unrepaired can be cytotoxic and mutagenic as they can block the replicating polymerase (107, 126, 127, 199, 214). While there are several different DNA glycosylases to excise the damaged base and generate AP sites, there is only one Ape1 protein, which is functionally involved in the SP-BER, LP-BER and the Neil-dependent BER pathways, thus emphasizing its significance in the BER pathway. Furthermore, importance of Ape1 to normal cellular functioning and development is highlighted by the embryonic lethality of Ape1 knockout mice at E3.5 to E9.5 (114, 208).

Other Repair Functions of Ape1

As discussed above, in addition to Ape1's strong 5' AP endonuclease activity, it also has a 3'-repair diesterase activity, which is important for the removal of lesions generated as a result of the β-lyase function of DNA glycosylases (Ogg1, Neil) (43, 47, 75) which are involved in the repair of oxidative or radiation-induced DNA damage (25, 26, 47, 54, 150). Lesions such as 3' phosphate and 3' phosphoglycolate moieties are generated by the action of oxidative agents such a bleomycin, radiation (IR) and are also formed at single-strand breaks. These 3' blocking lesions are removed by Ape1's phosdiesterase function so that the subsequent steps of BER can take place and repair can be completed (25, 26, 141, 150, 185). In addition to its hydrolytic and 3'-diesterase functions, Ape1 also possesses a 3'-5' exonuclease activity, which is important to process

3’-mispaired termini (28) and for the removal of unnatural deoxyribonucleoside analogs (27, 29), which can impede repair (25, 28, 29, 44). Additionally, Ape1 also possesses a weak RNase H function, which allows it to act on the RNA strand in a DNA-RNA hybrid (11).

The Redox Function of Ape1

In addition to its catalytic functions, Ape1 also functions as a reduction/oxidation (redox) signaling factor (Figure 4) and is therefore also referred to as Redox effector factor-1 (Ref-1) in the literature (1, 205-207). Ref-1 reduces key cysteine residues located in the DNA-binding domains of transcription factors such as AP-1 (Fos/Jun), p53, HIF-1α, NfκB and others (2, 62, 82, 113, 186, 188, 206, 213). This reduction of the critical cysteines in the DNA binding domains of the transcription factors increases their DNA binding ability thereby activating them and resulting in the transcription of several key genes important for cell survival and in cancer promotion and progression (47, 185) (Figure 4). Thus, the multifunctional Ape1 protein not only functions in and interacts with the proteins involved in the repair of damaged DNA, it also interacts with proteins involved in growth signaling pathways and pathways known to be involved in tumor promotion and progression. The redox function of Ape1 as a target in cancer has not been as extensively investigated as the DNA repair function of Ape1. However, given its role in activating transcription factors such as NFκB, AP-1 HIF-1α etc, inhibiting the redox ability of Ape1 should lead to decreased signaling via these transcription factors of the signaling pathways involved in cancer promotion and progression.

Other Functions of Ape1

In addition to Ape1's repair endonuclease and diesterase functions, Ape1 has also been shown to inhibit the activation of PARP1 (poly(ADP-ribose)polymerase 1) during oxidative damage repair thus preventing the cells from undergoing apoptosis (151). A relationship between Bcl2 and Ape1 resulting in decreased repair has been reported (99) in addition to negatively regulating the parathyroid hormone gene (PTH) (15, 33, 112, 144), being involved in granzyme A (GzmA) aided NK cell mediated killing (49, 135) and it has been implicated in nucleotide incision repair (NIR) (91, 92). Ape1 has also been suggested to play a role in negatively regulating the Rac1 / GTPase to prevent oxidative stress (147) and to regulate vascular tone and endothelial NO production (98) (Figure 5).

The Repair and Rexdox functions are disctinct from each other

Ape1 is a multifunctional protein with roles in DNA repair as well as in redox signaling in the cell besides making protein-protein interactions with a number of proteins. These two important functions of Ape1 are functionally distinct from each other and are encoded by distinct regions of the protein (207). The AP endonuclease or DNA repair activity, which is a critical component of the BER pathway, resides in the C-terminal portion of the protein (Figure 4). The AP endonuclease activity is mediated by the active site residues His 309, Glu 96, Asp 238 and Asp 308 where H309 is the catalytic residue. Asp 238 acts as a proton donor to donate a proton to a water molecule, which then functions as a nucleophile to cleave the phosphodiester bond (11, 13, 47, 58, 67, 128, 140). The redox regulatory activity of Ape1, which is important for the control

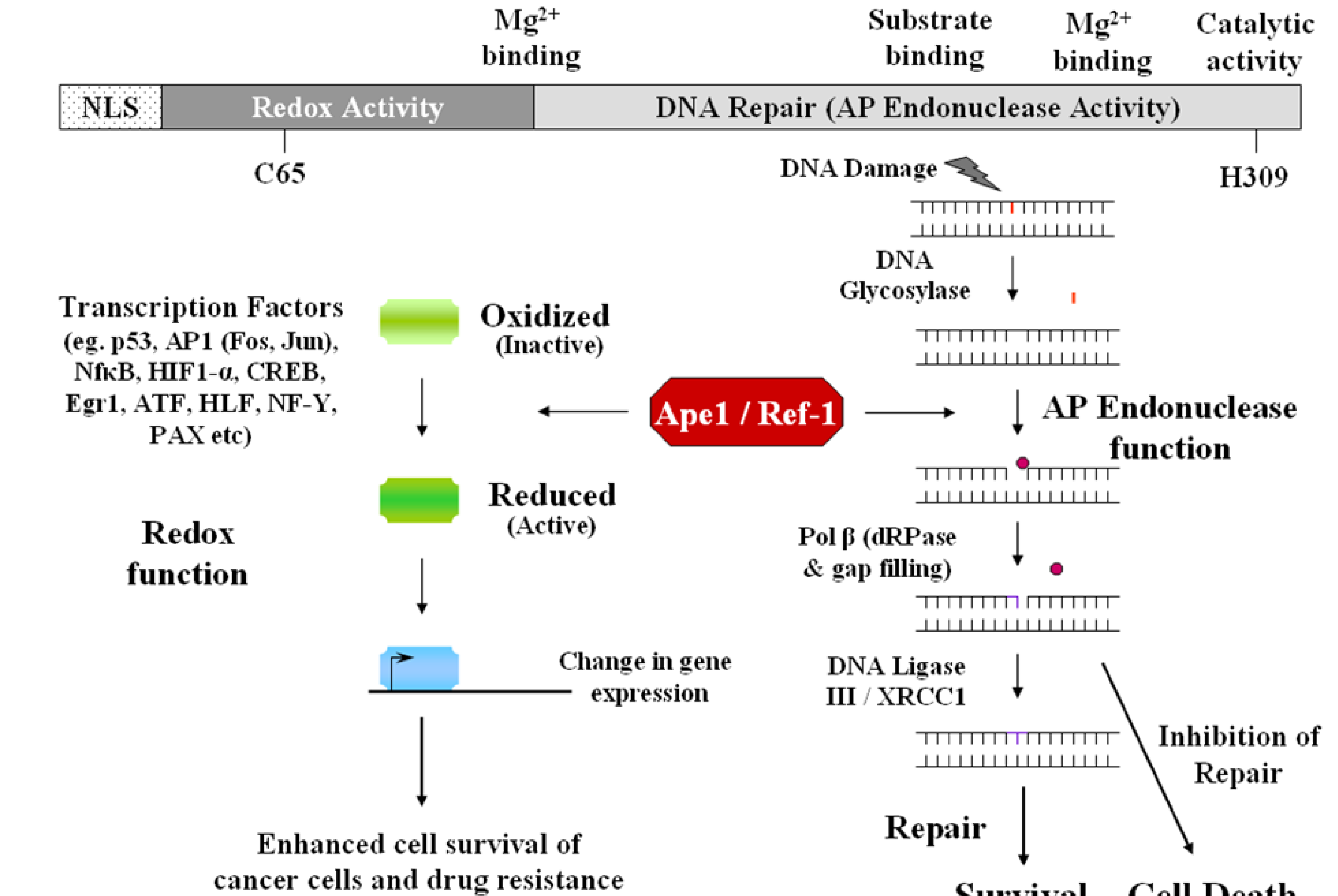


Figure 4: The Multifunctional Ape1 protein
Ape1 is a multifunctional protein with a role in DNA repair to maintain genomic integrity and a function in cellular redox signaling to activate key transcription factors leading to changes in expression of genes involved in crucial cellular processes

of transcription factors, resides in the N-terminal sequences of the protein. A conserved Cys 65 residue is crucial for this function of Ape1 (45, 47, 58) (Figure 4). These two activities of Ape1 can be functionally separated from each other, and disruption of either one of its activities does not affect the other. Several reports have shown that disruption of Cys 65 by site-directed mutagenesis (45) or by using a redox specific inhibitor, impairs the redox function of Ape1 but does not affect its DNA repair ability (158, 219).

Sub-cellular localization of Ape1 and its consequences in caner

Ape1 is ubiquitously expressed and though there are several reports showing that Ape1 is localized to the nucleus, cytoplasmic localization of Ape1 has also been reported (44, 102, 139, 160, 204). In addition to exhibiting a heterogeneous and complex pattern of staining, localization of Ape1 is tissue specific and even differs between neighboring cells (102, 160, 185, 204). Localization of Ape1 in the cytoplasm may be associated with its role as a mitochondrial DNA repair protein (47, 139, 185). Noting Ape1's role in redox control of transcription factors, the presence of Ape1 in the cytoplasm may be important to maintain these transcription factors in a reduced state prior to their transport to the nucleus (44). Ape1 has also been shown to accumulate in the nucleus and mitochondria in response to DNA damage (139). Thus, is appears that the intracellular localization of Ape1 is regulated; however, the significance of its sub-cellular localization is still not well understood.

Inhibition of DNA Repair as a Target in Cancer

DNA repair pathways are important to maintain the genomic integrity as highlighted by several cancer predisposing syndromes, which harbor germline mutations in DNA repair genes (32, 56, 83). Currently, surgery, hormone, chemo and radiation therapy are the mainstream treatment options available to treat cancers. The cytotoxic effects of many chemotherapeutic agents and radiation are related to their ability to induce DNA damage, and the ability of cancer cells to identify and efficiently repair such DNA damage undermines the efficacy of these agents (157). Therefore, inhibiting DNA repair proteins leading to reduced repair of damaged DNA in cancer cells is an attractive approach to combat chemotherapeutic resistance and to increase efficacy of therapy. Although it may sound contradictory to inhibit DNA repair pathway proteins since cancer promotion and deregulated cellular growth is aided by deficient DNA repair pathways, it actually makes sense to block DNA repair given the predominance of DNA damage during cancer treatments with chemotherapy and IR, which would allow for increased efficacy of the DNA damaging agent (14, 133). Thus, inhibiting specific proteins from selected DNA repair pathways in cancer cells could provide us with a selective way to sensitize cancer cells to chemotherapeutic agents and also combat their resistance to chemotherapeutic agents (131, 133).

Consequences of Inhibiting the BER Pathway Proteins in Cancer

In cancer cells, the upregulation of certain BER proteins results in imbalanced repair causing resistance to chemotherapeutic agents (65). Modulating or inhibiting the activities of these BER proteins can lead to deregulated repair resulting in sensitivity to

chemotherapy agents (52-54, 103, 115). The BER pathway proteins interact with each other to make the BER process efficient and a delicate balance exists between the levels of all the BER proteins, and disrupting this balance results in imbalanced repair (65) (Figure 5).

However, the central idea that the presence of robust DNA repair mechanisms leads to resistance to chemotherapeutic agents (14, 35, 42, 79, 116, 133, 171) has been challenged by some studies. Roth *et al* (166) showed that absence of Aag (3MeA DNA glycosylase) in the bone marrow (BM) cells of the myeloid lineage from Aag -/- mice are resistant to the alkylating agent methyl methane sulphonate (MMS) as compared to the wild-type BM cells. They speculated that initiation of repair by Aag and subsequent incomplete repair of the lesions in wild-type BM cells may be more toxic than the inability of Aag null BM cells to initiate repair of these damaged lesions. This effect was specific to the myeloid lineage of the Aag -/- mice and was not observed in embryonic stem cells (ES), primary embryonic fibroblasts (PEF) and cells from the lymphoid lineage in the BM, indicating that this effect is tissue-specific as well as likely lesion-specific. In general, it has been shown that the presence of DNA repair contributes to resistance to chemotherapeutic agents. DNA glycosylases show quite a bit of functional redundancy (114), and the action of all the DNA glycosylases results in the formation of AP sites. For instance, overexpression of 3MeA DNA glycosylases in *S. cerevisiae* and *E. coli* leads to increased sensitivity to alkylating agents and spontaneous mutations, possibly due to an imbalance between the levels of the DNA glycosylase and Ape1 proteins and also due to the build-up of unrepaired AP sites (166). Accumulation of these unrepaired AP sites can lead to (Figure 6) single strand breaks, increased apoptosis and

enhanced cytotoxicity (61). Ape1 is required to process these AP sites and along with the rest of the BER proteins can facilitate the ensuing completion of repair.

On the other hand, several reports have also shown that increased levels of the Ape1 protein in cancer cells is an indicator of poor prognosis and resistance to chemotherapeutic ministrations (108). Inhibition of the Ape1 protein using siRNA technology has been shown to alleviate some the resistance to chemotherapeutic agents (52-54, 103, 115).

Similarly, the importance of DNA Pol β in maintaining genomic integrity has been highlighted by the embryonic lethality of Pol β null mice (114, 171). It has been shown that increased levels of Pol β in cells are responsible for increased genomic instability and have also been implicated in tumorigenesis (3, 178). However, decreasing the levels of Pol β sensitizes cancer cell lines to laboratory and clinical chemotherapeutic agents (87, 171, 191).

The XRCC1 protein, although it has no activity of its own (87), plays an important role as a scaffold protein in the BER pathway where it interacts with several of the BER proteins to facilitate efficient repair (48, 202), and Horton and colleagues found that XRCC1 -/- mouse fibroblasts are hypersensitive to the alkylating agents ethyl methane sulphonate (EMS), MMS and TMZ and exhibits a delay in the repair of IR-induced DNA damage (87).

Increased expression of certain BER pathway proteins in cancer cells can result in efficient repair of damaged lesions and can reduce the effectiveness of chemotherapeutic agents. Therefore, keeping in mind the importance of the BER pathway in the repair of such damage induced by chemotherapy, exploiting the BER pathway and its proteins by

inhibiting them would increase the efficacy of chemotherapy thus making it an attractive target to develop novel means to combat chemotherapeutic resistance (171).

Inhibition of the DNA Repair Function of Ape1 as a Target in Cancer

There are several reasons why Ape1 is a rational target for chemotherapeutic agents: 1) overexpression of Ape1 leads to chemoresistance; 2) cells that lack Ape1 are not viable; 3) knockdown or blockage of Ape1 activity sensitizes cancer cells to chemo agents such as temozolomide (TMZ), bleomycin etc. Elevated levels of Ape1 in cancer cells have been postulated to be a reason for chemotherapeutic resistance (16, 18, 103, 108, 155, 162, 173, 195). The importance of Ape1's function in the DNA BER pathway is observed from the lethality of Ape1 knockout mice (114, 208). Specifically knocking down or inhibiting Ape1 using RNA interference and anti-sense oligonucleotide technology hypersensitizes mammalian cancer cells to several laboratory and clinical DNA damaging agents, such as methyl methane sulfonate (MMS), hydrogen peroxide (H_2O_2), bleomycin, TMZ, and gemcitabine (17, 52-54, 115, 121, 123, 136, 137, 159, 173, 194, 196) The decrease in cancer cell proliferation and survival after knocking down. Ape1 reiterates the importance of Ape1 function. Although these data demonstrate the uniqueness and feasibility of Ape1 as a target for inhibition in order to sensitize cancer cells, studies involving a reduction in Ape1 mRNA and protein do not allow us to dissect which function, repair or redox, of Ape1 is important for cell growth, cancer promotion and/or progression (17, 18, 103, 115, 127, 162, 173, 194, 195). Fung *et al* (61) demonstrated that depletion of Ape1 from cells using siRNA technology causes increased apoptosis and decreased cell growth of cancer cells. They further demonstrated that

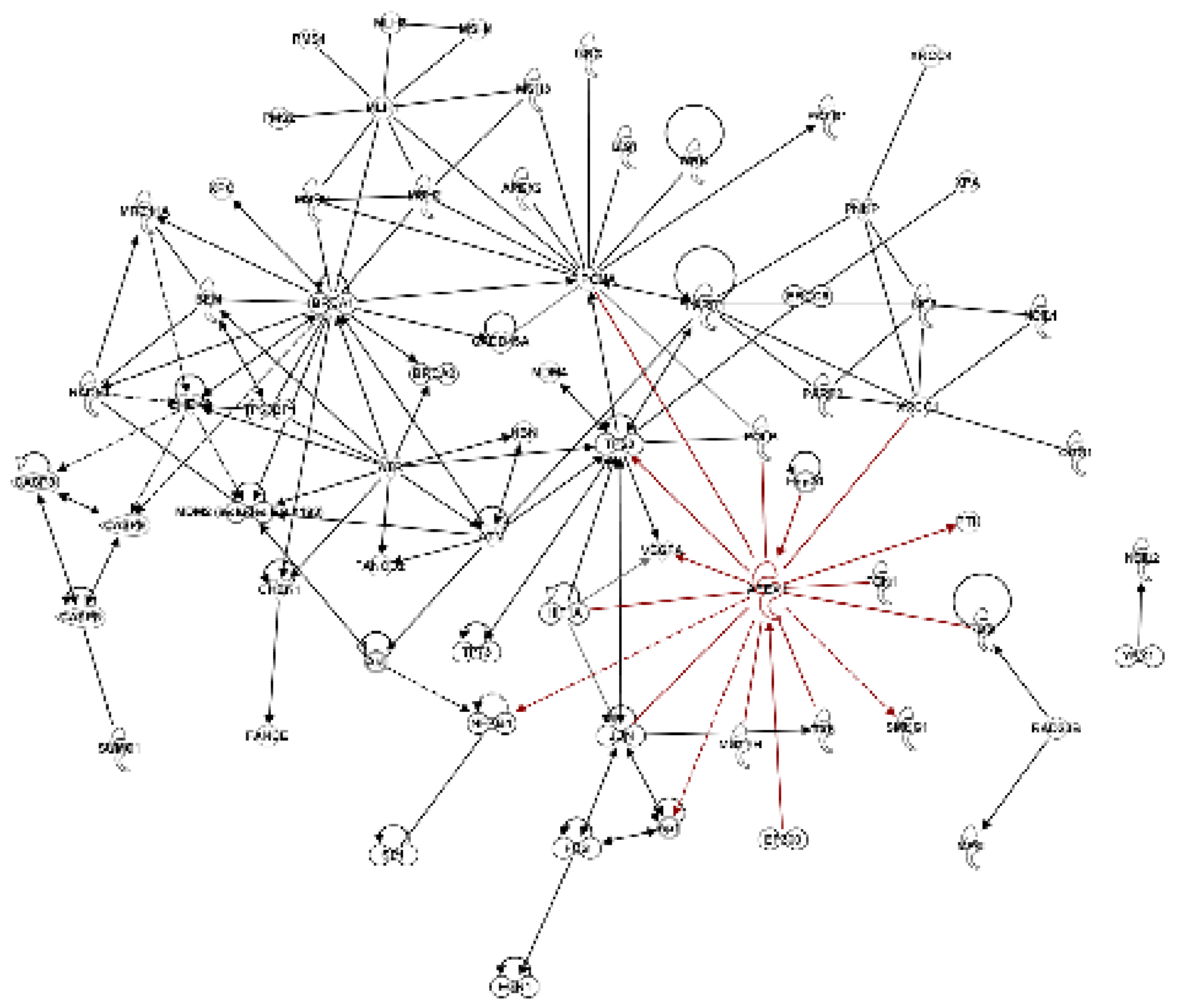


Figure 4: The protein – protein interaction network of Ape1
Ape1 interacts with several different proteins from the BER pathway and other DNA repair and signaling pathways to create a complex mesh of direct and indirect interactions. This matrix is a graphical representation of the molecular relationships between genes or gene products (proteins or complexes). Each gene or protein is represented as a node and the lines (edges) joining them represents the biological relationships between them. At least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity knowledge base corroborates each edge. Orthologs of a gene (human, mouse, and rat) are represented as a single node in the network but are stored as separate objects in the Ingenuity knowledge base. The various shapes of the nodes represent the functional class of that gene product. The nature of an edge is descriptive of the nature of the relationship between the nodes (e.g., solid line indicates a direct relationship, dashed line indicates an indirect relationship etc). This interaction network was generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com).

functional complementation with a yeast homologue (Apn1) of Ape1 deficient in redox activity could restore proliferation potential of the cells. Another report demonstrated that expressing a dominant-negative repair deficient Ape1 protein in cells sensitizes them to chemotherapeutic agents (137). Several studies conducted using a molecule methoxyamine (MX) that binds to AP sites in DNA and blocks Ape1's ability to cut the sugar-phosphate backbone sensitized cancer cells to chemotherapeutic agents (53, 121, 123, 184). Conversely, as we learn more about the redox function of Ape1, we appreciate its critical role in cell growth. Another recent study demonstrated that the redox function of Ape1 is important in hematopoietic differentiation (growth) by using a specific inhibitor of Ape1's redox activity, but did not cause the cells to undergo apoptosis (219).

These observations not only suggest a crucial role for both of Ape1's functions in cellular survival and tumor promotion and progression, but also demonstrate differences observed when the redox or repair functions are blocked. Developing specific inhibitors of the two functions of Ape1 would further allow us to discern which of the activities of Ape1 are important for cancer promotion and progression and normal cellular survival. Furthermore, the two functions may play different roles in different kinds of cancer allowing us to better understand tumor progression. Thus, the consequences of inhibiting Ape1 in cancer cells point to it being a logical target in cancer therapeutics. Identification of molecules that specifically inhibit Ape1's repair or redox activity should be an effective means to sensitize cancer cells to chemotherapeutic agents and thus impact the development of new and targeted cancer therapies (reviewed in (54)).

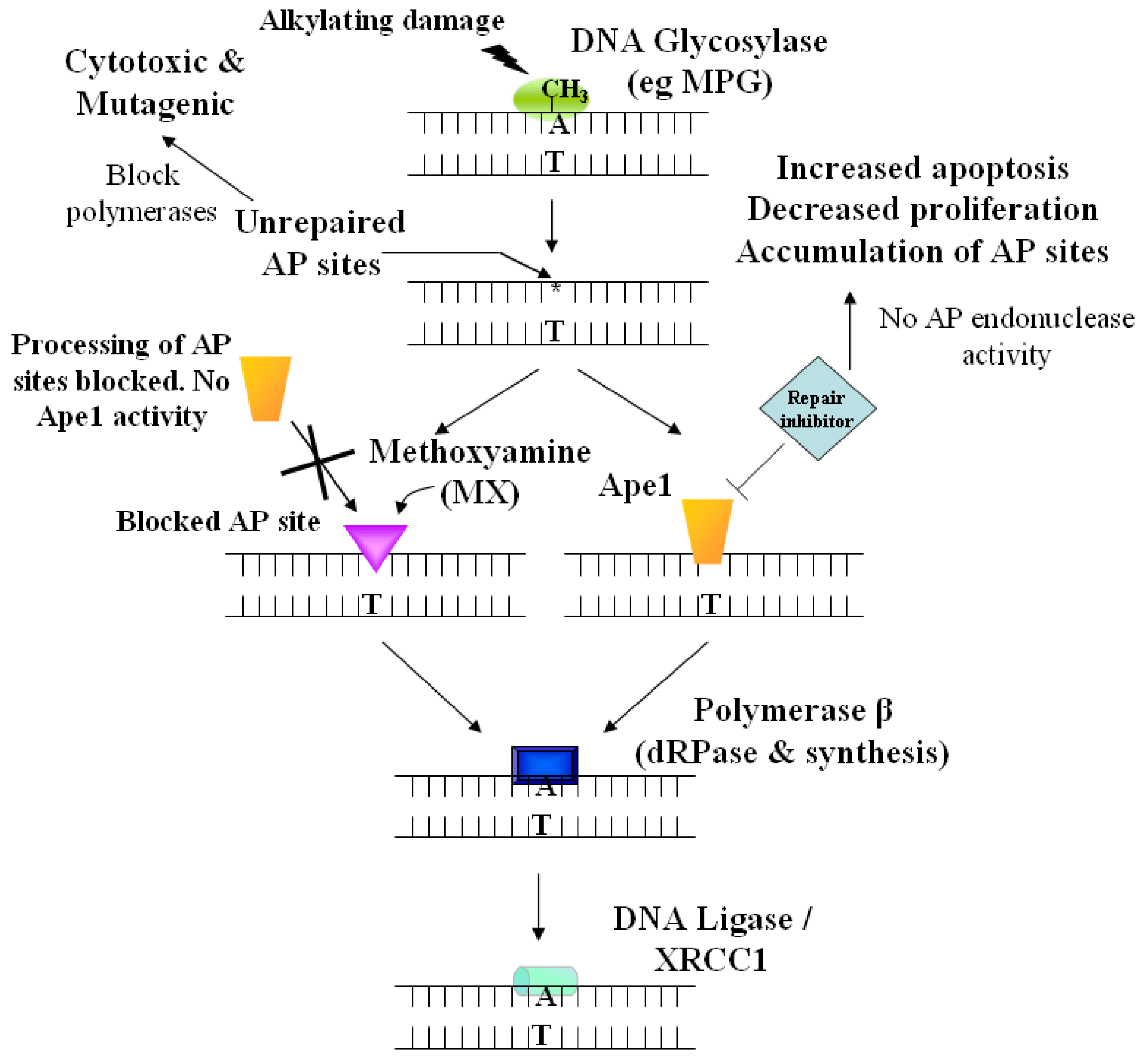


Figure 5: Consequences of Inhibition of the Repair Function of Ape1
Blocking Ape1's activity using an inhibitor of the BER pathway such as methoxyamine (MX) (125) leads to an accumulation of AP sites. Failure to repair such accumulated AP sites leads to cytotoxicity, increased apoptosis.

Existing Ape1 DNA Repair Inhibitors

As discussed above, Ape1's importance in the BER pathway and unique role supports the hypothesis that it is a strong target for cancer therapy; elevated levels in cancer cells and knocking down Ape1 using siRNA leads to an increased ability of cancer cells to undergo apoptosis and sensitization to chemotherapeutic agents (17, 47, 103, 108, 155, 162, 173, 195). Thus combining standard chemotherapeutic strategies with targeted inhibitors of Ape1's DNA repair function would increase the effectiveness of existing chemotherapeutic regimens. Currently compounds known to inhibit Ape1's DNA repair activity fall into two classes: negatively charged molecules including CRT0044876 or 7-Nitroindole-2-carboxylic acid (NCA) (132), an arylstibonic acid compound (169), pharmacophore based compounds (215), pharmacologically active compounds (174) and methoxyamine (MX) (125).

Methoxyamine (MX), an Indirect Inhibitor of Ape1's Repair Activity:

Methoxyamine (MX) is an inhibitor of Ape1 and is known to interact with the aldehydic C1\` atom of AP sites (125). This stable interaction between MX and the C1' aldehyde atom of AP sites results in the formation of an uncleavable covalent adduct (125). MX is considered to be an indirect inhibitor of Ape1 because it does not directly bind to Ape1 (125, 165). Instead it covalently modifies AP sites, and Ape1 is unable to readily cleave the resulting MX-AP site (59) thus preventing the subsequent BER proteins such as Pol β (86) from completing repair. MX can potentiate the cytotoxicity of alkylating agents such as TMZ and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (53, 121, 184). TMZ predominantly alkylates guanine at the N^7 and O^6 positions and adenine

at the N^3 position. The BER pathway repairs N^7 and N^3 alkylation damage and the action of the DNA glycosylases generate AP sites. MX binds to these AP sites thus preventing Ape1 from completing the repair and stabilizing the AP site intermediate (53, 121, 184). Clinical trials with MX in combination with TMZ are currently being pursued. However, MX is a simple compound, H_3CONH_2, with no obvious potential for improvement in efficacy through derivatization, and high concentrations of MX are required in cell-based assays (20-50 mM) in order to potentiate cell killing in combination with other agents (123, 137, 184, 210).

Lucanthone, a Direct Inhibitor of Ape1's Repair Activity

Lucanthone, originally identified as a Topoisomerase II poison (12) is considered to be a direct inhibitor of Ape1's DNA repair activity (138). Its extensive use to treat Schistosomiasis has shown it to be safe and nearly non-toxic from a clinical standpoint (37). Cancer cells treated with lucanthone exhibited a dose-dependent increase in AP sites seemingly due to inhibition of Ape1's repair activity and blocking an early step in the BER pathway (138). Patients with brain metastasis treated in combination with radiation and lucanthone showed increased regression of the tumors with the combination as compared to radiation alone (37). Additionally, lucanthone enhances the cell killing effect of MMS and TMZ in breast cancer cells by the inhibition of Ape1's DNA repair activity (130). However, the evidence of lucanthone also being a Topoisomerase II inhibitor raises the concern that the tumor cell killing observed could be partially attributed to the off-target effects of lucanthone, which again points to the need of a robust direct inhibitor of Ape1's repair function.

7–Nitroindole – 2–Carboxylic Acid (NCA), a Direct Inhibitor of Ape1's Repair Activity

Madhusudhan *et al* (132) identified CRT0044876 or 7-Nitroindole-2-carboxylic acid (NCA) in a high-throughput screen (HTS) of a library of 5000 drug-like compounds to be a direct inhibitor of Ape1's repair activity with an IC_{50} value of ~3 μM. NCA is negatively charged and is reported to inhibit all the DNA repair activities of Ape1 such as the AP endonuclease (repair activity), 3'-phosphodiesterase, 3'-5' exonuclease and 3'-phosphatase activities of Ape1. Survival analyses in HT1080 human fibrosarcoma cells showed that NCA potentiates the cytoxicity of MMS, TMZ, H_2O_2, and Zeocin (132). However, efforts to reproduce this repair inhibition have not been realized by our laboratory and by others (9), unpublished results.

Arylstibonic Acid Compounds as Inhibitors of Ape1's Repair Activity

Seiple *et al* (169) screened an NCI Diversity Set library of 2000 compounds to identify specific inhibitors of Ape1. The authors identified an arylstibonic acid compound 13755, which is negatively charged as an inhibitor of Ape1's DNA repair activity. This compound shows partial mixed type inhibition in that it binds both to the enzyme and the enzyme substrate complex. Even though these compounds have been reported to inhibit Ape1's DNA repair activity in vitro, they have virtually no cellular uptake and are less attractive as translational agents. Treatment of HOS osteosarcoma cells with 5 μM of the compound in the presence of MMS did not show decreased survival in cytotoxicity assays (169).

Pharmacophore Mediated Models to Identify Inhibitors of Ape1

A report by Zawahir *et al* (215) used a pharmacophore-based method of searching for inhibitors of Ape1 by mimicking the interactions that Ape1 makes with the AP site DNA. Three models were developed to search known small molecule databases for possible hits. The models represent the electrostatic environment and the chemical interplay between the residues on the Ape1 protein involved in repair and the AP site-containing DNA. From their investigations, they concluded that the compounds showing good inhibition of Ape1's DNA repair activity all contained negatively charged groups. Furthermore, the top selected hits were found to be selective for Ape1 as they inhibited the related *E. coli* exonuclease III but did not inhibit Endonuclease IV or HIV-1 integrase.

Identification of Pharmacological Inhibitors of Ape1

In another report by Simeonov *et al* (174) a library of 1280 pharmacologically active compounds (LOPAC) was tested to determine possible inhibitors of Ape1. Simeonov *et al* utilized a modified fluorescent based assay with a red fluorescent tag and its corresponding quencher instead of using the green fluorescent dye, fluorescein. The compounds identified through this screen were tested against the endonuclease IV protein, and the top hits did not inhibit it. These hit compounds were also able to enhance the cytotoxicity of MMS, and treatment with these inhibitors resulted in an increase of AP sites formed. These compounds are known to target other molecules and are therefore, not specific for Ape1 which may not make them the best choice of compounds to inhibit Ape1.

Need for Specific Inhibitors of Ape1's DNA Repair Activity

There is a clear need for a specific repair inhibitor of Ape1 in order to effectively determine the role of Ape1's repair activity in potentiating the effects of alkylating chemotherapeutic agents. This is required given the importance of inhibiting Ape1 using siRNA leading to sensitization of cancer cells to chemotherapeutic agents (17, 18, 103, 115, 127, 162, 173, 194, 195). However, these studies remove all of Ape1's functions (repair and redox) as well as Ape1's protein-protein interactions (48) making the data difficult to interpret. Thus, identifying specific and potent Ape1 repair inhibitors would facilitate the understanding of Ape1 not only in cancer, but also in dividing normal cells (bone marrow, gut etc), non-dividing normal cells (neurons) and other diseases where Ape1 has been implicated (22, 61, 84, 93, 185). In addition, these small molecule inhibitors will allow the specific inhibition of Ape1's repair function while keeping its post-translational modifications (23, 94, 97, 139) and subcellular location of Ape1 intact and in determining the effect of blocking Ape1's function on these subcellular events (39, 153, 185). In summary, identification of specific inhibitors of Ape1's repair activity will further our ability to determine the role it plays in cancer promotion and progression thus making a productive target of chemoprevention.

High-Throughput Screening (HTS) Methodology to Identify Specific Inhibitors of Ape1's DNA Repair Activity

High-throughput screening is a scientific method to assay large numbers of various chemical compounds against biological targets in a relatively short period of time. HTS assays are either entirely or partially automated and can be carried out in the

96-well or 384-well format. Robotic automation in HTS helps speed up the process of drug discovery and facilitates the generation of a large amount of scientific data in a short interval of time. Several different libraries of synthetic and drug-like compounds are available for HTS. Typically, the first round of HTS is carried out with a fixed concentration (1 μM – 10 μM) of the chemical compound. The positive 'hits' identified in the primary assay can be re-screened in the same assay and these hits are then followed up in secondary assays to validate the hits, determine an IC_{50} concentration and perform functional cellular assays. Thus, HTS is a promising and rapid methodology to identify potential modulators of the biological activity of the target from a large number of compounds.

Glioblastoma cell lines as models to study the effects of the Ape1 repair inhibitor

Malignant gliomas constitute a deadly group of cancer with a rather bleak outlook despite the advances in treatment and chemotherapy (173). Treatment options for gliomas usually includes surgical resection followed by chemotherapy with alkylating agents such as temozolomide (TMZ), procarbazine, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) given singly or in combination (180). Most therapeutic regimens do not induce complete remission and long-term survival is often accompanied by adverse toxic effects such as radiation-induced necrosis, alkylation-induced bone marrow suppression of alkylator-based chemotherapy. The levels of Ape1 have been shown to be elevated in gliomas, and these have been correlated to resistance to chemotherapy (16, 18, 19, 173).

Knowing that TMZ and radiation, which induce lesions repaired by Ape1, and the BER pathway is the standard of care for glioblastomas, makes it a good system to manipulate the activity of Ape1 in order to increase sensitivity to such chemotherapeutic agents (180). Therefore, the SF767 glioblastoma cell line was used to study the effects of my Ape1 repair inhibitors in a cell-based system.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

Chemicals and reagents for the experiments were purchased from Bio – Rad Laboratories (Hercules, CA), Corning Costar (Cambridge, MA), Dojindo Molecular Technologies (Rockville, MD), Eurogentec (San Diego, CA), Fisher (Pittsburg, PA), GE Healthcare (Piscataway, NJ), Gibco BRL Technologies, (Gaithersburg, MD), HyClone (Logan, UT), Invitrogen (Carlsbad, CA), LKT Laboratories Inc. (St. Paul, MN), Midland Certified Reagent Company (Midland, TX), NeoMarkers (Freemont, CA), Novus Biologicals Inc. (Littleton, CO), Nunc (Rochester, NY), Roche Applied Science (Indianapolis, IN), Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Sigma (St. Louis, MO), Thermo Scientific (Rockford, IL), TPP (Valley Park, MO) and Trevigen, Inc. (Gaithersburg, MD).

All the compounds from the High Throughput Screening Assay were purchased from Chemical Diversity Ltd (San Diego, CA)

METHODS

Purification of the Human Ape1 Protein

The human Δ40 Ape1 protein used in the high-throughput screening (HTS) assay and the gel–based AP endonuclease assay was purified as described before (64, 129) in Dr. Georgiadis's lab. The human Ape1 protein with the first 40 amino acids removed was expressed in the Rosetta *E. coli* strain using the pET15b expression vector containing an N-terminal hexa-His tag and the human Ape1 gene. The protein was purified from 6 liters of bacterial culture that was induced for 4 hours at 37°C. The cultures were pelleted and each 1 liter pellet was resuspended in 20 ml Buffer A (50 mM Phosphate pH 7.8, 0.3 M NaCl and 10 mM Immidazol) on ice. The cells were passed through a French Press twice to lyse them and the lysate was centrifuged at 35 K for 30 minutes at 4°C. The supernatant was loaded onto a Nickel column, and fractions were eluted using Buffer B (50 mM Phosphate pH 7.8, 0.3 M NaCl and 250 mM Immidazol). Fractions from the observed peak at 280nM were run on an SDS gel to confirm the presence of the protein. These fractions containing the protein were pooled and diluted 5-times with Buffer C (50 mM MES pH 6.5 and 1 mM DTT) to a salt concentration of 50 mM. This was loaded onto an S–sepharose column, and fractions were eluted with Buffer D (50 mM MES pH 6.5, 1 M NaCl and 1 mM DTT). Once again appropriate fractions were run on an SDS gel to confirm the presence of the protein. From the elution profile, concentration of Ape1 at 50% Buffer D (1 M NaCl) was estimated. The fractions containing the Ape1 protein were then digested overnight with thrombin to a final concentration of 2 U to remove the hexa-His tag. The next day the protein was run on an SDS gel to check for the removal of the His-tag. The fractions were then diluted 8-times to 50 mM NaCl using Buffer E (50 mM

MES pH 6.0 and 1 mM DTT). This was loaded onto an S-sepharose column and gradient eluted with Buffer F (50 mM MES pH 6.0, 1 M NaCl and 1 mM DTT). The appropriate fractions were run on an SDS gel and then collected. These fractions were then concentrated in a protein concentrator (Amicon, MA) with a cutoff limit of 10,000 Da. The concentration of the protein was then calculated and its activity determined using the AP endonuclease repair assay.

High Throughput Screening (HTS) Assay:

Oligonucleotides Used in the HTS Assay:

The pair of oligonucleotides used in the HTS assay were 30 base pairs in length and were synthesized via custom order from Eurogentec Ltd (San Diego, CA) (132). Of the pair of oligonucleotides, one of them has a Fluorescein label (**6-FAM**) at its 5' end (5'-**6-FAM-**GCCCCC*****GGGGACGTACGATATCCCGCTCC-3'). This same oligonucleotide also contains a synthetic AP site mimic, tetrahydrofuran (THF, represented as *****) at position 7 in the oligonucleotide. The complimentary strand has a quenching moiety (Dabcyl - **Q** in the oligonucleotide) at its 3' end (3'-**Q-**CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5'). The two single-stranded oligonucleotides were dissolved first in 1x TEN buffer (25 mM Tris pH 7.5, 1 mM EDTA and 50 mM NaCl) and also annealed in the same buffer at 95°C for 5 minutes in a 1:1 ratio at a concentration of 10 μM. The DNA was allowed to cool to room temperature overnight, and the DNA was then appropriately aliquoted and stored at -20°C. When the two oligonucleotides are annealed together, the resultant fluorescence of the oligonucleotide is diminished as the juxtaposition of the fluorescein and dabcyl tags

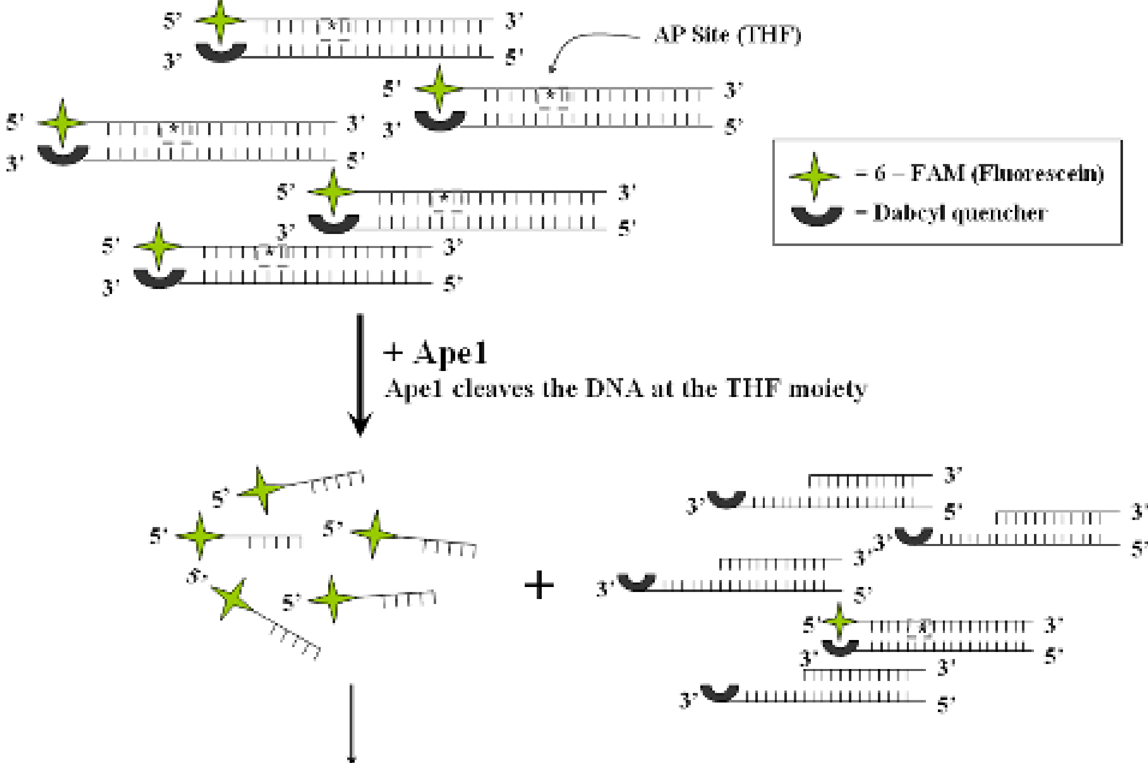


Figure 6: Principle of the High Throughput Screening (HTS) Assay
In this assay, which was modified from the one described by Madhusudhan *et al* (132), addition of Ape1 results in the cleavage of the THF residue to release the labeled piece of DNA. This results in a proportionate increase in fluorescence which is indicative of Ape1's DNA repair activity.

allows dabcyl to quench its fluorescence. However, on addition of Ape1, Ape1 cleaved the double-stranded DNA substrate at the THF moiety. This generates a short 7 base pair fluorescein fragment, which can then dissociate from the remaining oligonucleotide by thermal melting. The release of the fluorescein containing piece of DNA from dabcyl's proximity results in a proportionate increase in its fluorescence (Figure 7).

Optimization of the HTS Assay Conditions

The HTS assay used here was tweaked for our use from that described by Madhusudan *et al* (132), and several conditions for the assay were optimized before the actual screen was performed. First the fluorescence of the single stranded fluorescein containing THF oligonucleotide was tested (2-100 nM) to confirm that the background fluorescence of the THF oligonucleotide was in the detectable range. The reactions were carried out in a 50 µl volume, and a single end–point measurement was taken using the Ultra Plate Reader at the IU Chemical Genomics Core Facility (CGCF).

To select an optimal concentration of Ape1 protein in the linear range to use in the assay, a range of Ape1 protein concentrations (0.075-1.4 nM) in a 50 µl reaction volume with a 100 nM concentration of the double stranded oligonucleotide substrate and the HTS Assay buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM $MgCl_2$ and 2 mM DTT) were tested in the assay, and fluorescence measurements were taken over 15 minutes for each of the concentrations of Ape1 protein. By plotting the slopes (rates) of the time-dependent reactions against time of the assay indicated a steady and linear increase in fluorescence over time at 0.35 nM Ape1. The assay was performed at 37°C instead of at room temperature to ensure temperature consistency during the screen and to

aid in the spontaneous thermal dissociation of the cleaved piece of DNA from the rest of the substrate.

Z' Factor Measurement

A standard statistical value known as the Z' factor value, which reflects the reliability of the screen, was calculated before starting the screen. A Z' factor value between 0.5 and 1.0 is an indication of an excellent assay, and a value between 0 and 0.5 is an indication of a poor assay (216). To calculate the Z' factor measurement, positive and negative control reactions were carried out in 384-well plates. The positive reaction included 0.35 nM Ape1 along with 100nM of the oligonucleotide substrate and 1x HTS assay buffer in a 50 µl reaction volume. The negative reaction included all the above mentioned components including 50 mM EDTA. EDTA is a metal chelator that inactivates the Ape1 protein by chelating the Mg^{2+} ions, which are required for Ape1's activity thereby inactivating the Ape1 protein. The assay was carried out for 5 minutes, and then the rates of the positive and negative reactions (384 points for each of the positive and negative reactions) were used to calculate the Z' factor using the following formula:

$$Z'\text{-factor} = 1 - 3\,\frac{(\delta p + \delta n)}{(\mu p - \mu n)}$$

Where, δp = standard deviation of the positive reaction, δn = standard deviation of the negative reaction, µp = average of the points in the positive reaction and µn = average of the points in the negative reaction (216).

HTS Assay to Identify Potential Inhibitors of Ape1

A library of 60,000 diverse and drug-like compounds adhering to Lipinski's rules (119) from Chemical Diversity Ltd Inc (San Diego, CA) was tested at the IU CGCF. The library was aliquoted and screened at a concentration of 10 μM in black 384 well plates in a 50 μl reaction volume. The Genesis (Tecan) Workstation 150, TeMo with a 96-channel pipetting head was used to make all the additions in the following order:

- 20 μl of the inhibitor library (10 μM) already aliquoted into black 384 well plates.
- 20 μl of the Reaction mix which contains 100 nM oligonucleotide substrate and 1x HTS assay buffer (50 mM Tris pH 8.0, 1 mM $MgCl_2$, 50 mM NaCl and 2 mM DTT).
- 10 μl of the Ape1 protein at a final concentration of 0.35 nM.

Addition of the Ape1 protein to the plates initiates the reaction and change in fluorescence were measured at 37°C over 5 minutes using an Ultra384 Plate Reader (Tecan, Durham NC) in the Kinetic Mode with an Excitation frequency of 495 nm with an Emission frequency of 530 nm. Every assay plate included one column each for the positive and negative control for the assay. The positive control lane contained the Ape1 protein with no inhibitors, and EDTA served as the negative control for the assay as it chelates the Mg^{2+} required for Ape1 activity thereby inactivating the protein. The presence of Ape1 results in cleavage of the AP site mimic and a subsequent release of the short fluorescein labeled fragment, thus resulting in a proportionate increase in fluorescence. The rates of reaction were used to determine percent inhibition and the rate of reaction for Ape1 protein without inhibitors was considered as the 100% control and subsequent inhibition by the compounds was considered relative to that of the control.

$$\% \text{ Activity} = \frac{\text{Rate of reaction with inhibitor}}{\text{Rate of reaction with no inhibitor}} \times 100$$

Calculation of IC_{50} Values of the Compounds:

After two rounds of screening, IC_{50} values of the compounds selected for validation were determined. The assay used to determine the IC_{50} values was similar to the HTS assay, where a wide range of concentrations (0.1 µM-100 µM) of the compounds were tested with 0.35 nM Ape1, 100 nM of the annealed substrate and the assay buffer (50 mM Tris pH 8.0, 1 mM $MgCl_2$, 50 mM NaCl and 2 mM DTT) in a 50 µl reaction volume. Once again fluorescence readings were taken at 37^oC for 5 minutes and percent inhibition for each compound concentration was determined (as described above) as compared to the control with no inhibitor. IC_{50} values were calculated using the Sigma Plot graphing software with the following four-parameter logistic curve equation:

$$f = \text{Min} - \frac{\text{Max} - \text{Min}}{1 + (x / IC_{50})^{\text{Hill Slope}}}$$

Gel-based AP Endonuclease Assay:

This is another assay by which to determine the AP endonuclease activity of Ape1 (110). The 26 base pair oligonucleotides utilized in the gel-based AP endonuclease assay were obtained from the Midland Certified Reagent Company Inc (Midland, TX). The oligonucleotides comprise one strand with a hexa-chloro phosphoramidite (HEX) label and tetrahydrofuran (THF, represented as F) molecule, an AP site analog (5’-**HEX-**AATTCACCGGTACC**F**CCTAGAATTCG-3’) and its opposite strand

(3'-TTAAGTGGCCATGGTGGATCTTAAGC-5'). The single-stranded oligonucleotides were dissolved and annealed in 1x TEN buffer (25 mM Tris pH 7.5, 1 mM EDTA and 50 mM NaCl) at 95°C for 5 minutes at a 1:1 ratio at a concentration of 10 μM and then allowed to cool to room temperature overnight. The DNA was then diluted to 250 nM aliquots and stored at -20°C. A final concentration of 25 nM was used in the subsequent AP endonuclease assays.

The oligonucleotides used in the gel-based AP endonuclease assay differ from those used in the HTS assay. The size and sequence of the two oligonucleotides is different and as is the position of the THF moiety (at position 7 in the HTS oligonucleotides and at position 14 in the AP endonuclease oligonucleotides) (Table 1). The reactions were carried out in a 20 μl volume, and a typical reaction comprised of the following:

- 2 μl of the inhibitor compound (10x the final concentration)
- 2 μl of the 10x Ape assay buffer (50 mM HEPES pH 7.5, 50 mM KCl, 1 mM $MgCl_2$ and 2 mM DTT)
- 2 μl of the HEX labeled oligonucleotide substrate (25 nM final concentration)
- 2 μl of the Ape1 protein (0.175 nM final concentration)

The reaction mixture was then incubated at 37°C for 15 minutes, and the reaction was stopped by the addition of 10 μl of Formamide without dyes. 15 μl of the resultant reaction mixture was resolved on a 20% denaturing (7 M Urea) polyacrylamide gel in 1x TBE at 300 V for 35 minutes to reveal two bands: the longer full-length labeled strand and the shorter cleaved fragment with the HEX label (Figure 8). The gels were scanned using the Hitachi FMBio II Fluorescence Imaging System (Hitachi Genetic Systems,

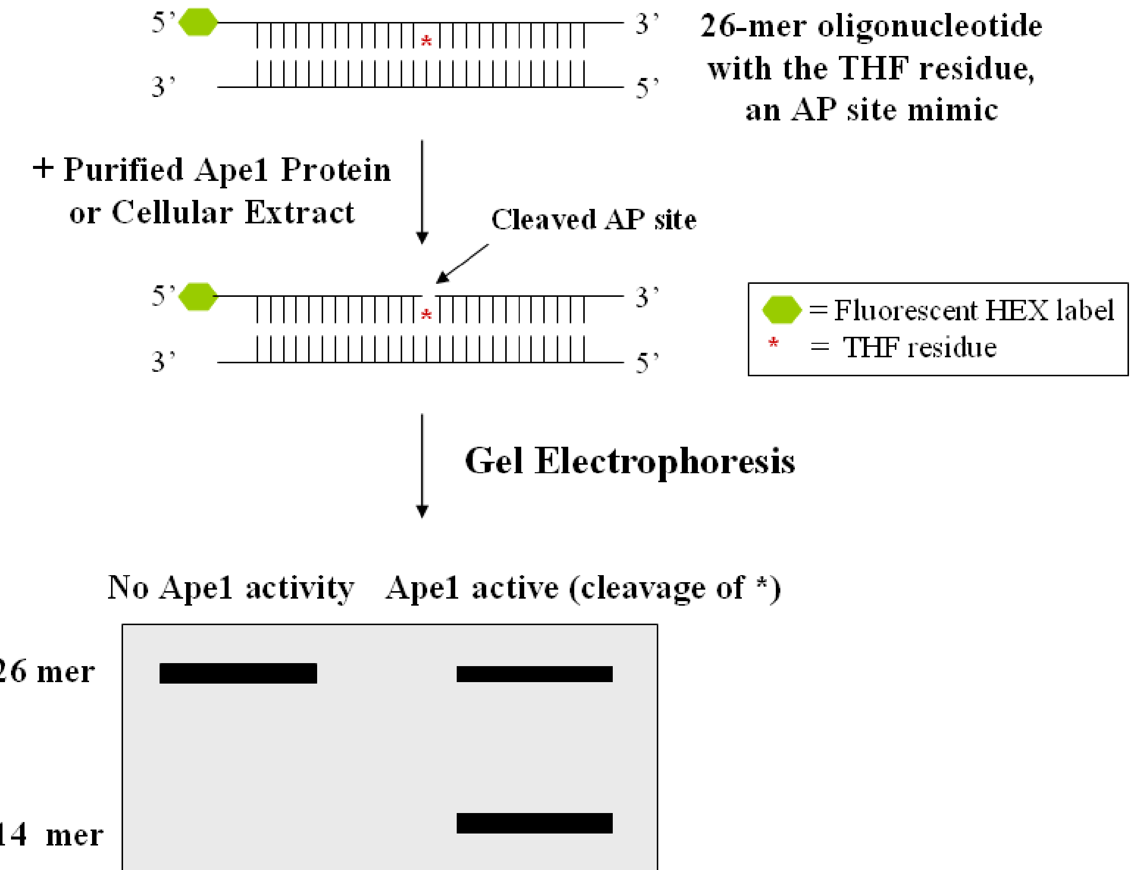


Figure 7: Principle of the gel-based AP endonuclease Assay
In this assay, Ape1 cleaves the THF residue and resolution of the samples on a denaturing gel results in two bands: the top band corresponds to the full length oligonuclotide whereas the bottom band corresponds to the cleaved band, which is an indication of Ape1's activity. Adapted from Kreklau *et al* 2001 (110).

South San Francisco, CA) and quantitated using the FMBio Software (Hitachi Genetic Systems). The amount of DNA cleaved was calculated as follows:

$$\% \text{ Cleaved} = \frac{\text{Lower (cleaved) band} - \text{background}}{\text{Upper (full-length) band} - \text{background} + \text{Lower (cleaved) band} - \text{background}}$$

Amount of DNA cleaved of the reaction with no inhibitors was considered to be 100% activity, and the subsequent % activity and inhibition for the reactions with inhibitors was calculated relative to this.

$$\% \text{ Activity} = \frac{\% \text{ Cleaved for a reaction with inhibitor}}{\% \text{ Cleaved for the reaction with no inhibitor}} \times 100$$

Gel-based AP Endonuclease Assay with pure Ape1 protein:

The potential inhibitors (18 compounds) identified after two rounds of screening and with IC_{50} values less than 50 μM were tested in this assay with the pure Ape1 protein. The ranges of inhibitor concentrations were based on the IC_{50} value and a few concentrations above and below the IC_{50} value were chosen. To find a concentration of Ape1 in the linear range to use in the assay, a range of concentrations (0.1-0.7 nM) of the Ape1 protein were assayed. 0.175 nM Ape1 was used to test the 18 compounds in this afore mentioned gel-based AP endonuclease assay.

Assay	Oligonucleotide substrate	Size (bp)	Cleavage product
HTS	5'-F-GCCCCCXGGGGACGTACGATATCCCGCTCC-3' 3'-QCGGGGGCCCCCTGCATGCTATAGGGCGAGG-5'	30	5'-F-GCCCCC (6-mer)
Ape1 repair	5'-HEX-AATTCACCGGTACCFCCTAGAATTCG-3' 3'-TTAAGTGGCCATGGTGGATCTTAAGC-5	26	5'-HEX-AATTCACCGGTACC (13-mer)

Table 1: Summary of oligonucleotides used in the HTS and gel-based assays

Gel-based AP Endonuclease Assay with the Endonuclease IV protein:

After validating the compounds in the HTS assay and with purified Ape1 in the gel-based AP endonuclease assay, the ability of these compounds to inhibit a related endonuclease, the *E. coli* endonuclease IV was determined. The endonuclease IV protein (100 Units/100 µl) used in the gel-based AP endonuclease assays was purchased from Trevigen (Gaithersburg, MD). Before picking an optimal concentration of endonuclease IV to use in the assay, a range of concentrations (3.75-60 nU) of the endonuclease IV protein were assayed. A concentration of 30 nU was found to be in the linear range of the reaction, and the consequent reactions and quantitation were carried out as described above with 30 nU of endonuclease IV and a range of concentrations of the inhibitor compounds.

Preparation of whole cell extracts from SF767 glioblastoma cells:

The SF767 cell extracts used in the gel-based AP endonuclease assay were prepared as described previously (110). Briefly, SF767 cells were trypsinized and collected. The cell pellets were then resuspended in no greater than 500 µl of cold 1x PBS + 2 mM DTT and sonicated on ice three times for 30 seconds with one minute in between each pulse. The tubes were then centrifuged at 13,000 rpm for 15 minutes twice in the cold room to ensure the removal of all the debris from the supernatant. The protein concentration of the SF767 cell extracts was determined using the Bio-Rad (Hercules, CA) Bradford Assay.

Gel-based AP Endonuclease Assay with SF767 cell extracts:

The SF767 cell extracts used in this assay were prepared as described above and the assay was performed as previously described. 3.75 ng of the SF767 cell extract along with the assay buffer, DNA substrate and inhibitor compounds was incubated at 37°C for 30 minutes and the reaction stopped by the addition of 10 µl of formamide without dyes.

Gel-based AP Endonuclease Assay to rescue the activity of SF767 cell extracts:

For the experiments where purified Ape1 protein was added back to the SF767 cell extracts treated with the inhibitor compounds, 3.75 ng of the SF767 cell extracts were first incubated with the compounds for 30 minutes at 37°C. The DNA substrate and pure Ape1 (0.4-3.5 ng) was added to the reaction mixture, and the reaction was allowed to proceed at 37°C for 30 minutes after which it was terminated by the addition of 10 µl of formamide without dyes.

Immunodepletion of Ape1 from SF767 WCE:

SF767 cell extracts were immunodepleted of the Ape1 protein using a polyclonal Ape1 antibody. 250 µg of SF767 cell extracts in 1x PBS were pre-cleared by adding 50 µl of washed (beads were washed with 450 µl of PBS twice and then re-suspended in 50 µl of PBS) protein A / G Plus-agarose beads (Santa Cruz Biotechnology Inc, Santa Cruz, CA) to the cell extracts and gently rocking them at 4°C for 1 hour. The extracts were centrifuged at 10,000 *g* for 1 minute and the supernatants collected. The supernatants were then incubated with 10 µg of the polyclonal Ape1 antibody (Novus Biologicals Inc, Littleton, CO) or normal rabbit IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at

4^oC with gentle shaking for 2 hours. After 2 hours, 50 µl of washed beads were added to the cell extracts and incubated for another 2 hours with gentle rocking at 4^oC. The cell extracts were then centrifuged at 10,000 *g* for 5 minutes and the supernatants collected. The protein concentration of the immunodepleted cell extracts was measured with the Bradford Assay (BioRad, Hercules, CA), and aliquots were stored at -80^oC.

Western Blot Analysis:

To determine Ape1 levels in SF767 cell extracts after immunodepletion, appropriate samples were mixed with equal amounts of 2X protein loading dye and boiled in boiling water bath for 5 minutes. The samples were then loaded onto a 12% Tris-HCl pre-cast gel (BioRad, Hercules, CA) and allowed to separate at 150 V for 40 minutes. The gel was then transferred onto a nitro-cellulose membrane at 90 V for 2 hours at room temperature. Following the transfer of proteins onto the membrane, the membrane was blocked in 5% blocking solution made from blotting grade blocker, non-fat dry milk (BioRad, Hercules, CA) dissolved in 1x TBS for 2-4 hours. Ape1 monoclonal antibody at a dilution of 1:1000 in 1% blocking solution was then added to the membrane and allowed to rotate overnight at 4^oC. The next day the membranes were washed with 1x TBST (1x TBS + 0.1% Tween 20) for 10 minutes, twice and a secondary anti-mouse HRP labeled antibody was added to the blots at a dilution of 1:1000 and allowed to rotate for 1-2 hours at room temperature. The membranes were washed once again with 1x TBST (1x TBS + 0.1% Tween 20) for 10 minutes, four times after which the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific,

Rockford, IL) was added to the blots. The blots were developed using the BioRad machine in the Chemiluminescence Hi-sensitivity mode.

The blots were processed as described above for the detection of actin, which was used as an internal loading control. The actin antibody (NeoMarkers, Fremont, CA) was used at a dilution of 1:1000 along with the anti-mouse secondary antibody also at a dilution of 1:1000. The actin membranes were detected using the ECL Western Blotting Detection reagent (GE Healthcare, Buckinghamshire, UK). The experiment was done three times, and the data is an average of three individual experiments with standard error. For both the gel assays with the immunodepleted extracts and the western blot analysis, comparisons were made between corresponding lanes in the IgG treated and the immunodepletd samples.

Gel-based AP Endonuclease Assay with immunodepleted SF767 cell extracts:

The AP endonuclease activity of the SF767 cell extracts immunodepleted of Ape1 was tested. A range of concentrations (0.9-15 ng) of the IgG controls and Ape1 antibody treated were tested in this assay as described above. The samples were incubated with the assay buffer and DNA substrate and kept at 37°C for 30 minutes and the reaction stopped by the addition of 10 μl of formamide without dyes.

Tissue culture with SF767 glioblastoma cells:

The SF767 glioblastoma cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) with high glucose supplemented with 10% Fetal Bovine Serum (FBS)

and 1% penicillin-streptomycin. The cells were passaged every two days to maintain them. All the cell based assays were done with these cells (111).

The MTT Assay to Measure Cell Survival and Proliferation:

The MTT assay is a quick and simple method to measure cell proliferation and can also be used to determine the cytotoxicity of laboratory and chemotherapeutic agents. The important component in the assay is a yellow tetrazole, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), which is reduced to purple formazan crystals in the mitochondria of living cells by the mitochondrial dehydrogenases. Dissolving these crystals in an appropriate solvent yields a purple solution, whose absorbance can be read at 570nm using a spectrophotometer. The amount of formazan crystals formed is proportional to the number of viable cells. Comparing the amount of formazan crystals produced by untreated control cells to those treated with an agent deduces the effectiveness of the agent in causing cell death by determining a dose-response curve (129). Cell survival after treatment with AR01, AR02 and AR06 was determined. Cells were treated with 0.05% trypsin and counted using a hemocytometer, and two thousand cells or media alone for the 72 hour time point were aliquoted into each well of a 96-well plate in triplicate and allowed to adhere overnight. The next day the cells were treated with the appropriate compounds and cell survival after 72 hours was ascertained by adding 20 µl of 0.5 mg/ml MTT to the wells. The cells were incubated at 37°C for 2 hours after which the solution in the wells was discarded by inverting the plates and firmly shaking them once to remove all the media. The plates with the formazan crystals in the wells were allowed to dry first face down for 30 minutes and then they were air-

dried for another 30 minutes. The resultant formazan crystals were then dissolved in 150 μl DMSO and the absorbance of the wells was read at 570 nm on the Versamax microplate reader (Molecular Devices, Sunnyvale, CA).

<u>Determination of Cell Survival and Proliferation using the xCELLigence System:</u>

The effects of the inhibitor compounds on the growth and survival of SF767 glioblastoma cells were determined using the xCELLigence DP System (Roche Applied Science, Indianapolis IN) (177, 209, 217). The xCELLigence system monitors in real time cell growth, attachment, and spreading based on an electronic system of impedance measurements. The attachment of cells in the wells results in an interference in continuous electronic current, which is read as impedance and is a measure of the property of the cells to attach and grow (101, 105, 146, 209) (Figure 9). For the xCELLigence DP System, 3000 SF767 glioma cells were plated in each well of the 16-well plates in 100 μl volume. Prior to plating the cells a background reading of the wells with 90 μl of appropriate media was recorded. After adding the cells to the wells, the plates were kept at room temperature for 30 minutes after which they were inserted into the cradles. The cells were allowed to grow for 20-24 hours before the cytotoxic agents were added. A range of MMS and AR03 concentrations were used to treat the cells and singly at 20x the final concentration in a 10 μl volume, and continuous impedance measurements were then monitored over a total of 96 hours. For the combined treatments with MMS and AR03, the additions were made in the same way as for MMS and AR03 alone. For TMZ alone and in combination with AR03, all the media from the wells was removed and the TMZ dilutions at final concentration were added in a 100 μl volume.

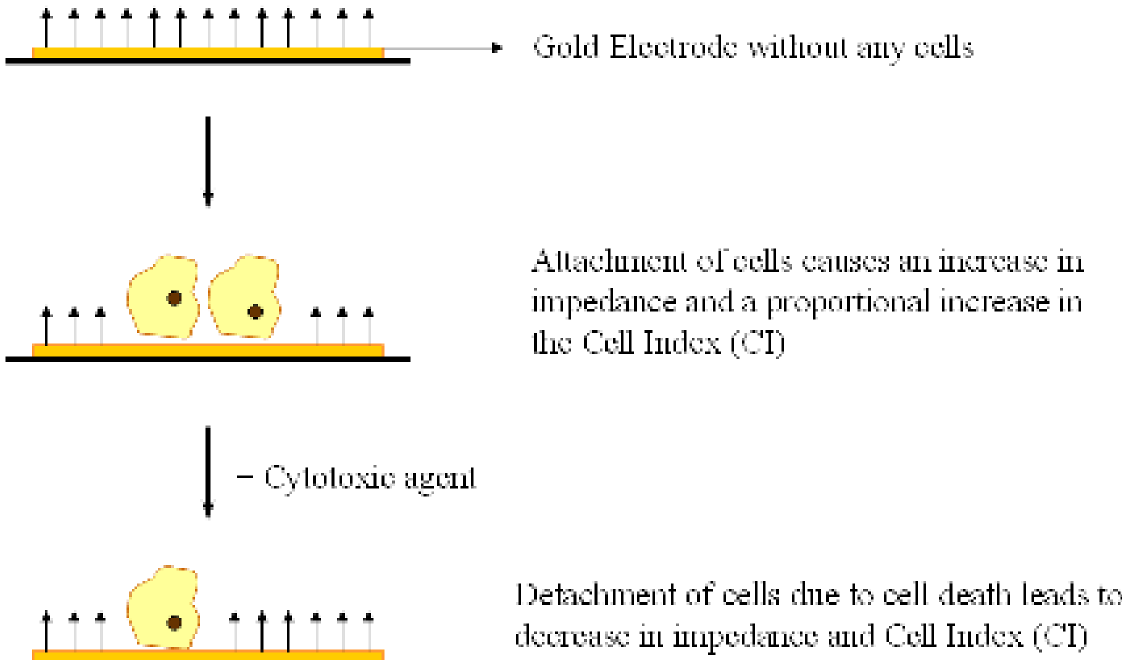


Figure 8: Principle of the xCELLigence assay
A schematic representation of the functioning of the xCELLigence assay. Adapted from the RTCA DP Instrument Operator's Manual (Roche Applied Science, Indianapolis, IN) and from Xing *et al* 2005 (209).

Once again, survival of the SF767 cells was monitored continuously. The assay was done in triplicate and was repeated three individual times.

Determination of AP Site formed using the Aldehyde Reactive Probe (ARP) Assay:

The ARP assay is a good assay to measure the number of AP sites generated in the DNA. The ARP reagent can specifically react with the aldehyde group of AP sites in the open conformation, and thus treating DNA containing AP sites with an excess of ARP reagent converts all AP sites to biotin-tagged AP sites. The amount of biotin can then be quantified by an ELISA-like assay (6, 109, 142, 143).

DNA Isolation:

To determine the number of AP sites formed, 2 x 10^6 SF767 cells were treated with 275 µM MMS and 3 µM AR03 alone or in combination for 24 hours after which the cells were collected and the genomic DNA was isolated using the Get-Pure DNA Isolation Kit (Dojindo Molecular Technologies, Rockville, MD). The cells were detached from the 10 cm^2 dishes using 2 ml of Trypsin and 5 ml of DMEM Media (with 10% FBS and 1% Penicillin & Streptomycin) and collected in 15 ml conical tubes. The cells were then centrifuged at 1200 rpm for 5 minutes. The media was then discarded and the cell pellet was re-suspended in 500 µl of 1x PBS (phosphate buffered-saline) and transferred to eppendorf tubes. This cell suspension was centrifuged at 2000 rpm for 5 minutes and once again the supernatant was discarded. The following steps were carried out using solutions provided in the Get – Pure DNA Isolation Kit (Dojindo Molecular Technologies, Rockville, MD). The cell pellets were then dissolved in 250 µl of the Lysis

solution by first vortexing them for 5 seconds and then pipeting the solution up and down 5 times after the additions of 10 µl of Proteinase K. The tubes were then incubated at 65°C for 10 minutes to completely dissolve any cell clumps present. 2 µl of RNAse A was added to the tubes and incubated at RT for 2 minutes in order to remove all the RNA present. 50 µl of Precipitation Solution I was added, and the tubes were vortexed for 5 seconds to result in a white precipitate. Addition of 50 µl of Precipitation Solution II with another 10 seconds of vortexing resulted in more white precipitate being formed. The tubes were centrifuged at 14,000 rpm for 10 minutes twice to clear the supernatants of the precipitate. The supernatants were transferred to new tubes, and equal amounts of 100% EtOH (ethanol) were added. The tubes were rocked gently to mix, and the DNA was visualized as a white precipitate in the tubes. The tubes were then centrifuged at 8000 rpm for 2 minutes, and the white pellet washed with 1 ml of 70% EtOH, centrifuged at 8000 rpm for 2 minutes, and the supernatant discarded. The DNA pellet was air dried by inverting the tube for 30 minutes. The DNA was then dissolved in 50 µl of TE buffer and allowed to dissolve completely overnight at 4°C. The next day, the concentration of the DNA was measured using the SmartSpec™ 3000 (BioRad, Hercules, CA) and the DNA was diluted to 100 µg/ml (25 µl). The concentration of the 100 µg/ml DNA dilution was confirmed and adjusted using the SmartSpec™ 3000 (BioRad, Hercules, CA).

AP Site Determination:

To determine the number of AP sites formed, 10 µl of the 100 µg/ml DNA dilution was mixed with 10 µl of the ARP solution from the DNA Damage Quantification, AP site Counting Kit (Dojindo Molecular Technologies, Rockville, MD)

and incubated at 37°C for 1 hour. The inside of the filtration tube was then washed with 100 µl of TE twice and after the incubation was complete, 380 µl of TE were added to the reaction mixture and transferred to the filtration tube. The tubes were centrifuged at 2500 *xg* for 15 minutes, and the filtrate solution was discarded. The DNA on the filter was then dissolved in 400 µl of TE by gently pipeting up and down. The tubes were once again centrifuged at 2500 *xg* for 15 minutes, and the filtrate solution was discarded. The ARP-labeled DNA on the filter was then resuspended in 200 µl of TE and transferred to a fresh eppendorf tube. The entire remaining DNA from the filter was transferred to the tube with another 200 µl of TE and 180 µl of this ARP-labeled DNA was then diluted with 220 µl of TE. 200 µl of this diluted ARP-labeled DNA was mixed with 333 µl of the DNA binding buffer in a new tube, and 160 µl of this mix was plated in triplicate in the coated ELISA plate provided with the kit, covered with a piece of parafilm. The DNA was allowed to attach to the plate overnight at RT. The next day, the DNA solution was discarded by inverting the plate once, and the plate was washed 5 times with 250 µl of the washing buffer, which is dissolved in 1L of distilled water. After the washes, using a multi-channel pipet, 150 µl of the diluted HRP-streptavidin (HRP-streptavidin stock diluted 1 / 4000 in washing buffer → 10 µl of the HRP-streptavidin + 40 ml of the washing buffer) was added to the wells. The plate was then covered with a piece of parafilm and was incubated at 37°C in the tissue culture incubator in the dark for 1 hour. Once again the solution in the plate was discarded by inverting the plate once, and the plate washed 5 times with 250 µl of the washing buffer. Finally, 100 µl of the substrate solution was added to the wells, and once again the plate was incubated at 37°C in the dark for 1 hour. Within 30 minutes of the end of the final incubation, the absorbance of

the plate was read at 650 nm using the Versamax microplate reader (Molecular Devices, Sunnyvale, CA). The number of AP sites formed was calculated based on the standard curve, and the number of AP sites is represented against 10^6 base pairs.

Statistics:

Each of the above mentioned experiments were carried out with the appropriate controls including vehicle controls (DMSO), and they were all repeated at least three individual times. The cell survival assays (MTT and xCELLigence experiments) and the AP site determinations were done in triplicate in addition to repeating them at least three individual times. P values of significance were calculated using the Student's t-test.

In case of the IC_{50} value calculations and the gel-based assays with pure Ape1 protein and SF767 cell extracts alone, the lanes with inhibitors were compared to the lane containing the vehicle control (DMSO) and without inhibitors.

For the experiments showing restoration of AP endonuclease activity of the cell extracts, the lanes treated with the Ape1 repair inhibitor are compared back to the vehicle (DMSO) treated control lane and the lanes with the cell extracts. Inhibitor and purified Ape1 protein were compared to the lane with cell extracts and inhibitor alone.

For both the gel-based AP endonuclease assays and the western analysis of the immunodepleted cell extracts, comparisons were made between corresponding lanes in the IgG treated control samples and the immunodepleted samples.

For the cell survival studies using the MTT assay for both experiments with single agent and combined treatment, columns with cells treated with the various compounds were compared back to the vehicle treated control. For the xCELLigence data,

representative experiments are shown. However, a similar trend was noted during the individual repeats of these experiments.

CHAPTER IV

RESULTS

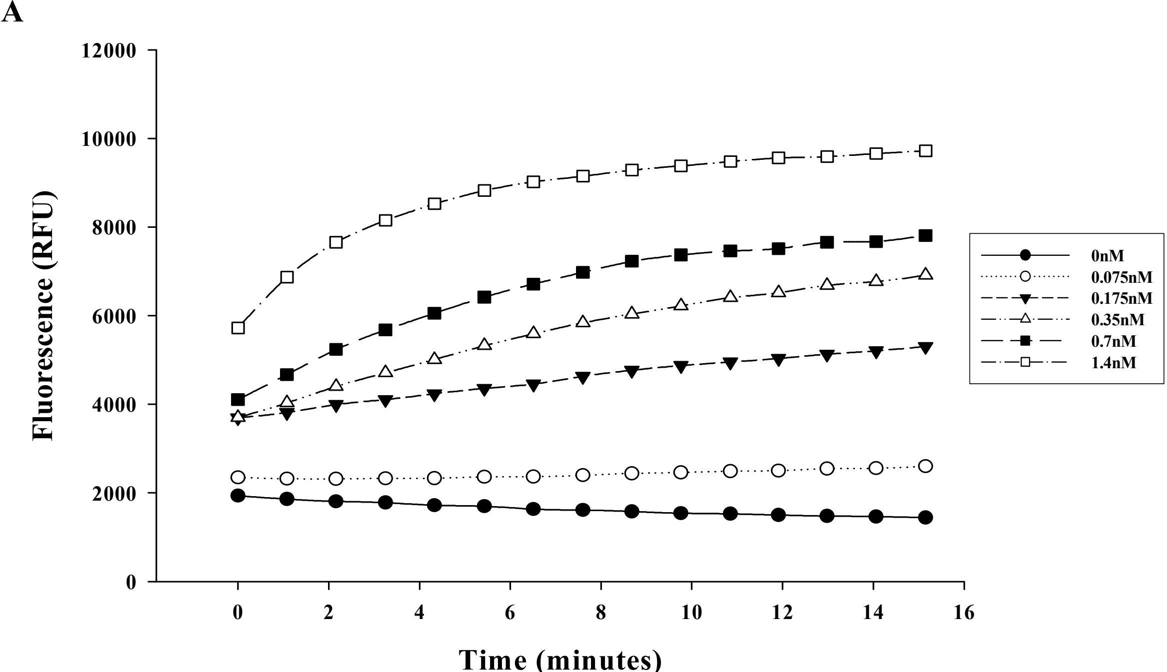
Optimization of the High Throughput Screening (HTS) Assay used to identify inhibitors of Ape1's DNA repair activity

We optimized and improved upon an HTS assay from that was described by Madhusudan *et al* (132) in our efforts to identify compounds, which inhibit Ape1's DNA repair activity. A library of 60,000 versatile and drug-like compounds from Chemical Diversity Ltd (San Diego, CA) was tested in this HTS assay. The assay is based on a system in which the measurement of the change in fluorescence is representative of the activity of the protein of interest. The assay employs two synthetic oligonucleotides annealed together, which are 30 base pairs long. One of the DNA strands has a unit of fluorescein (6-FAM) at its 5' end and a THF molecule at position 7 mimicing an AP site, the substrate that Ape1 acts upon (201). The 3' end of the complimentary DNA strand has a dabcyl group also known as a dark quencher and is able to quench the fluorescence of the fluorescein molecule (Figure 7). When the recombinant Δ40 Ape1 protein is added to the reaction mix containing the annealed strands of DNA along with the appropriate buffers, Ape1 cuts the DNA at the THF moiety resulting in the release of a short fragment tagged with fluorescein. This distance from the dabcyl quencher results in a concomitant increase in fluorescence, which I measured as an indicator of Ape1's activity (132).

Increasing concentrations of Ape1 were tested in this assay and changes in fluorescence were measured for each Ape1 concentration over a 15 minute time period to

Optimization of HTS assay conditions:

A



B

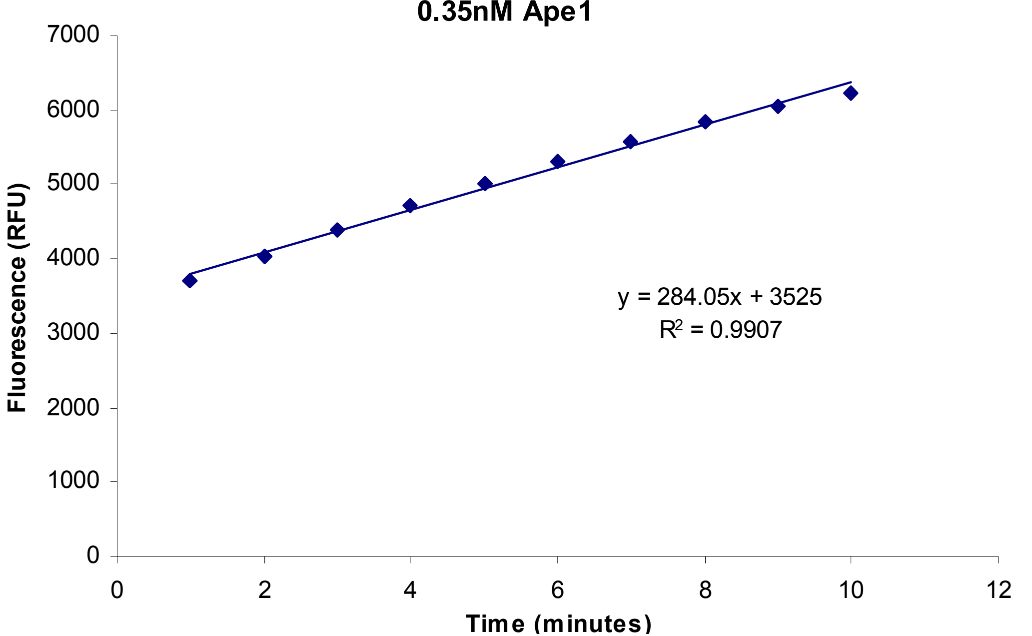


Figure 9: Optimization of the Conditions used in the HTS Assay
(A) Range of concentrations of Ape1 was tested in the HTS assay to determine a concentration of the Ape1 protein in the linear range. **(B)** 0.35 nM Ape1 shows a linear increase in fluorescence over time.

determine a concentration of Ape1 that would work in the linear range (Figure 10A). Figure 10B shows that 0.35 nM Ape1 shows a linear increase in fluorescence over time. Rates of reaction rather than absolute fluorescence were used to determine activity of the Ape1 protein and to compare a reaction in which Ape1 activity was inhibited from a control reaction. I wanted to have a distinct positive reaction with the 0.35 nM Ape1 alone so as to distinguish it from an inhibited reaction of Ape1. The reaction with 0.35 nM recombinant Ape1 protein was linear over 10 minutes and therefore, the library of compounds was screened with this concentration and fluorescence was monitored for 5 minutes.

Z' Factor Measurement

The Z' factor is a measure of the accuracy and precision of a high-throughput screening (HTS) assay (216). The Z'factor reflects: 1) the dynamic range between the positive and negative controls, 2) the reproducibility of the data and 3) reliability of the assay. This value indicates whether the window of opportunity to detect hits is distinct enough from the noise at the positive and negative controls. The four parameters required to calculate the Z'-factor, the averages (μp and μn) and standard deviation (δp and δn) of both the positive and negative controls, were obtained by performing the assay as described in the Materials and Methods. A Z' factor value between 0.5-1 is an indicator of a good and reliable assay with an ideal Z' factor value of 1.0, which is achieved with a large dynamic range and small standard deviations. However Z' factor values are rarely 1.0 and are never greater than 1.0 (216). 384 measurements each for the positive and negative controls were carried out (one 384-well plate) and the Z' factor calculated using

Z' factor measurement:

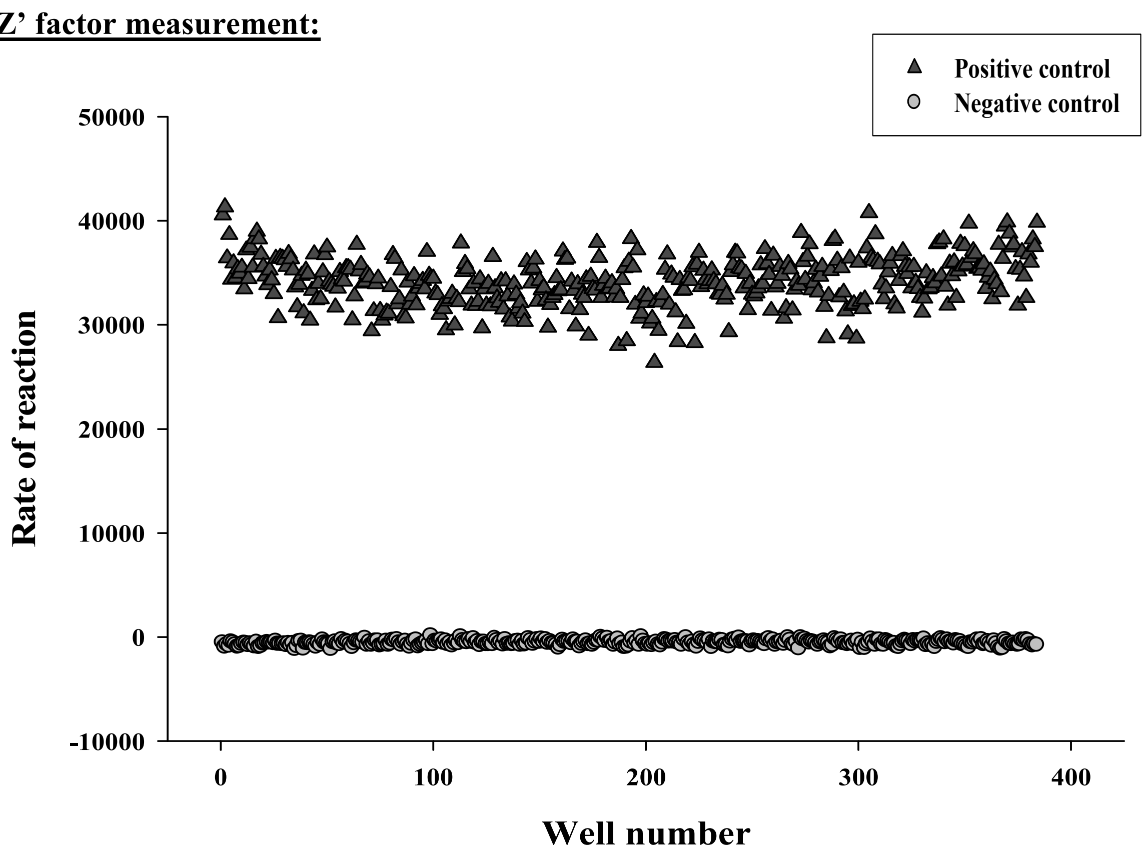


Figure 10: Z' factor measurement for the HTS assay
The Z' factor for the assay was measured using the HTS assay. The Z' factor value calculated for the screen was 0.78.

these values. The Z' factor value measured for the HTS assay was 0.78, which is within the range of acceptable Z' factor values (between 0.5-1) (Figure 11). The Z' factor value was calculated with each new batch of oligonucleotides and for all the measurements, the Z' factor was similar to the one reported.

<u>High Throughput Screen (HTS) to identify inhibitors of Ape1</u>

After optimizing the HTS assay and verifying that my assay was reliable, a 60,000 compound library from ChemDiv was screened to identify potential inhibitors of Ape1. This library conforms to Lipinski's rule of 5 (119) and is a varied collection of synthetic compounds. The compounds were screened using the following parameters and equipment: an inhibitor concentration of 10 μM, 50 μl reaction volume, black 384-well plates and the Genesis Workstation 150 TeMo with a 96-channel pipetting head (Tecan, Durham NC) at the IU Chemical Genomics Core (CGCF) facility. Assay additions to the plates containing the compound library were made such that 100 nM of the double stranded oligonucleotide substrate and the 1x assay buffer were added first, and the reaction was started by the addition of 0.35 nM Ape1 protein. Every assay plate included one column each of a positive and negative control for the assay. The positive control lane contained the Ape1 protein with no inhibitors, and as a negative control EDTA was added to the reaction mix. EDTA serves as the negative control for the assay as it chelates the Mg^{2+} required for Ape1 activity thereby inactivating the protein. Displacement of the short labeled piece of DNA and the resultant increments in fluorescence were measured over time. After an initial screen of the library, 190 hits showing ≥ 50% inhibition of Ape1's activity were identified (Figure 12A). Out of these

HTS Assay Results:

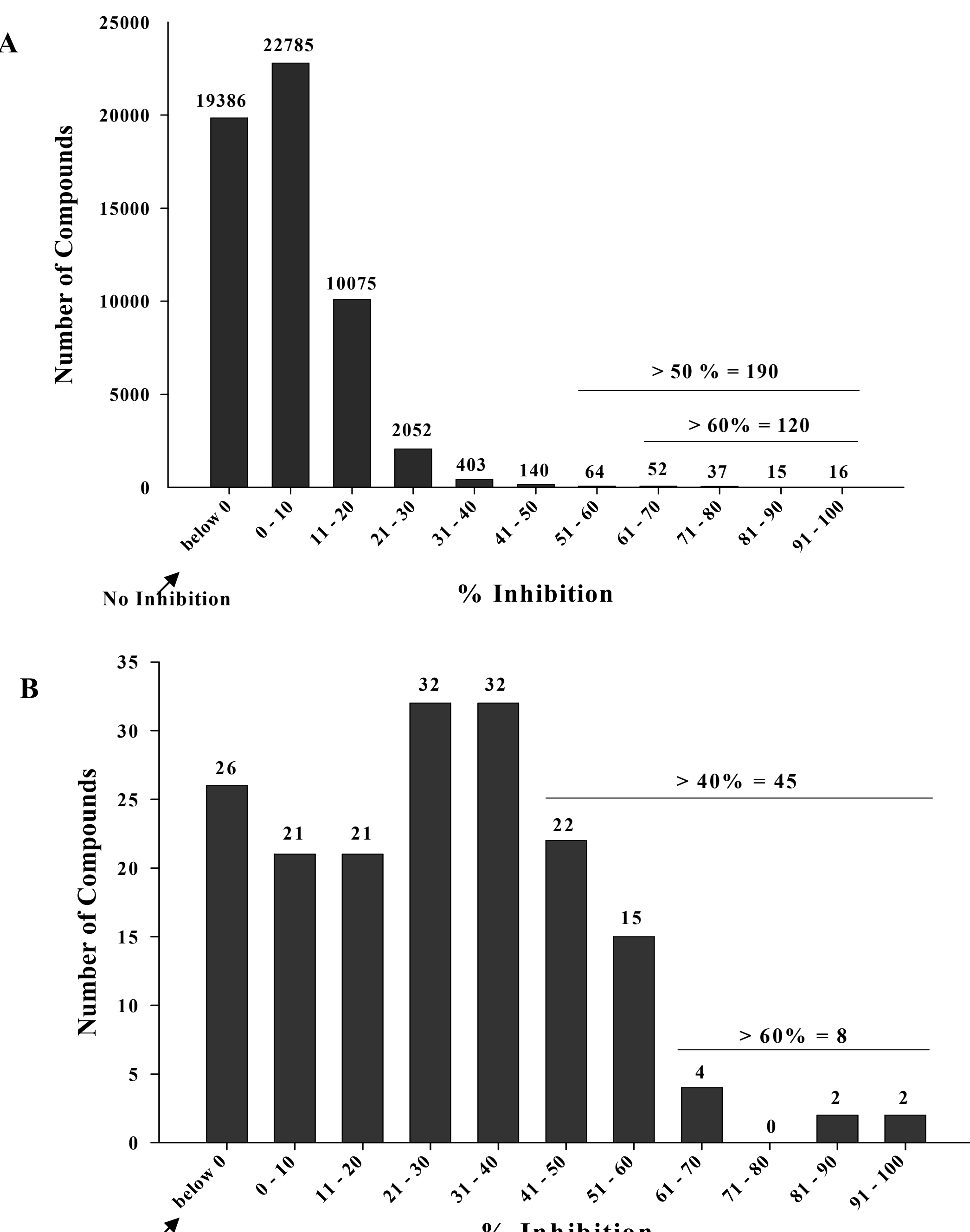


Figure 11: Results of the HTS assay of the compound library for inhibitors of Ape1 (A) Results of the initial screen identified 190 compounds that inhibited Ape1's activity by 50% or more. **(B)** Results of a secondary screen of the hits from the initial screen identified 45 compounds with ≥ 40% inhibition of Ape1's DNA repair activity, where the graphs are representations of the numbers of compounds plotted with their corresponding % inhibition of Ape1's activity.

List of compounds identified after two rounds of high-throughput screening:

Structure	ID	Mol Wt	HTS Assay (% Inhibition)		IC_{50} (µM)	50% inhibition of Ape1 - Gel assay (µM)	MTT Assay – ED_{50} (µM)
			1st	2nd			
	AR01	245.3	74	70	1.7 ± 0.3	0.4	>800
	AR02	278.4	54	40	6.4 ± 1.1	25	~7.5
	AR03	237.3	60	58	2.1 ± 0.1	5	~1
	AR04	350.4	58	45	14.9 ± 8.4	~25	~3
	AR05	351.4	75	85	6.6 ± 0.9	0.75	~50
	AR06	262.7	52	44	1.6 ± 0.1	0.7	>100
	AR07	400.5	80	41	~ 100	ND	ND
	AR08	259.3	71	21	>100	ND	~80
	AR09	322.4	51	52	42.9	ND	~8

	AR10	290.4	54	49	9.8	~50	~10
	AR11	278.4	53	50	4.7	~50	~10
	AR12	312.8	54	45	15.7	>50	~6
	AR13	308.4	62	57	15.9	>100	~10
	AR14	357.2	72	58	19.7	~100	~5
	AR15	312.8	70	57	17.7	~50	~8
	AR16	282.8	51	42	19.6	~50	~8
	AR17	290.8	50	52	~100	ND	ND

	AR18	341.5	83	55	7.1	>50	~6
	AR19	367.5	52	45	>100	ND	ND
	AR20	220.7	76	94	>100	ND	ND
	AR21	403.6	66	ND	50.9	ND	ND
	AR22	258.3	61	ND	>100	ND	ND
	AR23	479.7	52	48	>100	ND	ND
	AR24	376.5	77	92	>100	ND	ND
	AR25	372.5	78	19	~100	ND	ND
	AR26	370.5	79	20	~100	ND	ND
	AR27	358.5	87	3	>100	ND	ND

	AR28	412.6	83	29	>100	ND	ND
	AR29	386.5	80	30	>100	ND	ND
	AR30	386.5	94	36	>100	ND	ND
	AR31	400.5	95	35	>100	ND	ND
	AR32	370.5	82	26	42.4	>100	ND
	AR33	372.5	91	1	>100	ND	ND
	AR34	420.5	63	44	63.9	ND	ND
	AR35	398.5	80	35	>100	ND	ND
	AR36	398.5	93	43	>100	ND	ND

	AR37	373.9	52	28	~25	ND	ND
	AR38	399.9	68	29	>100	ND	ND
	AR39	371.9	53	22	>100	ND	ND
	AR40	419.9	77	34	>100	ND	ND
	AR41	297.1	64	58	0.8	0.75	~50
	AR42	256.3	53	25	26.6	ND	ND
	AR43	258.3	52	48	3.1	ND	ND

Table 2: A list of the preliminary compounds and their IC_{50} values

IC_{50} values (μM)	< 10	11 – 50	51 – 100	> 100
Number of compounds	9	9	6	17

Table 3: Range of IC_{50} values of the HTS assay compounds

190 compounds, 174 were re-screened in the same HTS assay. After two rounds of screening, 45 compounds exhibiting greater than or equal to 40% inhibition of Ape1's DNA repair activity were chosen for further validation assays (Figure 12B). A list of the 41 compounds validated is presented in Table 2.

Determination of IC_{50} values of the identified hits

After two rounds of screening, 41 target compounds were identified and considered for further validation. IC_{50} values of these 41 compounds were calculated using the previously described HTS assay. Each of the compounds was tested at several different concentrations (0.1-100 μM) with 0.35 nM Ape1 in the HTS assay and fluorescence readings were taken over 5 minutes. Percent inhibition of Ape1's activity at each compound concentration was ascertained as compared to the control with no inhibitor. NCA (132) was used as a control at a concentration of 10 μM, the same concentration at which the compound library was screened in the HTS assay. IC_{50} values were calculated using the Sigma plot software as described in the Materials and Methods. Out of the 41 compounds identified after two rounds of screening, 18 compounds showed an IC_{50} value less than or equal to 50 μM, and 9 of them had an IC_{50} better than or equal to 10 μM (Table 3). The IC_{50} value curves for the compounds AR01, AR03, AR06 and AR02 are presented in Figure 13.

For the sake of convenience from here on forward, I have grouped the data for

Determination of IC_{50} values of the top four compounds:

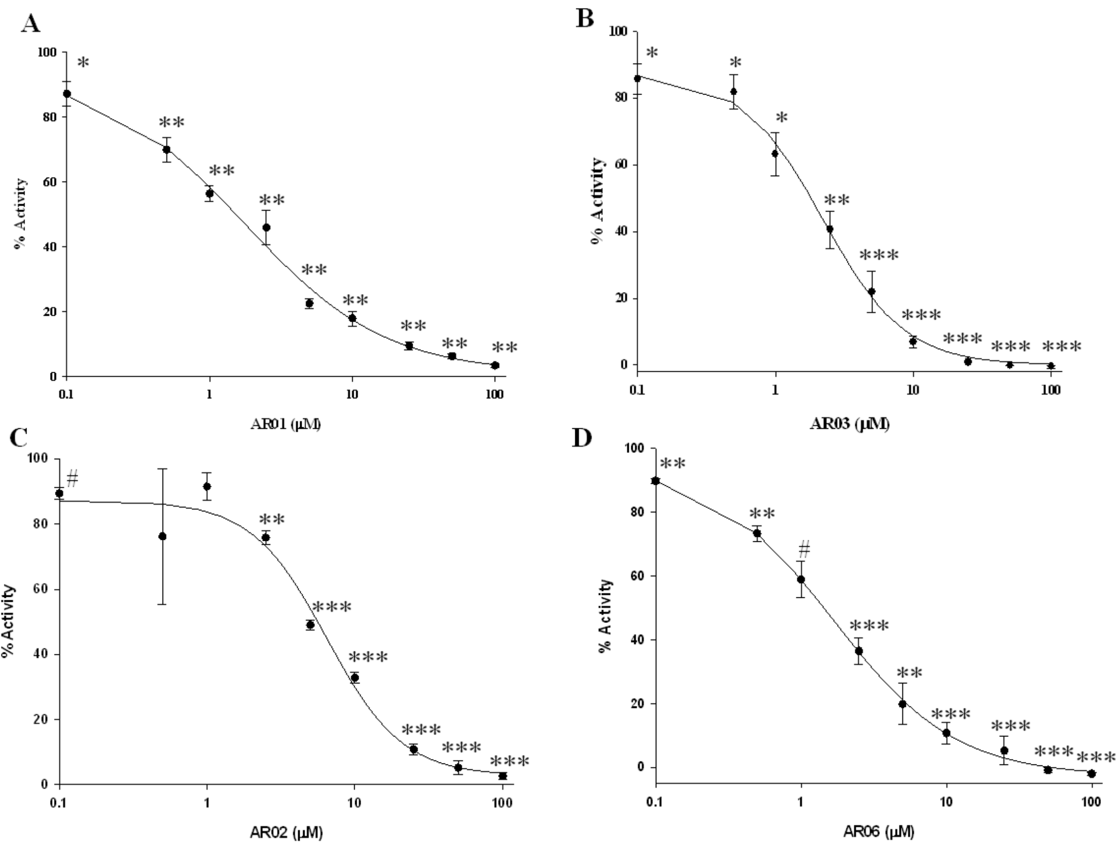


Figure 12: Calculation of the IC_{50} values of the top hit compounds
IC_{50} values of the top hit compounds were determined using the HTS assay. The assays for each compound were performed in triplicate. The Sigma Plot software (equation described in Materials and Methods) was used to calculate the values and presented here is a semi-log plot of the IC_{50} value determination. IC_{50} value curves for AR01 **(A)**, AR03 **(B)**, AR02 **(C)** and AR06 **(D)** are represented here. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = $p \leq 0.05$; # $p \leq 0.005$; ** = $p \leq 0.0005$; *** = $p \leq 0.0001$.

Gel-based AP endonuclease assay with purified Ape1 protein:

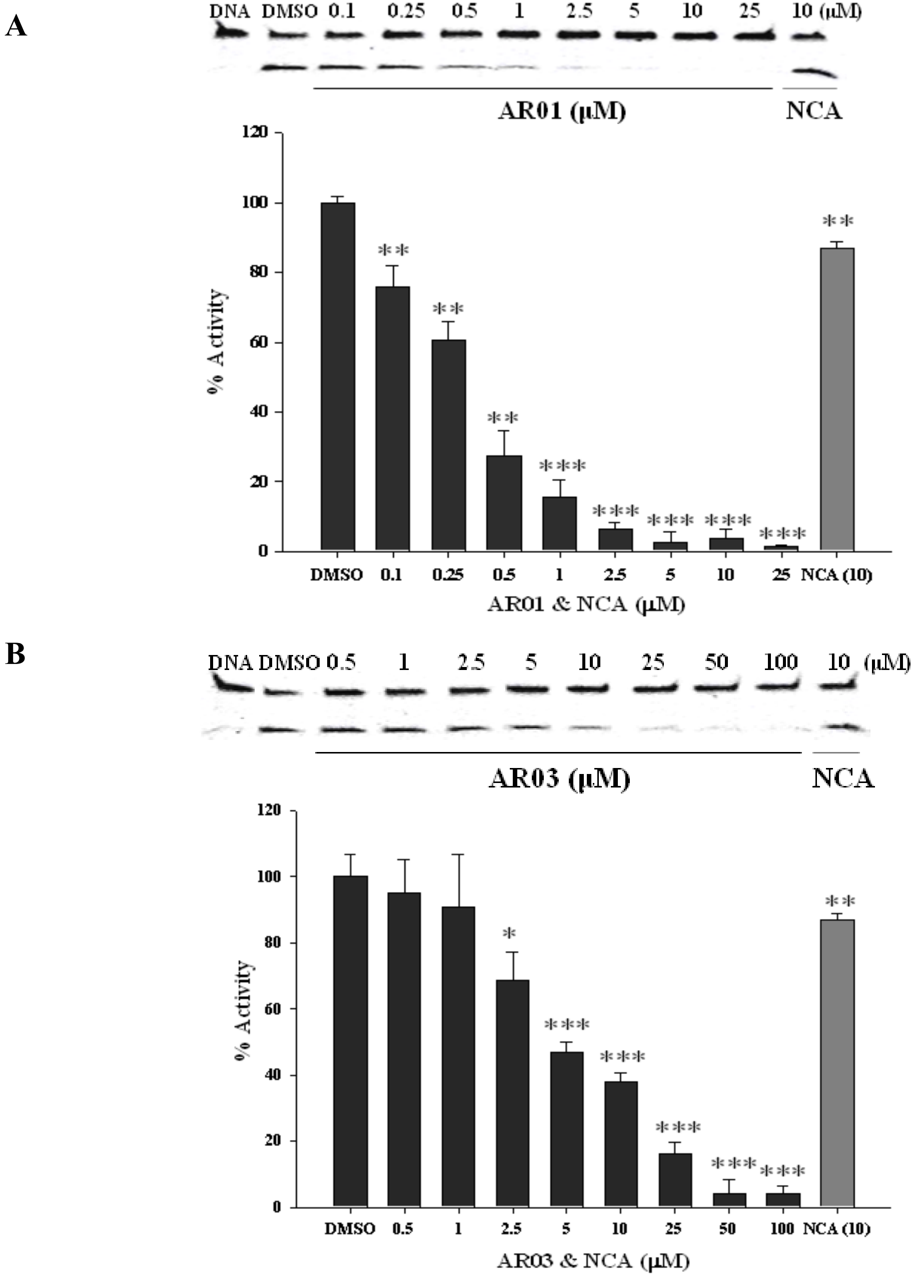


Figure 13: The compounds AR01 and AR03 can inhibit the activity of purified Ape1 protein in the gel-based AP endonuclease assay

A range of concentrations of AR01 **(A)** and AR03 **(B)** were tested with 0.175 nM Ape1 in the gel-based AP endonuclease assay. Representative gels are shown here for all the compounds. Each assay was performed in triplicate and is shown here as the average with standard error. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = $p \leq 0.05$; # $p \leq 0.005$; ** = $p \leq 0.01$; *** = $p \leq 0.0001$.

<u>Gel-based AP endonuclease assay with purified Ape1 protein:</u>

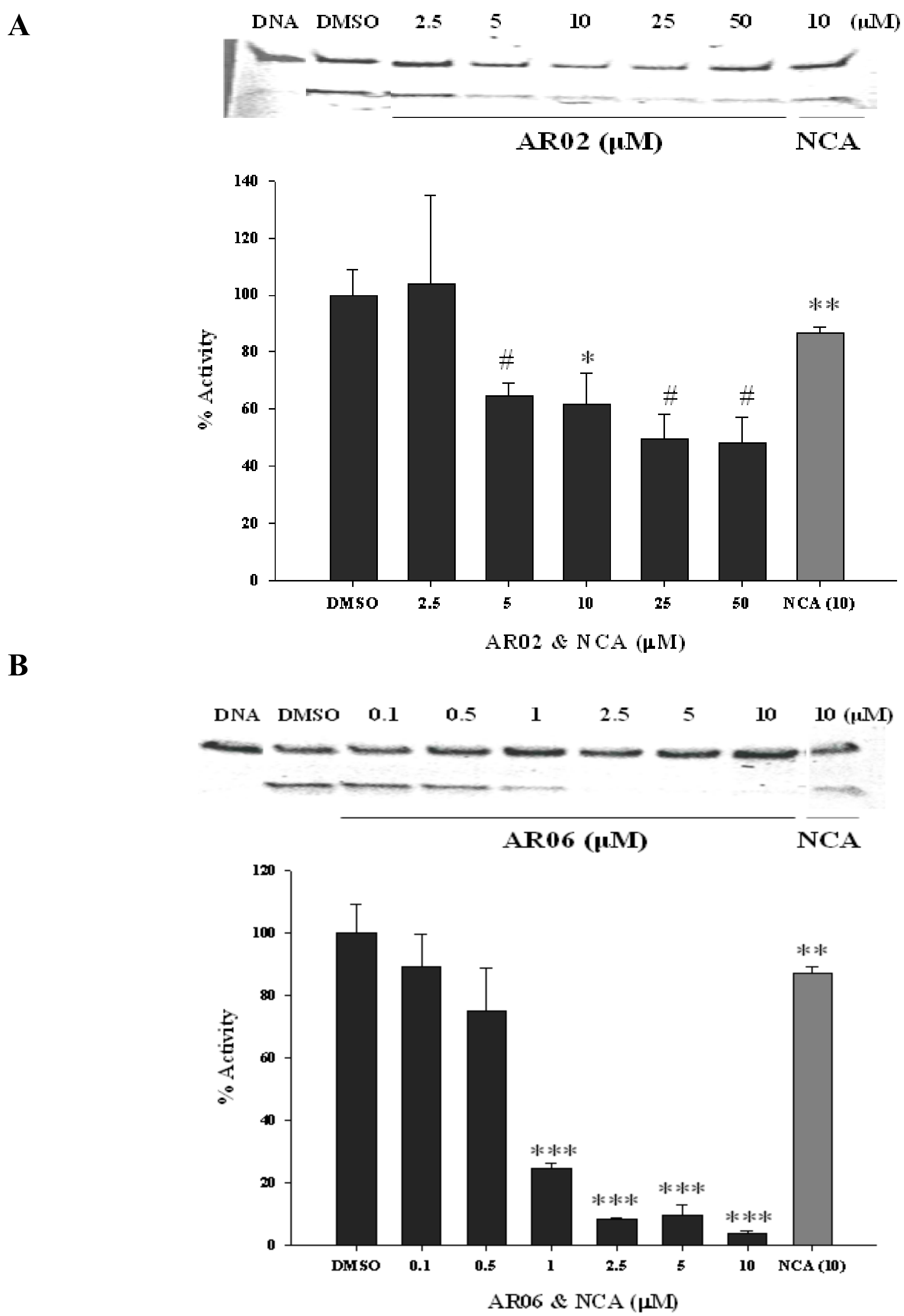


Figure 14: The compounds AR06 and AR02 can inhibit the activity of purified Ape1 protein in the gel-based AP endonuclease assay

A range of concentrations of AR02 **(A)** and AR06 **(B)** were tested with 0.175nM Ape1 in the gel-based AP endonuclease assay. Representative gels are shown here for all the compounds. Each assay was performed in triplicate and is shown here as the average with standard error. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = p≤ 0.05; # p≤ 0.005; ** = p≤ 0.01; *** = p ≤ 0.0001.

compounds AR01 and AR03 together in one figure as I consistently saw similar results with these compounds. The data for compounds AR02 and AR06 are grouped together.

Target validation to determine selectivity of the inhibitor compounds for Ape1's DNA repair activity in other *in vitro* assays

Two different assays to validate the 18 compounds with IC_{50} values less than 50 μM were performed: 1) the compounds ability to inhibit Ape1 in another distinct *in vitro* gel-based AP endonuclease assay (110) (Figure 8) and 2) inhibition of the functionally related and structurally unrelated AP endonuclease, the *E. coli*, endonuclease IV protein (63, 141, 199). The 41 compounds can inhibit Ape1's DNA repair activity both in the HTS assay and the gel-based assay (Table 2). Based on the confirmation of both assays that these compounds inhibit Ape1's repair activity, we chose four compounds from distinct families: AR01 (2-(4-(2,5-dimethyl-1H-prryol-1-yl)phenoxy acetic acid), AR02 (4-(2,6,8-trimethylquinolin-4-ylamino)phenol), AR03 (2,4,9-trimethylbenzo [b][1,8] naphthyridin-5-amine) and AR06 (N-(3-chlorophenyl)-5,6-dihyro-4H-cyclopenta [d] isoxazole-3-carboxamide) (Table 4).

After ascertaining that the four potential inhibitors do not bind genomic DNA (Table 2) and are able to inhibit Ape1 in two different AP site assays (Figures 13, 14 and 15), I tested the selectivity of these compounds for Ape1 by determining their effect on a functionally similar, but structurally different AP endonuclease protein, the *E. coli* endonuclease IV protein (63, 141, 199). The endonuclease IV protein was incubated with a range of concentrations of each of the four top compounds under similar conditions as described above. The gel-based assays was used to determine whether these four

Structure of the compound	ID	Mol Wt	HTS Assay (% Inhibition)		IC50 (µM)	ΔFID (% DNA binding)	Gel-based assay – 50% inhibition (µM)			Cell kill – MTT assay (ED50 µM)
			1st	2nd			Ape1	Endo IV	SF767 ext	
	AR01	245.3	74	70	1.7 ± 0.3	8	0.4	1.75	2.4	>800
	AR02	278.4	54	50	6.4 ± 1.1	~10	25	>50	~93	~7.5
	AR03	237.3	60	58	2.1 ± 0.1	~13	~5	~40	13	~1
	AR06	262.7	52	44	1.6 ± 0.1	~3	0.7	~10	~75	>100

Table 4: Top four compounds identified in the HTS assay

Gel-based AP endonuclease assay with Endonuclease IV protein:

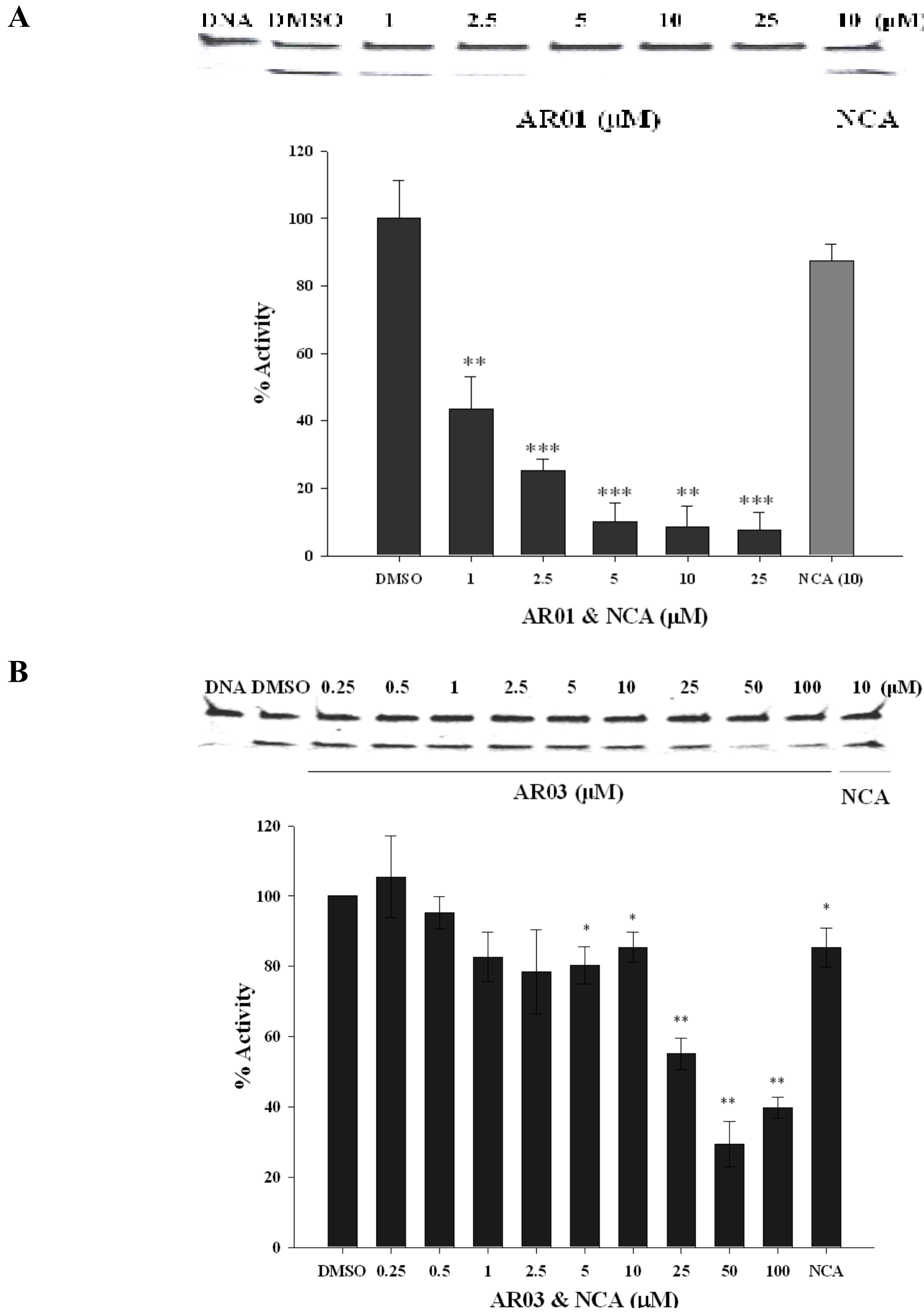


Figure 15: Effect of AR01 and AR03 on the activity of the endonuclease IV protein The ability of AR01 **(A)** and AR03 **(B)** to inhibit the activity of endonuclease IV was determined in the gel-based AP endonuclease. A range of concentrations of each of the compounds were tested with 6.25 units of endonuclease IV protein. Representative gels are shown for all the compounds. Each assay was performed in triplicate and is presented here as the average with standard error. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = p≤ 0.05; ** = p≤ 0.0005; *** = p ≤ 0.0001.

Gel-based AP endonuclease assay with Endonuclease IV protein:

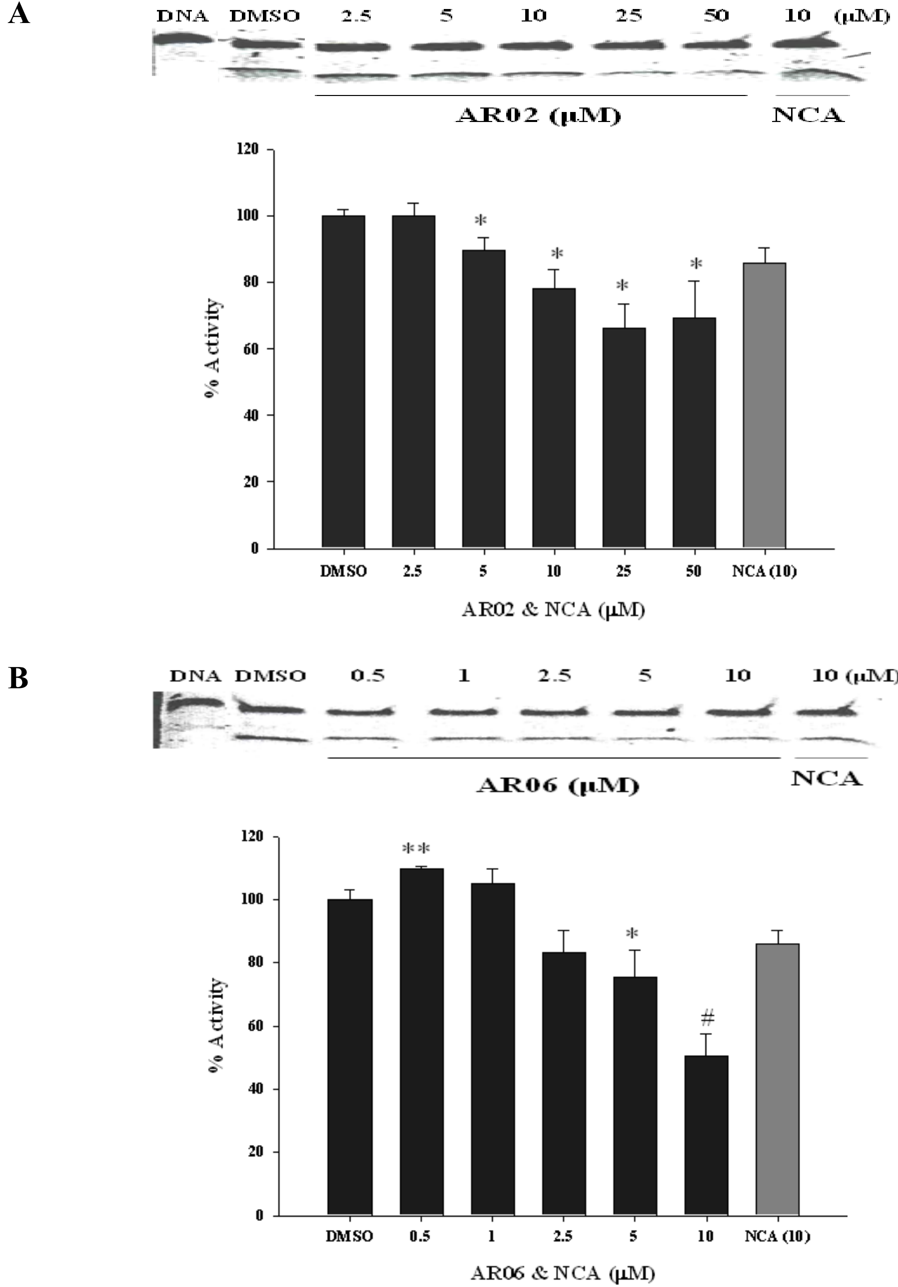


Figure 16: Effect of AR06 and AR02 on the activity of the endonuclease IV protein The ability of AR02 **(A)** and AR06 **(B)** to inhibit the activity of endonuclease IV was determined in the gel-based AP endonuclease. A range of concentrations of each of the compounds were tested with 6.25 units of endonuclease IV protein. Representative gels are shown for all the compounds. Each assay was performed in triplicate and is presented here as the average with standard error. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = p≤ 0.05; # = p≤ 0.005; ** = p≤ 0.0005.

compounds could inhibit endonuclease IV protein. The four compounds had an effect on the activity of endonuclease IV but at varying degrees. The concentration of AR01 required to inhibit the activity of endonuclease IV by 50% was similar to the concentration required to inhibit Ape1's activity by 50% (0.6 µM and 1.7 µM respectively), whereas a 16-fold higher concentration of AR03 (~40 µM) was required to inhibit the activity of Endonuclease IV protein by 50% (Figure 16) as compared to its IC_{50} value (2.1 µM). A concentration greater than 50 µM of AR02 and ~10 µM of AR06 were required to inhibit the activity of Endonuclease IV activity by 50% (Figure 17).

Therefore, although AR01 inhibits Ape1's activity, it also inhibits the activity of endonuclease IV at similar concentrations. The compounds AR03, AR02 and AR06 on the other hand required much higher amounts (16-fold, ~10-fold and ~6-fold respectively) as compared to their IC50 values to effect the same amount of inhibition of endonuclease IV's activity.

Compound ID	**IC_{50} values (µM)**	**Concentration required to inhibit activity by 50% in the gel assay (µM)**	
	Ape1	**Ape1**	**Endonuclease IV**
AR01	1.4	0.4	1.75
AR02	6.4	25	> 50
AR03	2.2	5	~40
AR06	1.6	0.7	~10

Table 4: Comparison of values of the top four compounds required to inhibit Ape1 and endonuclease IV proteins

Ability of the target compounds to inhibit Ape1 in whole cell extracts

Since the four target compounds inhibit recombinant Ape1 protein in the HTS assay and in the gel assay, I wanted to determine if these compounds could inhibit Ape1 in whole cell extracts. Whole cell extracts from SF767 glioblastoma cells were prepared as described previously (110), and inhibition of the AP endonuclease activity of these

extracts was determined in the gel-based assay. SF767 cell extracts (3.75ng) were treated with increasing concentrations of the four compounds to determine their effect on Ape1's DNA repair activity. As shown in Figure 18, AR01 and AR03 were able to inhibit the AP endonuclease activity of the cell extracts at concentrations comparable to those required to inhibit pure Ape1 in the gel-based assay (0.5 µM and 13.2 µM respectively). AR02 and AR06 (Figure 19) also inhibited Ape1's activity in the extracts, but where relatively low concentrations of AR01 and AR03 (Figure 18) were required for inhibition, ~15-fold and ~17-fold higher concentrations of AR02 and AR06 (~93 µM and 26 µM respectively for 50% inhibition) were required to inhibit Ape1's activity in the same cell extracts (Figure 19).

Compound ID	**IC_{50} value (µM)**	**Concentration required to inhibit activity by 50% in the gel assay (µM)**		
		Ape1	**Endonuclease IV**	**SF767 cell extracts**
AR01	1.4	0.4	1.75	2.4
AR02	6.4	25	> 50	~93
AR03	2.2	5	~40	13
AR06	1.6	0.7	~10	~75

Table 5: Values of the top compounds for inhibition of Ape1, Endonuclease IV and SF767 cell extracts

<u>**Further determination of selectivity of the top compounds**</u>

Purified Ape1 can resuce the AP endonuclease activity of SF767 cell extracts treated with the inhibitors

To further ascertain the selectivity of the inhibitors for Ape1 in the SF767 cell extracts, I assayed the ability of pure Ape1 protein to rescue AP endonuclease activity of these SF767 cell extracts treated with my top four inhibitors. To this end, the cell extracts

Gel-based AP endonuclease assay with SF767 cell extracts:

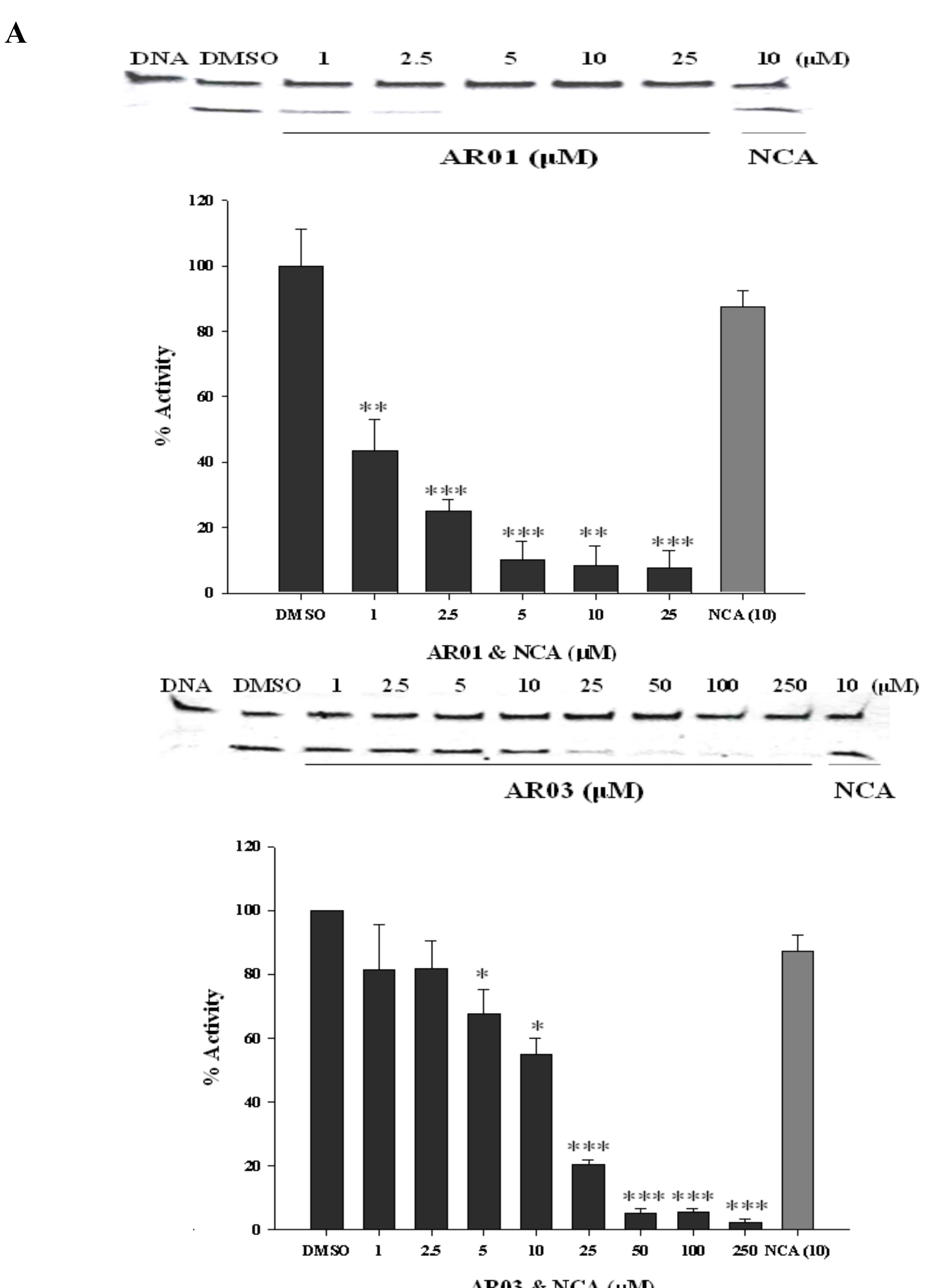


Figure 17: Ability of AR01 and AR03 to inhibit Ape1's activity in SF767 glioblastoma cell extracts
A range of concentrations of AR01 **(A)** and AR03 **(B)** were tested with 3.75 ng of SF767 cell extract in this assay. Representative gels are shown for all the compounds. Each assay was performed in triplicate and is shown here as the average with standard error. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = $p \leq 0.01$; ** = $p \leq 0.05$; $p \leq 0.005$; *** = $p \leq 0.0001$.

Gel-based AP endonuclease assay with SF767 cell extracts:

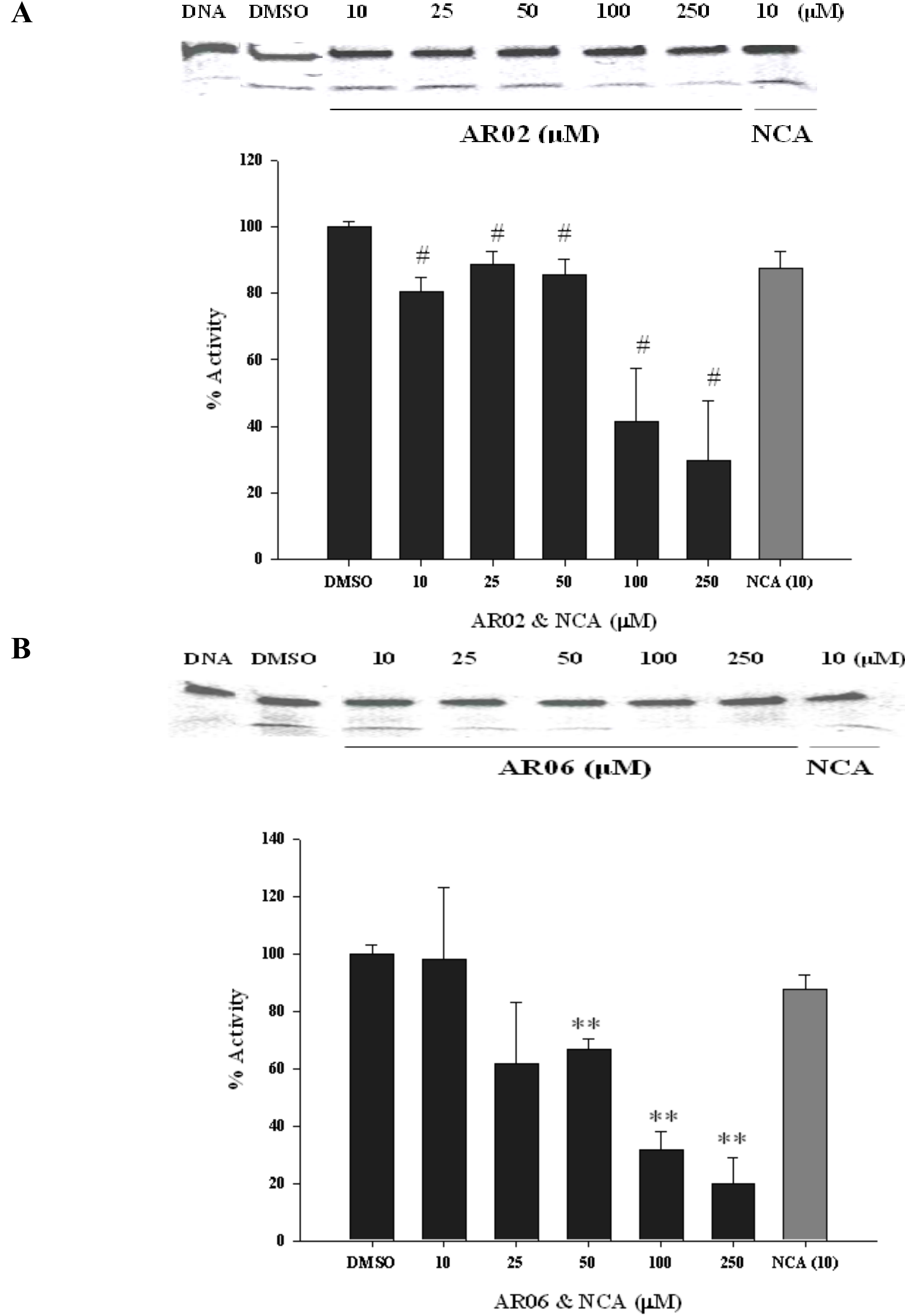


Figure 18: Ability of AR02 and AR06 to inhibit Ape1's activity in SF767 glioblastoma cell extracts

A range of concentrations of AR02 **(A)** and AR06 **(B)** were tested with 3.75 ng of SF767 cell extract in this assay. Representative gels are shown for all the compounds. Each assay was performed in triplicate and is shown here as the average with standard error. P values were calculated using the student's t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); # = $p \leq 0.05$; ** = $p \leq 0.005$.

were treated with a concentration of each of the four compounds that inhibited more than 50% of Ape1's activity. After incubating cell extracts with the four compounds for 30 minutes, varying amounts of purified Ape1 protein (0.7-5.6 nM) were added to them, and the reaction was allowed to proceed for another 30 minutes. When increasing amounts of purified Ape1 protein were added to SF767 extracts treated with 10 μM AR01 and 50 μM AR03, a dose dependent and linear increase in the AP endonuclease activity of the cell extracts was seen, whereas addition of the same amounts of purified Ape1 protein to plain SF767 cell extracts resulted in a saturation of the AP endonuclease activity of the extracts (Figure 20). Addition of a small amount (0.7 nM) of purified Ape1 to extracts treated with AR02 resulted in a slight increase in the AP endonuclease activity, but higher amounts (1.4-5.6 nM) of purified Ape1 resulted in AP endonuclease activity levels comparable to that with no inhibitors (Figure 21A). However, addition of 0.7nM of purified Ape1 to extracts treated with AR06 restored the AP endonuclease activity of these extracts to levels similar to that of the controls (Figure 21B).

Although, AR01 can inhibit Ape1's repair activity of purified Ape1 protein (Figure14A) and in SF767 cell extracts (Figure 18A), it acts as an equally good inhibitor of the *E. coli* endonuclease IV protein (Figure16A). A ~15-fold and ~17-fold respectively higher concentration than the IC_{50} values of AR02 and AR06 (Figure19) were required to inhibit Ape1's activity in cell extracts, and small amounts of purified Ape1 were able to restore the AP endonuclease activity of cell extracts comparable to that of the controls (Figure 21). I decided therefore, not to pursue the compounds AR01, AR02 and AR06 further at this juncture.

Rescue of the AP endonuclease activity of SF767 cell extracts with purified Ape1:

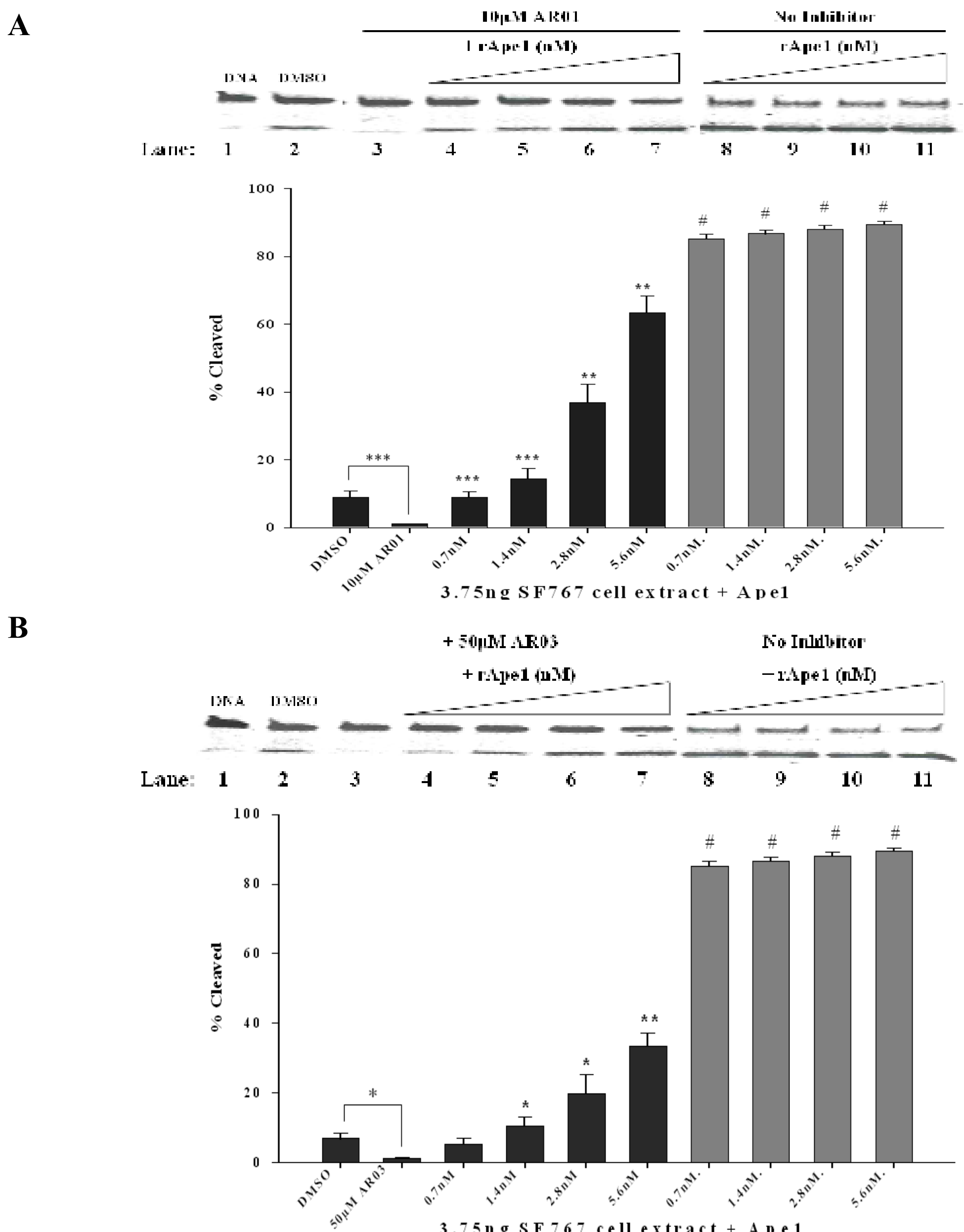


Figure 19: Purified Ape1 protein rescues the AP endonuclease activity of SF767 cell extracts treated with AR01 and AR03 in a linear range

Increasing amounts (0.7-5.6 nM) of purified Ape1 protein were added to SF767 cell extracts treated with 10 µM AR01 **(A)** and 50 µM AR03 **(B)**. Each assay was performed in triplicate and is shown here as the average with standard error and p values were calculated using the Student's t-test. **(A)** *** = $p \leq 0.01$ - comparing lane 2 with lane 1. ** = $p \leq 0.05$ and *** = $p \leq 0.01$ - comparing lanes 4-7 with lane 3. **(B)** * = $p \leq 0.05$ - comparing lane 2 with lane 1. * = $p \leq 0.05$ and ** = $p \leq 0.005$ - comparing lanes 4-7 with lane 3. # = $p \leq 0.0001$ - comparing lanes 8-11 with lane 2.

Rescue of the AP endonuclease activity of SF767 cell extracts with purified Ape1:

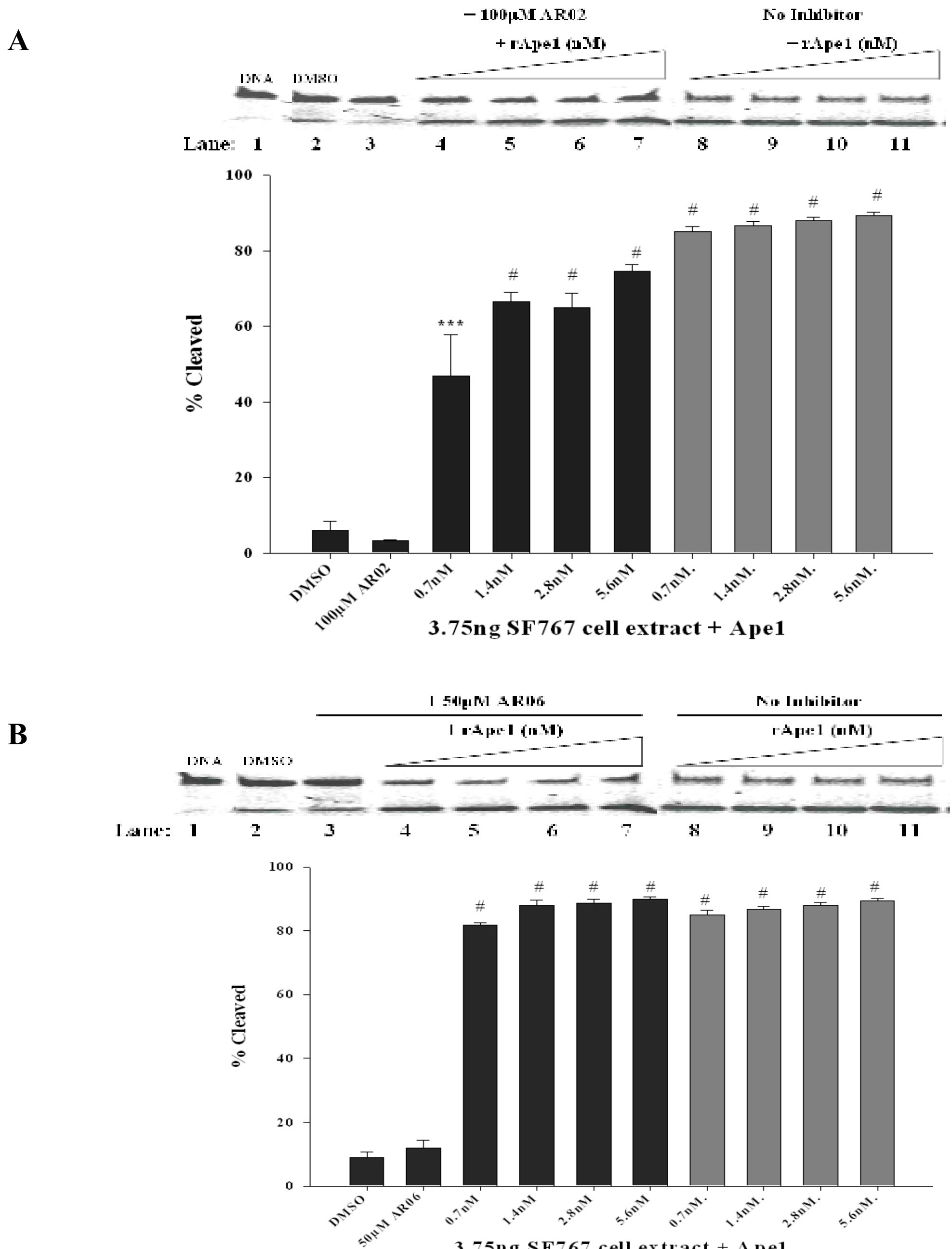


Figure 20: Purified Ape1 protein can rescue the AP endonuclease activity of SF767 cell extracts treated with AR06 and AR02

Increasing amounts (0.7-5.6 nM) of purified Ape1 protein were added to SF767 cell extracts treated with 100 μM AR02 **(A)** and 50 μM AR06 **(B)**. Each assay was performed in triplicate and is shown here as the average with standard error and p values were calculated using the Student's t-test. **(A)** # = p ≤ 0.0001 - comparing lanes 4-7 with lane 3. **(B)** *** = p ≤ 0.01 and # = p ≤ 0.0001 - comparing lanes 4-7 with lane 3. # = p ≤ 0.0001 - comparing lanes 8-11 with lane 2.

To determine selectivity of the gel-based AP endonuclease assay for Ape1

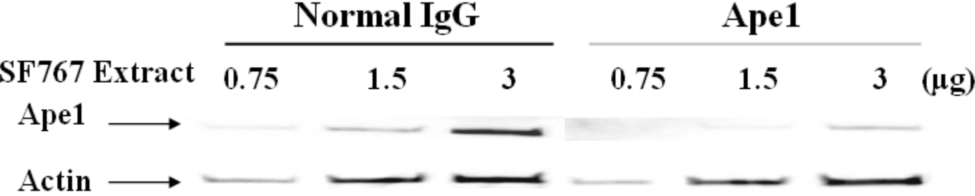
To confirm that the only enzyme in the cell extract participating in this assay to cleave the substrate is Ape1 and that the assay is specific for Ape1, the SF767 cell extracts were immunodepleted of Ape1 with a polyclonal Ape1 antibody (Novus Biologicals, Littleton CO) specific for Ape1. Western blot analysis shows that immunodepletion resulted in a 10-fold decrease in Ape1 protein in the SF767 whole cell extracts (Figure 22). This depletion of Ape1 from the extracts also resulted in a concurrent reduction in the ability of the cell extracts to hydrolyze the substrate in the gel-based AP endonuclease activity assay (Figure 23), thus confirming that the only enzyme from the extracts acting in my assay is Ape1.

Effect of the inhibitor compounds on the survival of SF767 glioblastoma cells

MTT Assays to determine survival of SF767 cells after treatment with the inhibitor compounds alone

After determining the effect of the top inhibitor compounds on the activity of Ape1 in the *in vitro* studies described above, the effect of these compounds on the proliferation of the SF767 human glioblastoma cancer cell line was determined using the MTT assay. SF767 cells were treated with increasing concentrations of the four inhibitors, and the survival of the cells after 72 hours was determined by the addition of MTT reagent (129). Even a concentration of 800 μM AR01 did not affect the survival of the SF767 cells (Figure 24A). One of the possible reasons for this effect could be that due to the charge on AR01, the cellular uptake of this compound may be impaired. Some of the other possible reasons for this observed effect of AR01 on the SF767 cells are

Immunodepletion of Ape1 from SF767 cell extracts – Western Blot:



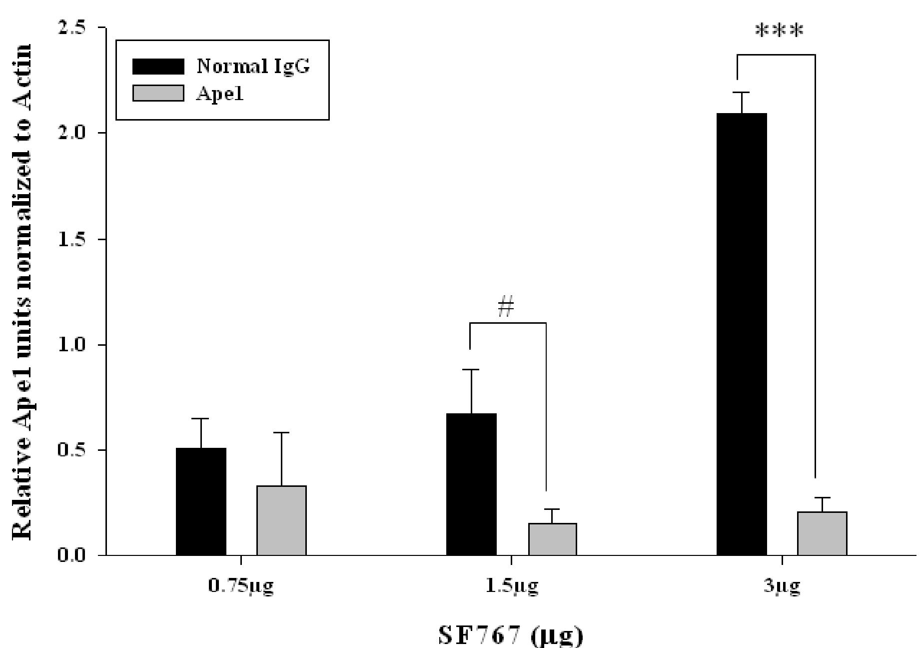
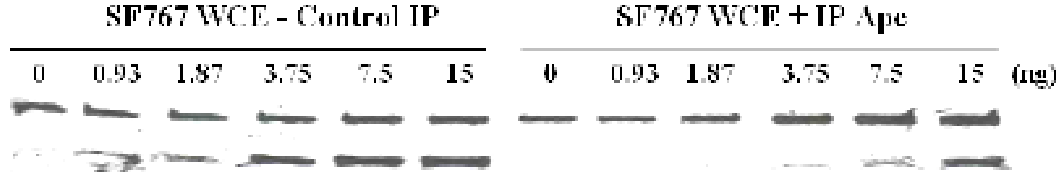


Figure 21: Immunodepleting Ape1 from SF767 cell extracts decreases Ape1's level from the cell extracts
Western blot analysis to show reduced levels of the Ape1 protein in the immunodepleted SF767 cell extracts. Actin was used as a loading control. The graph is a quantitation of the levels of Ape1 protein by normalizing back to Actin. A representative gel is shown, and the assays were performed three individual times and are presented here as averages with standard error. P values were determined using the student's t-test comparing the IgG control lanes to the lanes with the immunodepleted cell extracts # = $p \leq 0.08$; *** = $p \leq 0.0005$.

Immunodepletion of Ape1 from SF767 cell extracts – Ape1 activity assay:



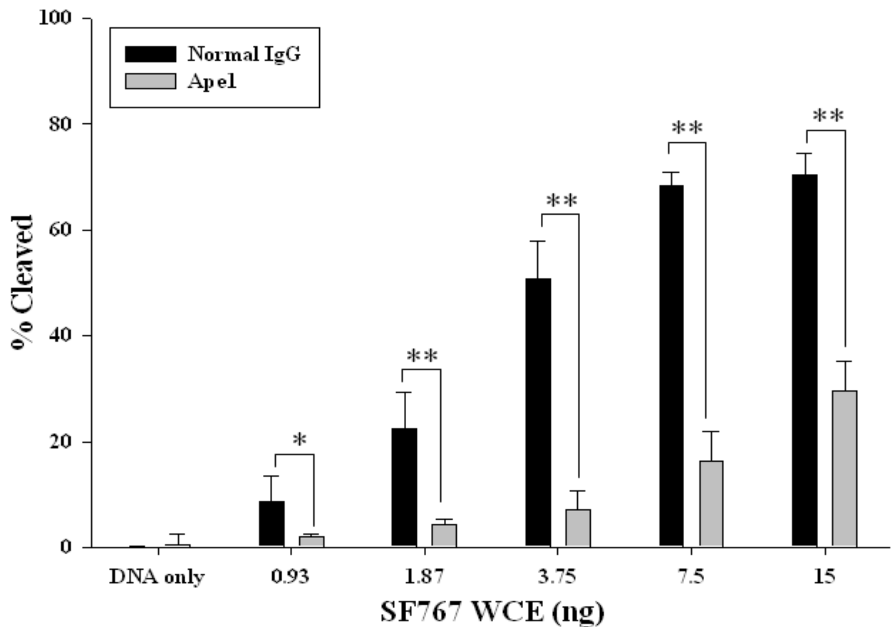


Figure 22: Immunodepletion of Ape1 from SF767 cell extracts decreases its AP endonuclease activity

The AP endonuclease activity of the immunodepleted SF767 cell extracts is significantly less than the IgG treated control extracts. The graph is a quantitation of the gel – based AP endonuclease assay results of normal IgG controls and Ape1 depleted SF767 cell extracts, and the activity of the cell extracts is represented as amount of DNA cleaved. A representative activity gel is shown, and the assays were performed three individual times and are presented here as averages with standard error. P values were determined using the student's t-test comparing the IgG control lanes to the lanes with the immunodepleted cell extracts. * = $p \leq 0.05$, ** = $p \leq 0.005$.

discussed further in the discussion section. AR03 and AR02 were cytotoxic to the SF767 cells with and LD_{50} values of ~1 μM and ~7.5 μM respectively, which are similar to their IC_{50} value of 2.2 μM and 6.4 μM respectively. On the other hand a concentration of ~100 μM of AR06 was required to kill 50% of the SF767 cells (Figure 24).

Based on the previous *in vitro* data and this cell killing data, the compounds AR01, AR02 and AR06 were not pursued further. AR01 was not considered further because it inhibits the activity of both Ape1 (Figure 14A) and endonuclease IV protein (Figure 16A) at similar concentrations (Table 6), and it does not appear to have much of an effect on the survival of the SF767 glioblastoma cell line (Figure 24A). Even though, AR02 and AR06 were cytotoxic to the SF767 glioblastoma cells (Figure 24 B&D), these compounds were also not pursued further because concentrations higher than their IC_{50} values (Table 6) were required to inhibit the activity of Ape1 in the SF767 cells extracts (Figure 19). All further experiments were therefore performed using the AR03 compound.

To determine whether AR03 can enhance the cytotoxicity of alkylating agents in SF767 glioblastoma cells using the xCELLigence system

The xCELLigence DP system (Roche, Indianapolis IN) monitors in real time cell growth, attachment and spreading based on an electronic system of impedance measurements. The attachment of a cell to the well results in an interference in continuous electronic current, which is read as impedance and is a measure of the property of the cells' ability to attach and grow (101, 105, 146, 209) (Figure 9).

I hypothesized that blocking Ape1's activity would affect the repair of alkylation

MTT assay to determine cytotoxicity of the four top compounds on SF767 glioblastoma cells:

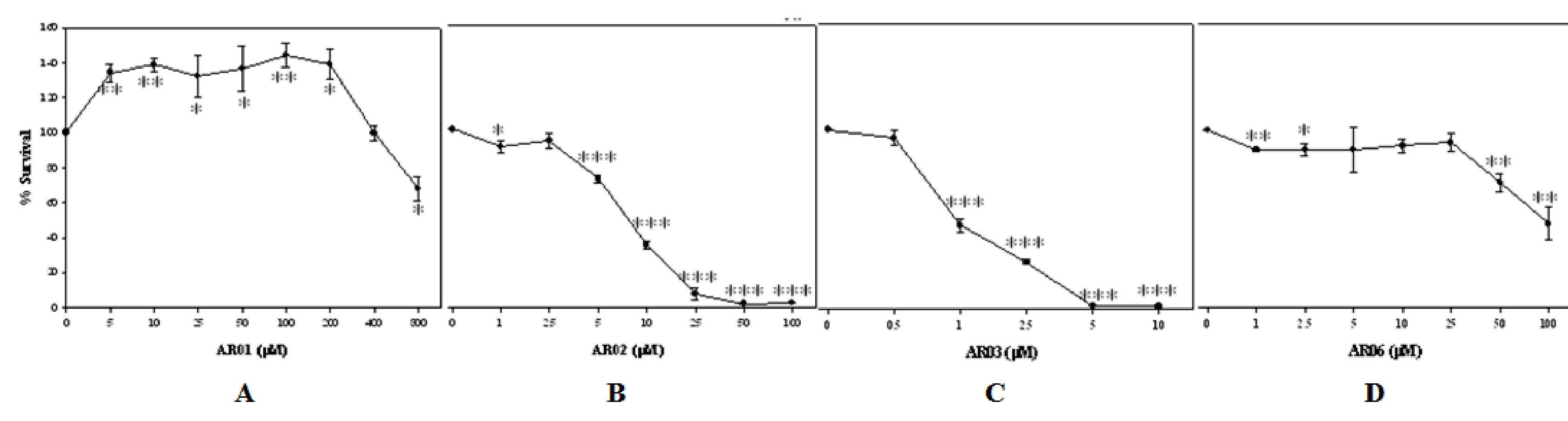


Figure 24: Survival of SF767 glioblastoma cells after treatment with the top inhibitor compounds using the MTT assay. The survival of the SF767 cells was determined using the MTT assay, 72 hours after treatment with the top inhibitor compounds. (A) AR01, (B) AR02, (C) AR03 and (D) AR06. p values were calculated using the Student's t-test. * = $p \leq 0.05$; ** = $p \leq 0.005$ and *** = $p \leq 0.0001$.

Compound inhibitors	AR01	AR02	AR03	AR06
ED_{50} (µM)	>800	~7.5	~1	~100

Table 7: ED_{50} values of the top four compounds using the MTT assay

damage as part of the BER pathway. Since AR03 selectively inhibits the repair activity of purified Ape1, of SF767 cell extracts (Table 6) and also inhibits the growth of the SF767 glioblastoma cells at relatively similar concentrations (Figure 24C), the ability of AR03 to enhance the cytotoxicity of a laboratory alkylating agent such as MMS and a clinical alkylating agent such as TMZ was determined using the xCELLigence DP system. The survival of SF767 glioblastoma cells treated with MMS, TMZ, and AR03 singly was monitored in real-time using the xCELLigence DP system (Roche, Indianapolis IN). AR03 as a single agent was efficient in killing the cells with an LD_{50} value of 1.2 μM (Figure 25A), which is comparable to the value obtained with the MTT assay (Table 6 & Figure 24 C) and a dose-dependent decrease in survival was also seen after treatment of the SF767 cells with MMS and TMZ (Figure 25 B&C).

In order to determine whether AR03 is able to enhance the cytotoxicity of MMS and TMZ, SF767 cells were treated with a dose of MMS or TMZ and AR03 showing >95% survival of the SF767 cells (Figure 25) in combination with the Ape1 repair inhibitor. Growth and survival of the SF767 cells was monitored in real time over 96 hours using the xCELLigence DP system (Roche, Indianapolis IN). Treatment of SF767 cells with a combination of the alkylating agent and the Ape1 repair inhibitor potentiated the cytotoxic effect of the alkylating agent (Figure 26). This data suggests that AR03 can enhance the cytotoxicity of MMS and TMZ further supporting my hypothesis that AR03 inhibits Ape1 and blocks the BER pathway from completing repair.

Determination of cytotoxicity of AR03, MMS and TMZ on SF767 cells using the xCELLigence system:

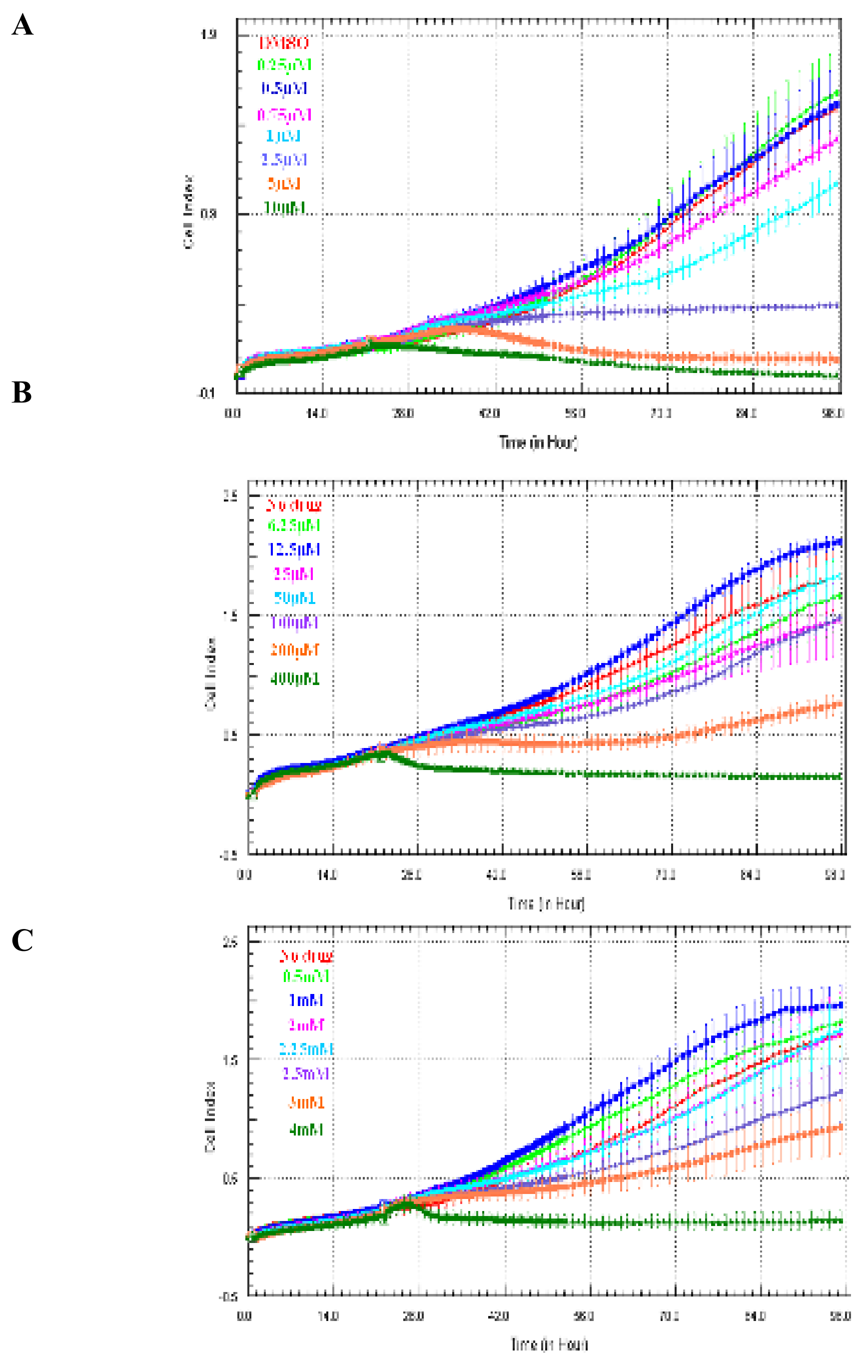


Figure 23: Cell survival analysis of SF767 glioblastoma cells after treatment with AR03, MMS and TMZ alone

The xCELLigence DP system was used to determine cell survival and growth after treatment with AR03 **(A)**, MMS **(B)** and TMZ **(C)** alone. The assay was performed in triplicate, three individual times and shown here is a representative experiment.

<u>Enhancement of MMS and TMZ cytotoxicity by AR03 in SF767 cells using the xCELLigence system:</u>

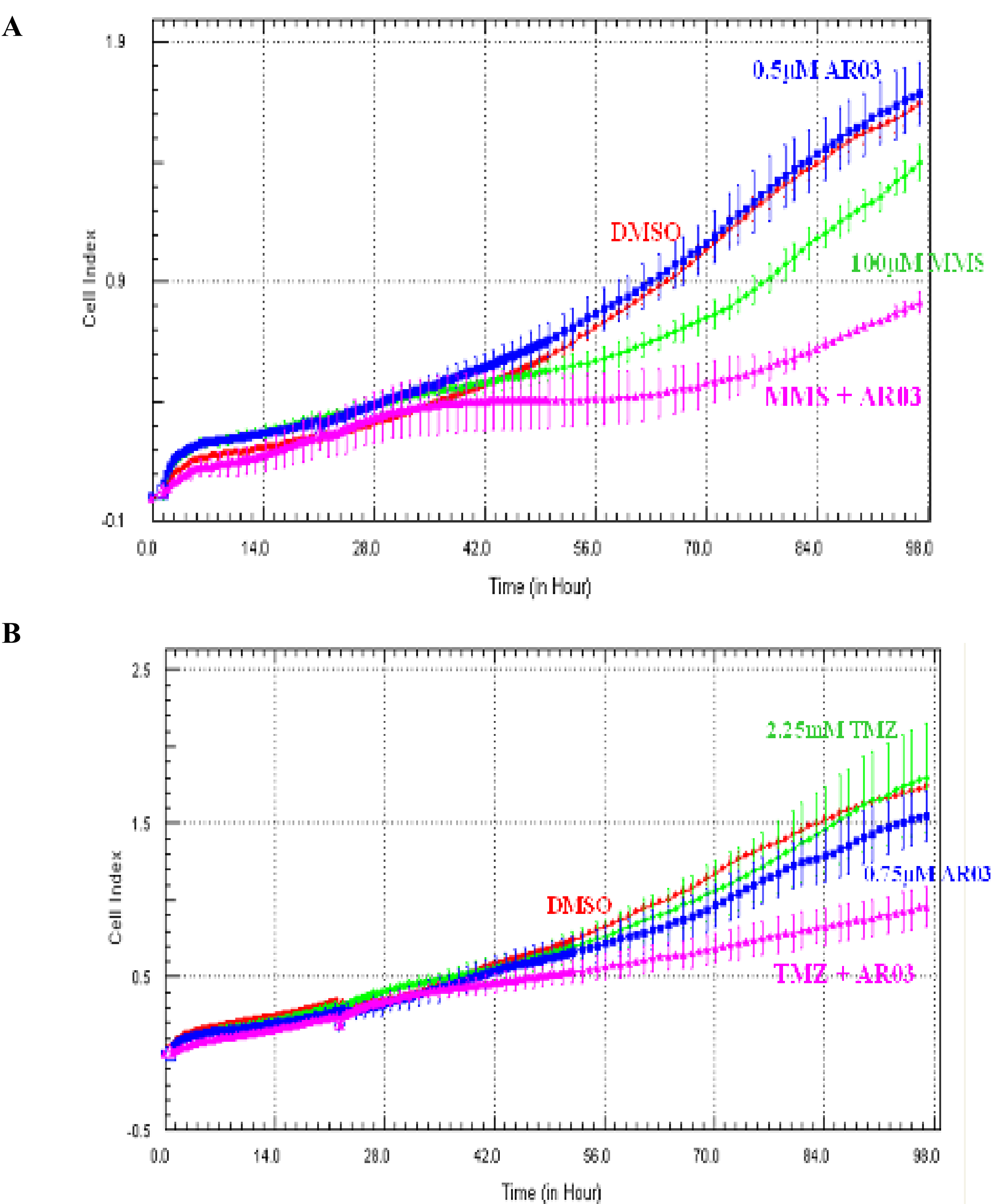


Figure 24: Cell survival analysis of SF767 glioblastoma cells after treatment with AR03 in combination with MMS and TMZ

The xCELLigence DP system was used to determine cell survival and growth after treatment with AR03 in combination with MMS **(A)** and TMZ **(B)**. The assay was performed in triplicate, three individual times and shown here is a representative experiment.

Calculation of the combination index (CI) values:

Since increased cytotoxicity of the SF767 cells was seen with a combined treatment of MMS and TMZ with AR03 as compared to either agent alone (Figures 25 & 26), the combination index (CI) values for the combined treatments were also calculated using the Calcusyn software based on the Chou-Talay method (30, 31, 152). By calculating the combination index values for a combination of two drugs one can determine whether they act additively, synergistically or antagonistically. A CI value less than 1 indicates synergy; equal to 1 indicates an additive effect and greater than 1 is indicative of antagonism (30, 31, 152). SF767 cells were treated with a combination of MMS and TMZ with AR03 and after 72 hours the combination index values were calculated using the Calcusyn software based on the survival curves for each agent alone and in combination. If AR03 inhibits the DNA repair activity of Ape1, then inhibition of Ape1 would potentiate the cytotoxicity of the alkylating agents due to its inability to repair the AP sites generated. CI values of 1.02 and 1.11 for the MMS with AR03 and TMZ with AR03 combinations respectively were obtained. Based on the description of the CI values, both values could be indicative of a nearly additive interaction (30, 31, 152) (Table 1). While nearly additive values were obtained with a combined treatment of MMS and AR03, the CI value for the TMZ and AR03 combined treatment borders on being slightly antagonistic. In this treatment, both the agents were added together at the same time, however, altering the treatment schedules of the two agents may yield different results. For example, inhibiting Ape1's DNA repair activity before adding MMS and TMZ might improve the efficacy of these alkylating agents. This can be done by pre-

treating the cells with AR03 to inhibit Ape1's DNA repair activity before adding the alkylating agents.

Drug Combination	CI value @ ED_{50}	Description
MMS + AR03	1.02	Nearly additive
TMZ + AR03	1.11	Nearly additive

Table 8: Combination Index (CI) values obtained for the combination treatments of MMS and TMZ with AR03 in SF767 glioblastoma cells

AP Site Determination in SF767 cells using the ARP Assay

To directly test that inhibition of Ape1 in cells by AR03 would lead to an increase in Ape1's substrate i.e. AP sites, I assayed the ability of the cells to repair AP sites generated as a result of treating the cells with the alkylating agent MMS. SF767 cells were treated with MMS and AR03 alone or in combination, and the number of AP sites formed was determined using the Aldehyde Reactive Probe (ARP) assay (109, 143). MMS treatment alone resulted in a ~4-fold increase in AP sites as compared to the control, whereas AR03 alone did not significantly increase the number of AP sites as compared to control. Treatment of the SF767 cellswith a combination of MMS and AR03 resulted in a statistically significant increase in AP sites as compared to the DMSO control ($p \leq 0.0001$), MMS alone ($p \leq 0.01$) and AR03 alone ($p \leq 0.0001$) (Figure 27). This result along with the previous data supports my hypothesis that AR03 inhibits the DNA repair activity of Ape1 in SF767 cells, leading to an increase in AP sites formed along with an enhancement of MMS-induced cytotoxicity.

AP site determination in SF767 cells after combined treatment with MMS and AR03:

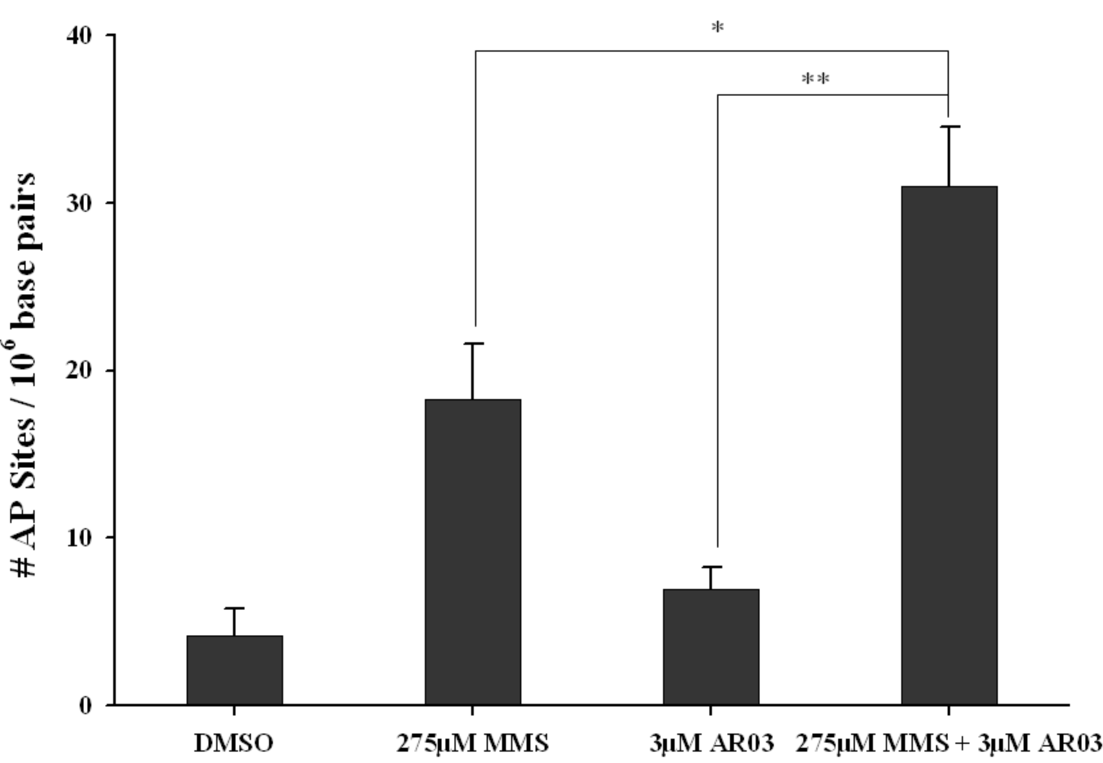


Figure 25: AP Site determination in SF767 cells after treatment with MMS and AR03 alone and in combination
In order to directly determine inhibition of Ape1 by AR03 in cells, AP site formation was measured using the ARP assay after treatment of the SF767 cells with AR03 and MMS in combination as compared to either agent alone. The assay was done in triplicate, four individual times, and the data presented here is an average of four individual experiments with standard errors. P values were determined for an n = 12 using the student's t-test where MMS + AR03 is compared to AR03 alone (** = $p \leq 0.0001$) and MMS alone (* = $p \leq 0.01$).

CHAPTER V

DISCUSSION

My thesis project was to identify a direct and selective inhibitor of Ape1's DNA repair activity using a high-throughput screening methodology with an eventual goal of utilizing this inhibitor to enhance the efficacy of cancer chemotherapy, specifically in glioblastoma. The targeting of DNA repair pathways to improve the efficacy of chemotherapy is an emerging strategy to combat some resistant cancers (14, 35, 42, 79, 131). Elevated levels of the Ape1 protein are thought to contribute to the resistance of several cancers to chemotherapy and are also a presage of poor prognosis and survival (16, 18, 103, 108, 155, 162, 173, 195). Therefore, modulating or inhibiting the activity of Ape1 would result in increased sensitivity of such cancers to chemotherapeutic agents. Our efforts and those of others have shown that blocking the DNA repair activity of Ape1 can sensitize cancers and make chemotherapy more competent and effective (17, 52-54, 115, 121, 123, 136, 137, 159, 173, 194, 196). However, none of these compounds has yet entered the clinic. Ape1 is a multifunctional protein with not only a critical role in the repair of damaged DNA, but it also functions as a redox factor to reduce cysteine residues of critical transcription factors. The DNA repair activity of Ape1 has been implicated in resistance to chemotherapy (17, 52-54, 115, 121, 123, 136, 137, 159, 173, 194, 196) and the redox activity of Ape1 has been shown to be essential for cell growth and proliferation (68, 70, 71, 77, 129, 211, 212, 218-220). My hypothesis here was that a selective inhibitor of Ape1's DNA repair activity would allow us to specifically target and inhibit its DNA repair activity while maintaining its redox function and other

interactions intact. Such an inhibitor of Ape1 would prove to be a good tool in the efforts to overcome drug resistant cancers and make them more sensitive to chemotherapy. Furthermore, I decided to focus my cell-based studies on a glioblastoma cell line (SF767) as a model system. Glioblastoma is one of the most fatal and common brain cancers with a 26% 2-year survival rate (78, 179, 182).

As presented in this thesis, I have identified an inhibitor of Ape1's DNA repair activity, AR03, which selectively inhibits Ape1's repair activity without affecting its redox activity (Figures 13B, 14B and 17B). This compound inhibits Ape1's DNA repair activity in *in vitro* assays and in cells as observed by the significant increase in the number of AP sites after treatment of the SF767 cells with AR03 and the alkylating agent MMS. AR03 is also able to enhance the cytotoxicity of the laboratory and clinical alkylating agents MMS and TMZ, respectively (Figures 18B, 20B, 24A, 25 and 26). Thus, the increased sensitivity of the SF767 cells to the alkylating agents together with the increase in AP sites and the inability of Ape1 to repair them presents further proof of target inhibition by AR03 and emphasizes the selectivity of AR03 for Ape1.

<u>High Throughput Screening (HTS) assay for inhibitors of Ape1</u>

Described here are the results of an HTS assay to identify inhibitor compounds of Ape1's DNA repair (AP endonuclease) activity. For my analyses, I optimized several conditions of the HTS assay from the one previously described by Madhusudan *et al* (132) to screen a library of 60,000 synthetic, drug-like compounds from ChemDiv. This library of compounds was screened at the Chemical Genomics Core Facility (CGCF). 41 compounds, which inhibited Ape1's DNA repair activity by 40% or greater were

identified after two rounds of screening. In order to organize the positive hits, I first determined the IC_{50} values of these compounds. The compounds were tested in the HTS assay and the IC_{50} values were calculated using the Sigma Plot software (San Jose, CA) as described in the Materials and Methods.

<u>The four top compounds can inhibit the activity of purified Ape1 protein in another distinct AP endonuclease assay</u>

After determining the IC_{50} values of the compounds, all further experiments were performed with 18 compounds that had an IC_{50} value less than 50μM (Table 3). As a second validation assay, I tested the ability of these compounds to inhibit purified Ape1 protein in a separate gel-based AP endonuclease assay (Figure 8) (110). This gel-based AP endonuclease assay differs from the HTS assay in several ways. The oligonucleotides used in the two assays differ from each other in size, sequence and position of the THF moiety, at position 7 in the HTS assay and at position 14 in the gel-based AP endonuclease assay oligonucleotides respectively (Table 1). In the HTS assay, activity of Ape1 was monitored continuously over a period of time (5 minutes), while the gel-based AP endonuclease assay is an end-point assay such that after 15 minutes the activity of Ape1 was determined. As previously discussed, following the determination that these compounds can inhibit the repair function of purified Ape1 protein both in the HTS and the gel-based AP endonuclease assay, I chose four compounds to pursue in all further experiments: AR01, AR03, AR06 and AR02 (Figures 13, 14 and 15). The four distinct compounds, which are listed in Table 4: AR01 (2-(4-(2,5-dimethyl-1H-prryol-1-yl)phenoxy acetic acid), AR02 4-(2,6,8-trimethylquinolin-4-ylamino)phenol), AR03

(2,4,9-trimethylbenzo [b][1,8] naphthyridin-5-amine) and AR06 (N-(3-chlorophenyl)-5,6-dihyro-4H-cyclopenta [d] isoxazole-3-carboxamide), belong to separate compound families and have IC_{50} values less than 10 μM (Figure 13).

<u>Determination of selectivity of these top four compounds for Ape1</u>

While absolute selectivity and specificity is always difficult to obtain, I proceeded to identify which of these compounds were selective for Ape1. Since the substrate for Ape1 is DNA and the *in vitro* assays also utilize oligonucleotides, it was important to determine that the inhibitors directly target Ape1's function. I propose that there can be four ways in which these compounds can effect the observed inhibition of Ape1.

The compounds could bind DNA

First, these compounds could bind DNA and thus inhibit Ape1's activity. Binding of the compounds to DNA or the oligonucleotides in the assays would show up as positive for repair inhibition (Figure 28B). However, since the observed inhibition would be due to the compound binding DNA and not Ape1, it would count as a false positive hit. This possibility was eliminated through the testing or all the positive hits from the screen for DNA binding. The DNA binding ability of the compounds was determined using the Fluorescence Intercalator Displacement (FID) assay (66, 192). The top four compounds were tested in this assay by LaTeca Glass from Dr. Georgiadis's lab. Any compounds that tested positive in this FID assay and my HTS assay were excluded from further analyses as they would not be specific for Ape1. The four top compounds chosen AR01, AR02, AR03 and AR06, did not bind DNA supporting my contention that they did inhibit Ape1's AP endonuclease activity.

The compounds could directly bind Ape1

Secondly, the four top compounds could directly bind Ape1 and thereby inhibit Ape1's DNA repair ability (Figure 28C). All four of the compounds showed inhibition of Ape1 in the HTS and gel-based AP endonuclease assay (Figures 13, 14 and 15). In this case the compounds could be specific for Ape1 and not other AP endonucleases. This was confirmed by determining whether these compounds could inhibit another related AP endonuclease. While Ape1 is the only mammalian AP endonuclease in cells, the endonuclease IV family of enzymes functions as the AP endonucleases in *E. coli* and yeast (47, 154). The *E. coli* endonuclease IV protein is also a part of the Class II family of endonucleases, but it belongs to a different subfamily and is structurally distinct from Ape1. Endonuclease IV also cuts DNA at AP sites, but not using the same active site as found in Ape1 (63, 141, 199). This *E. coli* AP endonuclease serves as a good measure to determine the selectivity of these potential inhibitors. Thus, one would want compounds that block Ape1's DNA repair activity but not endonuclease IV activity. AR03 requires a 16-fold higher concentration and AR06 and AR02 both require approximately 10-fold greater concentrations to inhibit 50% of endonuclease IV's activity suggesting that these compounds could be selective for Ape1 (Figures 16B, 17A and 17B). AR01, however, inhibits the activity of the *E. coli* endonuclease IV protein at a concentration of ~1.75 μM (Figure 16A) which is similar to its IC_{50} value. If a compound inhibits the activity of both Ape1 and endonuclease IV, one possibility is that it binds to the AP site rather than binding to Ape1. This possibility will have to be confirmed before a definite conclusion about the method of inhibition of this compound can be drawn as some aspects of the active sites of Ape1 and endonuclease IV are similar (141).

The compounds may bind AP sites

A third possible way that these compounds could inhibit Ape1 occurs through their binding to the AP sites in DNA thereby blocking Ape1 from acting on them (Figure 28D). This type of inhibition would be similar to that seen with methoxyamine (MX), which binds to the AP site and blocks Ape1's and DNA Polymerase β's activity by preventing these proteins from processing the AP sites and completing repair (85, 88). Such an interaction of the compounds with the AP sites would affect not only Ape1's activity but also of any other enzyme acting on AP sites including the *E. coli* endonuclease IV in my assays. Figures 16B and 17 demonstrate that compounds AR03, AR06 and AR02 require 16-fold and 10-fold greater concentrations respectively, to inhibit the activity of endonuclease IV as compared to Ape1 and more than likely, these compounds are not AP site binders. Although AR01 inhibits Ape1's activity, it also inhibits endonuclease IV's activity at a concentration comparable to its IC_{50} value (Figure 16A). This inhibition may be due to a number of reasons, one of which is that AR01 binds to the AP sites and is therefore able to inhibit both Ape1 and endonuclease IV. As AR01 is a negatively charged compound, it may also effect this inhibition by mimicking the negatively charged phosphate groups present in the DNA backbone.

One possible way to further dissect out whether AR01 is specific for Ape1 or whether it binds to AP sites can be determined by modifying the Aldehyde Reactive Probe (ARP) assay, which measures the formation of AP sites in the DNA. The ARP reagent binds to the aldehyde group present in an open configuration AP site thereby tagging all the AP sites in the DNA with biotin. These biotin tagged AP sites can then be measured using a streptavidin bound indicator enzyme such as HRP (109, 142, 143).

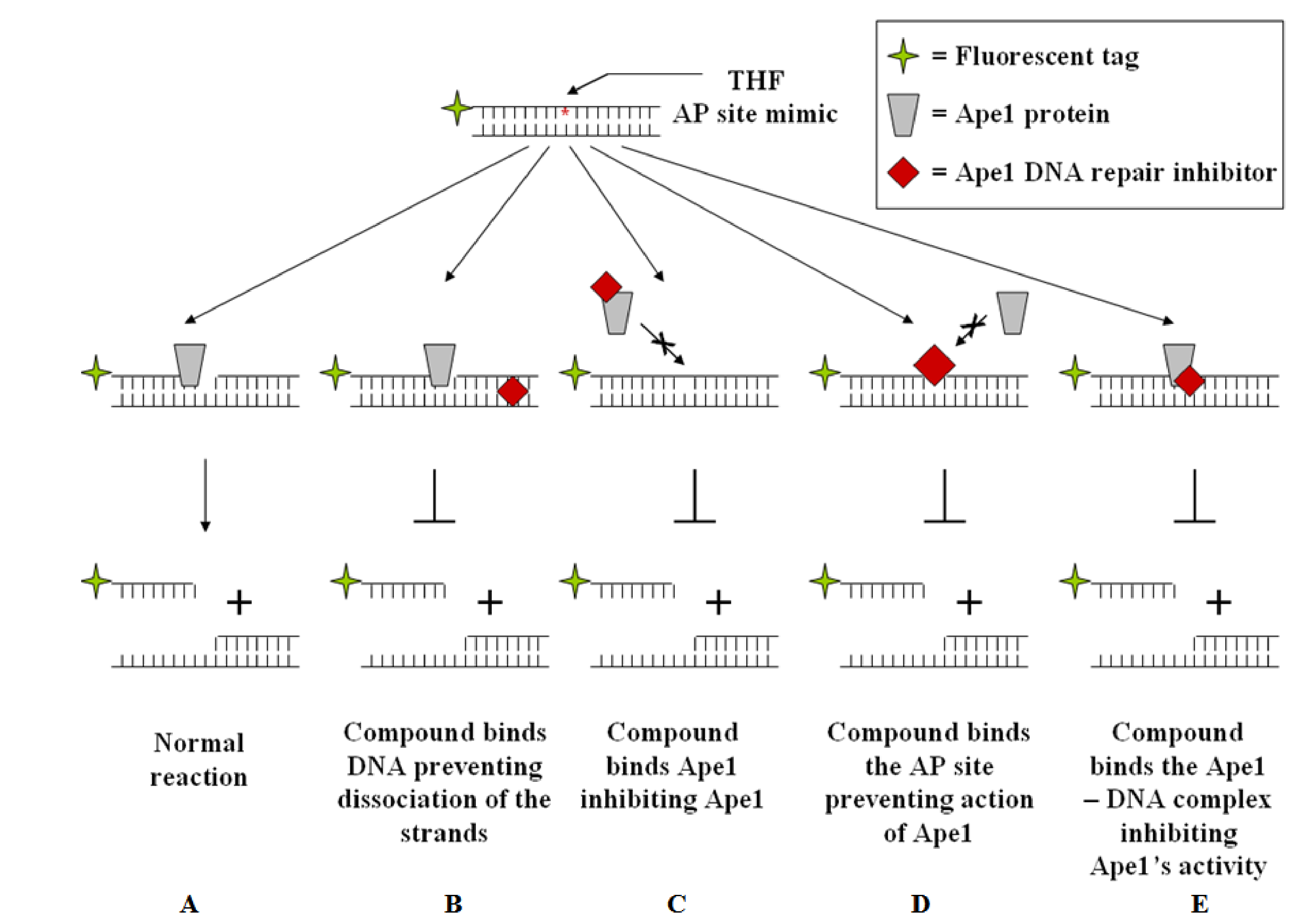


Figure 28: Possible ways of inhibition of Ape1's DNA repair activity by the inhibitor compounds

Genomic DNA can be treated with AR01 and after a certain period of incubation, AP sites formed in the DNA can be measured. If AR01 binds to AP sites then the ARP reagent will not be able to bind to the AP sites resulting in a decrease in the number of AP sites measured in this assay. This would be similar to the result observed with Methoxyamine (MX) (54, 59, 122, 125, 165, 184), an inhibitor of the BER pathway, which covalently binds to AP sites and disrupts the functioning of the BER pathway. As an AP site binder, MX can be used as a positive control to show that there is a decrease in the number of AP sites measured after treatment with MX.

The compounds may bind the enzyme-substrate complex of Ape1 on the DNA

A fourth possible way for these compounds to elicit inhibition of Ape1's activity may be through their binding to the enzyme-substrate complex of Ape1 and AP site DNA. This type of interaction may immobilize Ape1 on the DNA, which may be due to one or both of the following reasons: the binding of the compounds may inhibit Ape1 from acting on the AP sites or this interaction may prevent the dissociation of Ape1 from the AP sites and impedeng the access of the subsequent BER proteins to these AP sites in order to complete repair (Figure 28E). This possibility would be more likely in the cellular extracts as the extracts would contain the rest of the BER proteins required to complete repair. In the *in vitro* gel-based assay however, the binding of the compounds to this protein-DNA complex would prevent the dissociation of the labeled DNA from the rest of the oligonucleotide, cleaved as a result of Ape1's activity. Therefore, a possible explanation for the inhibition of both Ape1 and endonuclease IV by AR01 may be that

AR01 binds AP sites or may bind to the enzyme-AP site DNA complex. However, since AR01 is negatively charged, it is unlikely that it will bind to the AP sites in the DNA.

The top inhibitor compounds, inhibit Ape1's DNA repair activity in SF767 cell extracts

After determining that these potential 'hit' inhibitors are effective against Ape1, their capacity to inhibit Ape1 in a cellular environment was tested. The AP endonuclease activity of the SF767 cell extracts was inhibited in a dose-dependent manner after treatment with these inhibitor compounds. AR01 and AR03 inhibited 50% of Ape1's activity in the extracts at concentrations comparable to that required to inhibit pure Ape1 (Figure 18). On the other hand, although AR02 and AR06 inhibit the activity of purified Ape1 protein (Figure 15), a concentration greater than 50 μM of AR02 (~93 μM) and AR06 (~75 μM) was required to inhibit Ape1's repair activity in the cell extracts (Figure 19), which may suggest off-target interactions.

As another way to determine inhibition of Ape1's activity by the inhibitor compounds, the ability of purified Ape1 to rescue the AP endonuclease activity of SF767 cell extracts treated with the inhibitors was determined. If the compounds inhibit Ape1's DNA repair activity, then addition of increasing amounts of purified Ape1 would result in a gradual and linear increase in the AP endonuclease activity of the cell extracts. This is what I observed. The rescue of the AP endonuclease activity of the SF767 cell extracts treated with AR01 and AR03 was linear and dependent on the amount of Ape1 protein added (Figure 20). These results indicate that AR03 not only inhibits Ape1 in the cellular extracts, but can also inhibit the activity of the added purified Ape1 protein. This further

demonstrates that Ape1 is the primary target of AR03 in the processing of AP sites and supports my hypothesis that AR03 can selectively target Ape1. However AR01, despite being able to inhibit the AP endonuclease activity of the SF767 cell extracts (Figure 18A) also inhibits the activity of endonuclease IV at similar concentrations (2.4 μM and 1.75 μM respectively) (Figure 16A). As mentioned above, a further clarification is warranted to estimate the mechanism of inhibition of AR01. Furthermore, the addition of the smallest amount of purified Ape1 elicited saturating levels of the AP Endonuclease activity even in the presence of 100 μM AR02 and 50 μM AR06 as compared to controls without inhibitors (Figure 21), insinuating that these compounds may interact with other molecules in the celluar extract.

The gel-based AP endonuclease assay is specific for Ape1 and no other Ape1-like enzyme in the cell extracts can function in this assay

As an additional verification of the assay specificity, I wanted to confirm that the gel-based assay used to test Ape1's inhibition in cell extracts with the inhibitor compounds was specific to Ape1 and that no other Ape1-like enzyme in the cell extracts could compensate for Ape1. Removing Ape1 from the cell extracts resulted in a significant decrease in the AP endonuclease activity of these cell extracts, confirming that Ape1 is the only protein in the cell extracts that can function in this assay (Figures 22 and 23).

Ape repair inhibitor-AR01

Although AR01 can consistently inhibit the activity of purified Ape1 (Figure 16A) as well as in cellular extracts (Figure 18A), it also inhibits the activity of the related AP endonuclease, endonuclease IV (Figure 16A) at a concentration similar to its IC_{50} value (1.4 μM and 1.75 μM respectively) and does not kill SF767 cells (Figure 24A). There can be several reasons for AR01 not killing the SF767 cells: First, AR01 is negatively charged, so it might not be getting into the cells. Second, AR01 may get into the cells and just not effectively target Ape1. Third, if it enters the cells, it may be rapidly pumped out of the cells. Fourth, it could be degraded by cellular enzymes rendering it inactive. Alternatively, AR01 may bind to the serum proteins present in the cell culture media and become sequestered in the media and may not enter the cells or it may degrade in the media itself. One way to alter AR01 to pursue in cell-based assays would be derivitize the compound and change the negatively charged acetic acid to a neutral ester and then test the effect of this AR01 derivative on the SF767 cancer cells. Additionally, AR01 can be mixed with liposomes to deliver it into the cells and then measure its effect.

Therefore, although initially, AR01 seemed like a very promising inhibitor of Ape1's DNA repair function, I decided not to pursue it further due to the above mentioned complicating factors.

Ape repair inhibitors-AR02 and AR06

The AR02 and AR06 compounds can inhibit the activity of purified Ape1 (Figure 15) at concentrations comparable to their IC_{50} values (Figure 13) but require ~ 10-fold higher concentrations to inhibit the activity of the endonuclease IV protein (Figure 17).

However, a concentration of ~93 μM of AR02 and ~75 μM of AR06 (Figure 19) is required to inhibit 50% of Ape1's activity in the SF767 cell extracts. If these compounds were selective for Ape1, they would be able to inhibit the AP endonuclease activity of the SF767 cell extracts and would also be able to inhibit the activity of the exogenous purified Ape1 protein. However, addition of low concentrations of purified Ape1 protein resulted in the rescue of the AP endonuclease activity of SF767 cell extracts treated with 100 μM AR02 and 50 μM AR06 to saturating levels as compared to controls without inhibitors (Figure 21). These two compounds are cytotoxic to the SF767 cells (Figure 24B and D respectively), albeit at concentrations much lower than those required to inhibit Ape1's activity in the cell extracts (Figure 19). This data together with the previous *in vitro* data suggests that these compounds may not be selective for Ape1, and the inhibition seen by AR02 and AR06 may be due to off-target effects. Additionally, there were problems with the solubility of AR06 when it was diluted for my assays. Therefore, due to all these reasons, I decided not to develop AR02, AR06 or AR01 as inhibitors of Ape1, and my efforts were focused on the AR03 compound.

AR03 can act as a single agent against human cancer cells

In order for the inhibitors to be clinically and therapeutically relevant, they should be biologically active at physiologically attainable concentrations. Several clinical chemotherapeutic agents such as TMZ and radiation (IR) are used to treat a variety of cancers. TMZ is an alkylating agent, which induces DNA damage repaired by the BER pathway, while IR produces DNA strand breaks as well as reactive oxygen species (ROS), which generate oxidative damage also repaired by Ape1 and the BER pathway

(43, 56, 57, 75). Additionally, elevated levels of Ape1 in a variety of cancers including glioblastomas have been correlated to resistance to chemotherapy and poor survival, and knocking down Ape1 with antisense oligonucleotides enhanced alkylation and radiation sensitivity of several mammalian cancer cell lines (16-18, 173). I selected the SF767 glioblastoma cell line to test my inhibitor compounds, as most therapeutic regimens for gliomas consist of alkylating agent–based chemotherapy and/or radiation therapy (180).

As shown in Figures 24C and 25A, AR03 effectively kills the SF767 glioblastoma cells in a dose dependent manner. As ~10,000 AP sites are generated per cell per day by the spontaneous depurination of bases due to the action of endogenous damaging agents resulting in the formation of AP sites, blocking Ape1 repair activity might be predicted to increase tumor cell killing (199). Persistence of these AP sites can be mutagenic and cytotoxic to the cells as it can stall replicative polymerases at AP sites giving rise to strand breaks (107, 126, 127, 199, 214). Also, error-prone translesion bypass polymerases can insert inappropriate bases opposite the AP site leading to an alteration in the nucleotide sequence, which are mutagenic. Additionally, single strand breaks created from AP sites can turn into double strand breaks leading to cytotoxicity (107, 126, 127, 199, 214). Hence, inhibiting the DNA repair activity of Ape1 with a repair inhibitor would lead to an accumulation of AP sites in the cells leading to cytotoxicity (47, 199).

While AR03 is effective as a single agent, if AR03 does inhibit Ape1's DNA repair activity, it should also enhance the cytotoxic effects of the laboratory and clinical alkylating agents MMS and TMZ respectively. In this case, the AP sites generated by the action of MMS and TMZ would be left unrepaired as AR03 would inhibit Ape1's ability to process them, leading to increased cell death (47, 199). Treatment of SF767

glioblastoma cells with a combination of MMS or TMZ and AR03 resulted in increased killing of SF767 glioblastoma cells (Figure 26) compared to either agent alone (Figure 25). These data strengthen my hypothesis that AR03 targets Ape1 and AR03 could be effective a single agent against the cancer cells in addition to enhancing the efficacy of chemotherapy.

Inhibition of Ape1 in SF767 glioblastoma cells by AR03 results in an increase of unrepaired AP sites

Finally, as a direct estimation of inhibition of Ape1 by AR03 in the cells, I assayed for the formation of AP sites. This is the only assay that directly measures the substrate of Ape1, AP sites in the DNA. I hypothesized that blocking Ape1's repair activity by AR03 would lead to an increase AP sites as they would not be processed by Ape1. A significant increase in AP sites was observed after treating SF767 cells with AR03 and MMS compared to either agent alone (Figure 27) as a result of Ape1's inability to repair the AP sites induced by MMS. This reinforces the selectivity of AR03 for Ape1. AR03 appears to be a promising hit compound as it is effective not only as a single agent, but can also be combined with existing chemotherapeutic agents to improve outcome in a variety of cancer cell lines.

In conclusion, I describe here a selective novel small molecule, AR03 that was identified using the high-throughput screening (HTS) methodology. AR03 has an IC_{50} value of 2.2 μM for the inhibition of Ape1 in this HTS assay. AR03 inhibits the activity of purified Ape1 protein in two distinct assays (the solution-based HTS assay and the gel-based AP endonuclease assay) (Figures 13C and 14B). AR03 also inhibits Ape1's DNA

repair function in SF767 glioblastoma cell extracts as evidenced in Figures 18B and 20B. A concentration of AR03 16-fold greater than its IC_{50} is required to inhibit the structurally unrelated *E. coli* endonuclease IV (141), which is indicative of AR03's selectively to inhibit Ape1's DNA repair activity (Figure 16B). In addition, AR03 functions as a single agent to kill SF767 glioblastoma cells and to enhance the cytotoxicity of the alkylating agents MMS and TMZ also leading to an increase in AP sites (Figures 25, 26 and 27). This inability of Ape1 to repair the AP sites due to inhibition by AR03 presents further proof of target inhibition by AR03 and emphasizes the selectivity of AR03 for Ape1.

Ability of AR03 to target the redox activity of Ape1

As mentioned earlier, Ape1 is a multifunctional protein that has two major functions: a DNA repair activity and a redox signaling role (45, 55, 187). Since Ape1 has these two important functions and AR03 inhibits the DNA repair activity of Ape1, knowing its effect on the redox function of Ape1 would be interesting. The two functions of Ape1 are distinct from each other and lie far apart from each other in two different regions of the protein (Redox function in the N terminal and the DNA repair activity in the C terminal portion) (1, 205-207). Not only are these two functions physically separate, each function has a distinct active site and catalytic residue required for activity (11, 13, 45, 47, 58, 67, 128, 140). Therefore, I would not expect the redox activity of Ape1 to be affected by the AR03 compound. Nevertheless, the effect of AR03 on the redox activity of Ape1 can be determined using a redox electrophoretic mobility shift assay (EMSA) (129). In this assay, reduced Ape1 protein will be treated with AR03, and

the ability of Ape1 to reduce the cysteine residues on the transcription factor AP-1 (Fos/Jun), a known redox target of Ape1, will be determined. If AR03 does not affect the redox activity of Ape1, the reduction of cysteine residues promotes binding of AP-1 to a consensus sequence in an EMSA gel-shift assay, which results in a shift in the position of AP-1 bound DNA as compared to the control (205, 206).

Future Directions

Effect of combining other chemotherapy agents with AR03 in multiple cancer cell lines and the effect of AR03 on primary cells

The standard of care chemotherapy regimens for glioblastomas includes surgery followed by treatment with TMZ and irradiation (IR) (176, 179-182) and both of these agents generate lesions that are repaired by the BER pathway. My cell survival experiments with SF767 glioblastoma cells show that the Ape1 repair inhibitor can enhance the cytotoxicity of the laboratory alkylating agent MMS as well as the clinical agent TMZ (Figure 25). IR, which is part of the treatment for glioblastomas, also generates reactive oxygen species (ROS), which generate oxidative lesions (8-oxoG for example) in the DNA. The BER pathway in addition to repairing alkylation damage also repairs oxidative DNA damage (24, 200). Therefore, the ability of AR03 to enhance the efficacy of IR could also be determined. Additionally, the ability of AR03 to function as a single agent as well as to enhance chemotherapeutic efficacy will need to be determined in other glioblastoma cell lines. For AR03 to be clinically relevant, it should be able to enhance the efficacy of the clinical chemotherapeutic agents in more than one glioblastoma cell line. Eventually, the AR03 inhibitor will also be evaluated in animal mouse models of brain tumor using orthotopic and xenograft models.

In addition to glioblastomas, elevated levels of Ape1 have been shown to be implicated in chemotherapeutic resistance of several other cancers such as breast, ovarian, colon, pancreatic and other diseases (16, 18, 39, 52, 53, 84, 103, 108, 130, 134, 155, 162, 173, 195, 198, 220). AR03 can be used to improve therapeutic efficacy in the treatment of such cancer diseases where Ape1 has been implicated.

Ape1 functions in normal and cancer cells to maintain the cell's genomic integrity, and while Ape1 levels are elevated in human cancers (16, 18, 103, 108, 155, 162, 173, 198), the effect of AR03 on normal cells will also need to be assessed. The bone marrow compartment is most affected after the administration of a chemotherapeutic agent, and the regeneration of the bone marrow cells are responsible for reducing the side effects of chemotherapy. Determining whether AR03 has any potential negative effects on such a population of the normal dividing bone marrow cells (CD34+ bone marrow progenitor cells) would need to be tested (219).

To further characterize the repair response and DNA damage induced by AR03 with and without treatment of chemotherapeutic agents in glioblastoma cell lines

In order to further characterize the effects of the Ape1 DNA repair inhibitor AR03 in glioblastomas, the DNA damage and repair response will be analysed in the SF767 glioblastoma cell line. Cell growth analysis using the MTT and xCELLigence assays has shown that AR03 can enhance the cytotoxicity of MMS and TMZ (Figures 24, 25 and 26). In order to further determine that the enhanced cytotoxicity seen with TMZ and AR03 is due to inhibition of Ape1's ability to process the AP sites formed, the amount and type of DNA damage will be determined. If AR03 inhibits Ape1's DNA repair function, then treating the cancer cells with a combination of both agents would result in an increase in the AP sites (Figure 27). However, a failure to repair the persistant AP sites would result in these AP sites being converted to single-strand breaks (SSBs) during replication (20, 47, 149, 187, 202). The SSBs would ultimately be converted to double strand breaks (DSBs) as a result of incomplete repair (47, 69, 187, 202), and the

accumulation of such DNA damage would result in the cells undergoing apoptosis. The ability of cells to undergo apoptosis after treatment with TMZ and MX (125, 165), an inhibitor of Ape1's DNA repair activity has been reported previously (53, 120, 121, 123, 184, 191, 210).

The Comet assay can be used to determine the formation of SSBs and DSBs. Electrophoresis of the cells under different conditions allows one to assay for SSBs and DSBs: Under alkaline conditions, both SSB and DSB can be detected and only DSBs can be detected under neutral electrophoresis conditions (5, 145, 189, 190). This assay is based on the ability of broken DNA fragments (alkaline-sensitive AP sites or single/double strand breaks) to migrate out of a cell upon applying an electric current, whereas supercoiled undamaged DNA will not migrate out of the cells, or at a much lesser extent under these same conditions (5, 145, 189, 190). As an additional assay to measure the formation of DSBs, the levels of the phosphorylated H2A.X (γH2A.X), which is a marker of DSBs (164), will be determined using Western blotting techniques.

Apoptosis will be determined using Flow cytometry by staining the cells with Annexin-V and PI. Annexin-V positive and PI-negative cells will be considered positive for apoptosis (195).

Chemical knockout of Ape1 using an inhibitor of Ape1's DNA repair activity, AR03 and an inhibitor of Ape1's redox activity

As described previously, Ape1 is a multifunctional protein with two major activities, a DNA repair and a redox activity (207). In addition to these main activities, Ape1 interacts with a variety of proteins not only from the BER pathway but also from

other signaling pathways (9, 33, 41, 48, 92, 98, 112, 124, 147, 148, 151, 172). Therefore knocking down the levels of Ape1 in cells using the antisense oligonucleotide technology, not only inhibits its DNA repair activity, but also downregulates its redox function. The sensitization of mammalian cancer cells to a variety of chemotherapeutic agents after knocking down Ape1 has been previously reported by our laboratory and others (52, 103, 115, 130, 162, 195). Furthermore, removal of Ape1 from the cells would also prevent Ape1 from maintaining its protein-protein interactions and may alter its sub-celllar location (34, 39, 44, 47, 48, 102, 139, 185) and its ability to be post-translationally modified (48, 50, 97, 139). This makes it difficult to attribute the observed effects on cellular survival definitely to the DNA repair function. Similarly, expressing a dominant-negative form of the Ape1 protein inhibits the normal DNA repair activities of Ape1 in the cells (137). However, these types of studies imbalance the cellular system and make an interpretation of the results difficult. Being able to selectively inhibit one activity of Ape1 would help separate out the effects of these two activities on tumor cell survival and response to chemotherapy, without removing Ape1 from the cellular milieu and maintaining Ape1's interactions. E3330, a small molecule compound that inhibits the redox function of Ape1 has been shown to inhibit the growth of human cancer cell lines (129, 218, 220). Therefore, combining the DNA repair inhibitor AR03 with the redox inhibitor, E3330 would enable us to selectively target the two major functions of Ape1 known to be involved in tumor promotion and progression, while maintaining other activities.

Therefore, understanding the role of Ape1's DNA repair function is crucial from both a basic science and translational viewpoint, as is an understanding of its redox

function. A further understanding of the interplay between the two major functions of Ape1 will let us better grasp the cellular response to both endogenous and exogenous stress and DNA damage.

CHAPTER VI

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EDUCATION

08/1997-05/2000	B.Sc., Zoology, Fergusson College, University of Pune, India
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PUBLICATIONS / ABSTRACTS

Bapat A, Fishel ML and Kelley MR. Going Ape as an Approach to Cancer Therapeutics. *Antioxidants and Redox Signaling*. Epub ahead of print August 20th 2009 (Peer reviewed Review article)

Bapat A, Glass LS, Luo M, Fishel ML, Long EC, Georgiadis MM and Kelley MR. Novel small molecule inhibitor of Ape1 endonuclease blocks proliferation and reduces viability of glioblastoma cells. Manuscript submitted to Molecular Cancer Therapeutics.

Glass LS, **Bapat A**, Kelley MR, Georgiadis MM and Long EC. Semi-automated high-throughput fluorescent intercalator displacement-based discovery of cytotoixc DNA binding agents from a large compound library. Manuscript submitted to Bioorganic and Medicinal Chemistry Letters.

Bapat A, Fishel ML, Georgiadis MM and Kelley MR. Inhibition of Ape1's repair activity as a target in cancer. **American Association for Cancer Research (AACR)** 2008; Mini-Symposium Talk, DNA Repair and Mutagenesis, April 11th – 16th 2008, San Diego CA.

Bapat A, Fishel ML, Georgiadis MM and Kelley MR. High Throughput Screen for Inhibitors of Ape1. **MidWest Society for Pediatric Research (MWSPR),** Poster Presentation; October 18th-19th 2007.

Bapat A, Fishel ML, Georgiadis MM and Kelley MR. High Throughput Screen for Inhibitors of Ape1. **IU Simon Cancer Center's Annual Research Day**, Indiana University School of Medicine (IUPUI), Indianapolis IN, Poster Presentation; May 2nd 2007 and May 7th 2008.

Bapat A, Fishel ML, Georgiadis MM and Kelley MR. High Throughput Screen for Inhibitors of Ape1. **Biochemistry Research Day,** Department of Biochemistry and Molecular Biology, Indiana University School of Medicine (IUPUI), Indianapolis IN, Poster Presentation; September 28th 2007 and October 2nd 2008.

HONORS AND ACTIVITIES

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