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Novel small molecule inhibitor of Ape1 endonuclease blocks proliferation and reduces viability of glioblastoma cells

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Running Title: Inhibitors of the Repair function of APE1

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Abbreviations List:

Ape1 AP endonuclease 1
AP sites Apurinic / apyrimidinic sites
AR Ape1 Repair Inhibitor
ARP Aldehyde Reactive Probe
BER Base Excision Repair
CGCF Chemical Genomics Core Facility
CI Cell Index
dRP Deoxy ribose phosphate
dsDNA  Double stranded DNA

*E. coli*  *Escherichia coli*

EMSA  Electrophoretic Mobility Shift Assay

EtBr  Ethidium Bromide

FBS  Fetal Bovine Serum

FID  Fluorescence Intercalator Displacement

HEX  Hexachloro phosphoramidite fluorescein

HTS  High-throughput Screen

IUSM  Indiana University School of Medicine

LD<sub>50</sub>  Lethal Dose at 50% kill

MMS  Methyl Methane Sulfonate

MX  Methoxyamine

NCA  7-nitro indole 2-carboxylic acid

THF  Tetrahydrofuran

TMZ  Temozolomide

WCE  Whole Cell Extracts

XRCC-1  X-ray Cross-species Complementing Factor - 1

**Recommended Section Assignment:**  Cellular and Molecular
Abstract

Ape1 is an essential DNA repair protein that plays a critical role in repair of apurinic/apyrimidinic (AP) sites via base excision repair. Ape1 has received attention as a druggable oncotherapeutic target, especially for treating intractable cancers such as glioblastoma. The goal of this study was to identify small molecule inhibitors of Ape1 AP endonuclease. For this purpose, a fluorescence-based high-throughput assay was used to screen a library of 60,000 small molecule compounds for ability to inhibit Ape1 AP endonuclease activity. Four compounds with IC$_{50}$ values less than 10 $\mu$M were identified, validated and characterized. One of the most promising compounds designated AR03, inhibited cleavage of AP sites in vivo in SF767 glioblastoma cells, in vitro in whole cell extracts and inhibits purified human Ape1 in vitro. AR03 has low affinity for dsDNA and weakly inhibits the Escherichia coli endonuclease IV, requiring a 20-fold higher concentration than for inhibition of Ape1. AR03 also potentiates the cytotoxicity of methyl methanesulfonate and temozolomide in SF767 cells. AR03 is chemically distinct from the previously reported small molecule inhibitors of Ape1. AR03 is a novel small molecule inhibitor of Ape1, which may have potential as an oncotherapeutic drug for treating glioblastoma and other cancers.
DNA repair pathways maintain the integrity of eukaryotic and prokaryotic genomes. The Base Excision Repair (BER) pathway repairs bases damaged by endogenous and exogenous alkylating and oxidizing agents (Christmann et al., 2003). The BER pathway is initiated by a damage-specific DNA glycosylase, which recognizes and excises the damaged base to generate an apurinic/apyrimidinic site (AP) site. Alternatively, AP sites can also be generated by spontaneous depurination (Wilson and Barsky, 2001). AP endonuclease 1 (Ape1), cleaves the phosphodiester backbone 5’ to the AP site generating 3’-hydroxyl and 5’-deoxy ribose (dRP) phosphate termini. Polymerase β removes the 5’ dRP, fills in the one base gap, and the nick is ligated by DNA ligase III/XRCC1 (X–ray cross-species complementing 1) to complete repair (Evans et al., 2000; Robertson et al., 2009).

Ape1 belongs to the xth family of class II endonucleases, whose prototypical member is the *Escherichia coli* (*E. coli*) exonuclease III. xth-related proteins account for almost 95% of the cellular AP endonuclease activity in most eukaryotic species (Evans et al., 2000). Ape1 exhibits a prominent 5’ hydrolytic AP endonuclease, a weak 3’-diesterase and a 3’-5’ exonuclease activity (Chou and Cheng, 2003; Parsons et al., 2004). In *Saccharomyces cerevisiae* (yeast) however, Apn1 is the most abundant AP endonuclease (Popoff et al., 1990) and along with *E. coli* endonuclease IV belongs to the nfo family of the class II endonucleases (Evans et al., 2000). Although the *E. coli* endonuclease IV and Ape1 proteins have similar biological roles, their protein sequences are dissimilar (Wilson and Barsky, 2001; Garcin et al., 2008).

Ape1 is also a redox factor, which stimulates the DNA binding capacity of several transcription factors [p53, AP1 (Fos/Jun), HIF1-α, NFκB etc] by reducing cysteine residues in their DNA binding domains (Xanthoudakis, 1994; Tell et al., 2008; Luo et al., 2009). Through its DNA repair and redox functions, Ape1 is involved in protein-protein interactions within the BER pathway.
pathway and several other signaling pathways (Fan and Wilson, 2005). Most likely, both these functions of Ape1 contribute to the resistance of cancer cells to chemotherapeutic agents. While Ape1’s repair function likely plays a direct role in resistance, the redox function may play an indirect role through its modulation of transcription factors, which control the expression of genes with important roles in cell survival, tumor promotion and tumor progression (Fishel and Kelley, 2007; Luo et al., 2009; Reed et al., 2009).

Malignant glioma is a common and often fatal brain cancer, with a 2-year survival rate of ~26%. Treatment options for gliomas include surgical resection followed by chemotherapy with temozolomide (TMZ) or radiation both of which generate lesions repaired by the BER pathway (Evans et al., 2000; Chaudhry, 2007; Stupp et al., 2007). These treatments rarely result in complete remission and are often accompanied by adverse toxic effects in longer-surviving patients (Stupp et al., 2007). High expression levels of Ape1 in cancer cells including glioblastomas, compared to normal cells have been reported and altered sub-cellular localization of Ape1 correlates with cellular resistance to chemotherapeutic agents such as methyl methane sulfonate (MMS), TMZ, hydrogen peroxide, bleomycin and gemcitabine in several cancer cell lines (Bobola et al., 2001; Kelley et al., 2001; Robertson et al., 2001; Silber, 2002). Congruously, treatment with Ape1-targeted siRNA or expression of dominant-negative Ape1 increases cellular sensitivity to ionizing radiation and alkylating and oxidizing agents thereby suggesting Ape1 as a potential therapeutic target in cancer cells (Evans et al., 2000; Fishel et al., 2007; Fishel and Kelley, 2007; McNeill and Wilson, 2007).

Known inhibitors of Ape1’s repair activity include methoxyamine (MX) as well as small molecules identified in high-throughput screens. MX reacts with and covalently modifies AP sites to non-specifically inhibit enzymes that bind to AP sites (Rosa et al., 1991), including
Ape1, DNA polymerase β (Horton and Wilson, 2007) and other BER enzymes (Taverna et al., 2001). MX enhances the sensitivity of several cancer cell lines to TMZ and radiation (Fishel et al., 2007). Recent studies identified small molecule inhibitors of the endonuclease activity of Ape1: NCA (7-nitro-indole-2-carboxylic acid; CRT0044876) (Madhusudan et al., 2005), arylstibonic acid compounds (Seiple et al., 2007), pharmacophore-based compounds (Zawahir et al., 2009) and Reactive Blue 2, 6-hydroxyl - DL-DOPA (RB-DOPA) and myricetin (Simeonov et al., 2009). The arylstibonic compounds effectively inhibit Ape1 in vitro but are ineffective in vivo due to poor cellular uptake (Seiple et al., 2007), while the pharmacophore compounds were not tested in cell based assays (Zawahir et al., 2009). RB-DOPA and myricetin have numerous other targets besides Ape1 in cells and therefore are not very promising agents with selectivity or specificity as Ape1 inhibitors (Simeonov et al., 2009).

Here we aimed to identify and characterize specific inhibitor(s) of the Ape1 AP endonuclease, using a high-throughput assay (HTS) to screen a library of 60,000 small molecule compounds, which is the largest HTS performed to date. Four compounds with IC₅₀ values less than 10μM were identified, validated, and characterized. The most promising compound, designated Ape1 Repair inhibitor 03 (AR03) inhibits the AP endonuclease activity of purified human Ape1 in vitro and inhibits cleavage of AP sites in SF767 cell extracts in vitro and in SF767 glioblastoma cells in vivo. AR03 also potentiates the cytotoxicity of MMS and TMZ in SF767 cells.
Methods

Enzymes:

The human Δ40 Ape1 protein was purified as described before (Georgiadis et al., 2008). Briefly, the Δ40 Ape1 protein in the pET15b vector with an N-terminal hexa-His tag was expressed in the Rosetta E. coli strain. The cell pellets were re-suspended in Buffer A (50 mM Phosphate pH 7.8, 0.3 M NaCl and 10 mM Immidazol) and lysed using a French Press. The supernatant was first eluted from a Nickel column with Buffer B (50 mM Phosphate pH 7.8, 0.3 M NaCl and 250 mM Immidazol) and the pooled Ape1 fractions were diluted 5x with Buffer C (50 mM MES pH 6.5 and 1 mM DTT) to a salt concentration of 50 mM which was eluted a second time from an S-Sepharose column with Buffer D (50 mM MES pH 6.5, 1 M NaCl and 1 mM DTT). The Ape1 fractions digested overnight with Thrombin (2 U) to remove the hexa-His tag, were diluted 8x to 50 mM NaCl using Buffer E (50 mM MES pH 6.0 and 1 mM DTT) and gradient eluted from an S-Sepharose column with Buffer F (50 mM MES pH 6.0, 1 M NaCl and 1 mM DTT). Ape1 fractions were concentrated using a 10,000 Da-cutoff protein concentrator and protein concentration and activity of the protein were determined.

The E. coli endonuclease IV protein (100Units) used in the gel-based AP endonuclease assays was purchased from Trevigen (Gaithersburg, MD).

Cell extracts from SF767 glioblastoma cells were prepared as described previously (Kreklau et al., 2001). The protein concentration of the SF767 cell extracts was determined using the Bio-Rad Bradford Assay.

Oligonucleotides:

The pair of oligonucleotides used in the high throughput screening (HTS) assay were 30 base pairs in length (5’-6-FAM-GCCCCC*GGGGACGTACGATATCCCGCTCC-3’ and
3’-Q-CGGGGGCCCCCTGCATGCTATAGGGCGAGG–5’) and were synthesized via custom order from Eurogentec Ltd (San Diego, CA) (Madhusudan et al., 2005). Of the pair, one of the oligonucleotides has a Fluorescein label (6-FAM) at the 5’ end and contains an AP site mimic called Tetrahydrofuran (THF, represented as * in the oligonucleotide). The complimentary strand has a quenching moiety (Dabcyl-Q in the oligonucleotide) at its 3’ end. A stretch of G and C base pairs were used before and after the THF moiety in the HTS assay to prevent spontaneous dissociation of the short labeled fragment prior to cleavage by Ape1. 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 (5’-HEX-AATTCACCGGTACC*CCTAGAATTCG-3’) with a hexa–chloro phosphoramide (HEX) label and Tetrahydrofuran (THF, represented as *) molecule, and its opposite strand (3’-TTAAGTGGCCATGGTGGATCTTAAGC-5’) (Kreklau et al., 2001).

For both the HTS and the Gel-based AP endonuclease assay, 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 allowed to cool to room temperature overnight. The DNA was appropriately diluted, aliquoted and stored at –20°C.

**High Throughput Screening (HTS) Assay:**

The HTS assay used here was modified for our use from that described by Madhusudan et al (Madhusudan et al., 2005). A library of 60,000 diverse compounds adhering to Lipinski’s rules (Lipinski and Hopkins, 2004) from Chemical Diversity Ltd Inc (San Diego, CA) was tested at the Chemical Genomics Core Facility, Indiana University School of Medicine (IUSM). The library was aliquoted and screened at a concentration of 10 μM in black 384 well plates in a 50
μl reaction volume including 0.8% DMSO. The Genesis (Tecan) Workstation 150, TeMo with a 96-channel pipetting head was used to first add 20 μl a reaction mix containing the annealed fluorescein-dabcyl containing oligonucleotide substrate (100 nM) and the assay buffer (50 mM Tris pH 8.0, 1 mM MgCl₂, 50 mM NaCl and 2 mM DTT) to the plates containing the compound library (10 μM) in a 20 μl volume. 10 μl of the Ape1 protein at a final concentration of 0.35 nM was added to the reaction mix in the assay plates to initiate the reaction and changes 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. 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 Ape1 protein without inhibitors was considered as the 100% control and subsequent inhibition by the compounds was considered relative to that of the control.

**Calculation of IC₅₀ values of the compounds:**

After two rounds of screening, IC₅₀ values of the compounds selected for validation were determined. The assay used to determine the IC₅₀ 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 MgCl₂, 50 mM NaCl and 2 mM DTT) in a 50 μl reaction volume with a final DMSO concentration of 0.8%. Once again, fluorescence readings were taken at 37°C for 5 minutes and percent inhibition for each compound concentration was determined as compared to the control with no inhibitor. IC₅₀ values were calculated using the Sigma Plot graphing software using the four parameter logistic equation shown in Equation 1:
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\[ y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x}{\text{EC}_{50}}\right)^{\text{Hill Slope}}} \]

Each data value is an average of three independent experiments with standard errors. Statistical analysis of the data was done using the student’s t-test with p values representing the comparison between lanes with inhibitor and without inhibitor.

**Gel-based AP endonuclease assay:**

An annealed 26 base pair oligonucleotide substrate containing a THF moiety at position 13 is used in this assay, such that cleavage by Ape1 results in two 13 mer fragments of DNA, one with the fluorescent HEX label and one unlabeled. 0.175 nM Ape1 was added to the reaction mix also containing assay buffer (50 mM HEPES pH 7.5, 50 mM KCl, 1 mM MgCl$_2$ and 2 mM DTT), 25 nM of the HEX labeled DNA substrate and the inhibitor compounds in a range of concentrations in a total reaction volume of 20 μl. The reaction mixture was then incubated at 37°C for 15 minutes and the reaction 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 (Tris-Borate EDTA) 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.

For the endonuclease IV protein (100Units), the assay was performed as described above with 6.25 units of endonuclease IV protein and different concentrations of the inhibitor compounds.

The assay with SF767 cell extracts was also performed as described above. 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. For the experiments where pure 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 (0.7 - 5.6 nM) 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. Similarly, for SF767 whole cell extracts (WCE) immunodepleted of Ape1, the reaction was carried out as described above (Kreklau et al., 2001).

For each of the above gel-based assays (with pure Ape1 protein, endonuclease IV protein and SF767 cell extracts), assays were performed three times and the data presented as the average of three individual experiments and standard error. The Student’s t-test was used to calculate p values. For the gel assays with pure Ape1 and SF767 cell extracts alone, lanes with inhibitor are compared to the lane without inhibitors. For the experiments showing restoration of AP endonuclease activity of the cell extracts, lanes which contain pure Ape1 protein are compared to the lane with the cell extract and inhibitor alone.

**Fluorescence Intercalation Displacement (FID) assay to determine DNA-binding:**

All validated Ape1 inhibitors were tested for DNA-binding using an FID assay that takes advantage of the greatly increased fluorescence observed for ethidium bromide (EtBr) when bound to DNA (Fox, 1997; Glass et al., 2010), in this case using calf thymus DNA to present all possible DNA-binding sites. Compounds that effectively compete with EtBr for binding to DNA will result in a decrease in the fluorescence measured for EtBr. Triplicate measurements were made using a Perkin Elmer Envision 2102 multilabel plate reader for 1, 10, and 100 µM compound (final concentration of 0.8 % DMSO) in a total of 50 µl including 21 µM calf thymus DNA, 6.5 µM ethidium bromide, 100 mM NaCl, and 10 mM Tris pH 7.4 in 384 well black Nunc plates. The fluorescence was measured for each well at 530 nm excitation and 615 nm emission.
and compared to the negative control (no added compound) in order to calculate percent fluorescence decrease for each well and standard deviations. Positive controls included known DNA-binding agents, the minor groove binder, netropsin and an intercalator, actinomycin D.

**Immunodepletion of Ape1 from SF767 WCE:**

SF767 cell extracts were immunodepleted of the Ape1 protein using a polyclonal Ape1 antibody (Novus Biologicals, Littleton CO). 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 re-suspended in 50μl 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 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°C with gentle shaking for 2 hours. 50 μl of washed beads were added to the cell extracts and incubated for another 2 hours also with gentle rocking at 4°C. The cell extracts were 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°C.

**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 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 transferred onto a nitro-cellulose membrane at 90V 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 added to the membrane and allowed to rotate overnight at 4°C. The next day the membranes were washed with 1x TBST (1x TBS + 0.1% Tween 20) 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. After washing the membranes with 1x TBST (1x TBS + 0.1% Tween 20) the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL) was added to the blots and the blots were developed using the BioRad machine in the Chemiluminescence Hi-sensitivity mode. Actin was used as the internal loading control and the actin antibody (NeoMarkers, Fremont, CA) was used at a dilution of 1:1000 and actin was detected using the ECL Western Blotting Detection reagent (GE Healthcare, Buckinghamshire, UK). The experiment was performed three times and the data is an average of three individual experiments and 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 immunodepleted samples.

**Cell culture based survival assay:**

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 effects of the inhibitor compounds on the growth and survival of these SF767 glioblastoma cells was determined using the xCELLigence DP System (Roche Applied Science, Indianapolis IN) (Solly et al., 2004). 3000 SF767 glioblastoma cells were plated in each well of the 16-well plates in 100μl volume and 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. MMS (100 µM) and TMZ (2.25 mM) and AR03 (0.5 µM & 0.75 µM) in a 10 µl volume at a concentration 20x the final concentration, were added to the wells, alone or in combination. Continuous impedance measurements were then monitored over 72 hours. The assay was performed in triplicate.

**AP Site determination (ARP) assay:**

To determine number of AP sites formed SF767 cells were treated with 275 µM MMS and 3 µM AR03 alone or in combination for 24 hours. Cells were collected after 24 hours and the genomic DNA was isolated using the Get-Pure DNA isolation kit (Dojindo Molecular Technologies, Rockville, MD). The ARP reagent specifically reacts with the aldehyde group of AP sites in the open conformation converting them to biotin-tagged AP sites. The amount of biotin can then be quantified by an ELISA-like assay (Kow and Dare, 2000). Number of AP sites formed was determined using the AP Site Quantitation kit (Dojindo Molecular Technologies, Rockville, MD) as per the kit instructions. The experiments were repeated in triplicate and the data presented is an average of four independent experiments with standard error. An n = 12 was considered while calculating the p values for this experiment and p values were obtained using the student’s t-test where the combined treatment group was compared to AR03 inhibitor and MMS treated group alone.

**Redox Electrophoretic Mobility Shift Assay (EMSA):**

Redox EMSA assays were performed as described previously (Georgiadis et al., 2008).
Results

Optimization of the Fluorescence-based Assay for Ape1 AP Endonuclease Activity

A fluorescence-based AP endonuclease assay described by Madhusudan et al. (Madhusudan et al., 2005) was adapted in this study for high throughput screening (HTS) of a library of 60,000 small molecule compounds (see Materials and Methods). Briefly, the assay employs two complementary 30 bp synthetic oligonucleotides, one of which carries 5’-fluorescein and a tetrahydrofuran (THF) AP site mimic (Wilson and Barsky, 2001), while the complementary oligonucleotide carries a 3’-dabcyl fluorescence-quenching moiety (Figure 1A). After cleavage at the THF moiety, a short ssDNA fragment 5’-tagged with fluorescein is released from the duplex DNA substrate, resulting in increased fluorescence. In the linear range of this assay, the increase in fluorescence over time is proportional to the amount of AP endonuclease activity in the reaction (Madhusudan et al., 2005) (Figure 1A). To optimize the HTS screen, increasing concentrations of Ape1 were added to the reaction mixture, and a concentration of 0.35 nM Ape1 was selected for screening at 37°C for 5 min (data not shown). Under these conditions, the Z’ factor was 0.78, which indicates that the assay provides a statistically valid measure of inhibition of the Ape1 AP endonuclease (Zhang et al., 1999).

Identification and validation of Ape1 inhibitors from a 60,000-compound library

The HTS protocol used in this study is described in detail in Materials and Methods (see Supplemental Figure 1). In the first HTS cycle, 190 compounds were found to inhibit ≥ 50% of the Ape1 endonuclease activity. In a parallel effort, the same 60,000 compound library was screened for DNA-binding activity using a high-throughput fluorescence intercalator displacement assay (Glass et al., 2010). Compounds identified as Ape1 endonuclease inhibitors that also demonstrated strong DNA-binding were not further evaluated; these compounds would
not be expected to be specific inhibitors of Ape1 endonuclease activity. After two rounds of re-screening, 45 compounds were identified that achieved ≥40% inhibition of Ape1 endonuclease activity (Figures 1B, C). Forty-one of these compounds were available from ChemDiv at that time and were further validated as described below.

The concentration dependence for inhibition of AP endonuclease activity was determined for the 41 compounds that emerged as hits from the secondary HTS. NCA (Madhusudan et al., 2005) was used as a positive control, and IC$_{50}$ values were calculated using Sigma plot software. Eighteen compounds had an IC$_{50}$ value ≤50 μM, and 9 compounds had an IC$_{50}$ value ≤10 μM (Supplementary Table 1-data not shown in text). Representative data are shown for AR01 and AR03, including four-parameter logistic curves for determining IC$_{50}$ values, in Figures 2A and 2B, respectively. From the 41 compounds, seven chemically distinct classes of compounds were selected. Shown in Table 1 is the most potent inhibitor of each chemical class representing the chemical diversity of inhibitors identified in the HTS.

For comparison, a similar IC$_{50}$ analysis was carried out for previously characterized inhibitors of Ape1 AP endonuclease: these compounds included two arylstibonic acid compounds (13755 and 13793) reported by Seiple et al (Seiple et al., 2007) and the pharmacophore compound 18 (A1NI2-A3NI1) reported by Zawahir et al (Zawahir et al., 2009). Previously reported inhibitory effects on Ape1 activity of NCA (Madhusudan et al., 2005), resveratrol (Yang et al., 2005), and arylstibonic acids 13755 and 13793 (Seiple et al., 2007) were not reproducible or were quantitatively different in the present study than in previous studies (Supplementary Table 2-data not shown in text). Although the precise reason for these discrepancies is not known, they may reflect differences in the AP endonuclease assay conditions used in each study.
The seven chemically distinct AP endonuclease inhibitors were retested for DNA-binding using a fluorescent intercalator displacement (FID) assay (Table 1; Supplemental Figure 2) (Glass et al., 2010). In the FID assay, 10 µM netropsin or 10 µM actinomycin D (positive controls), caused a 32% or 63% decrease in fluorescence, respectively. A decrease in fluorescence of <20% indicated no significant DNA-binding activity. The FID data confirm that inhibitors AR01-AR07 do not exhibit significant DNA binding (Table 1).

At this point in the study, five inhibitors, AR01, 02, 03, 05, and 06 appeared to be the most promising of the chemically distinct inhibitors with IC_{50} values less than 10 µM. However, when diluted in aqueous solutions required for cell-based assays, AR05 was found to be completely insoluble. Thus, four chemically distinct inhibitors, AR01 (2-(4-(2,5-dimethyl-1H-pyrrol-1-yl)phenoxy acetic acid), AR02 (4-(2,6,8-trimethylquinolin-4-ylamino)phenol), AR03 (2,4,9-trimethylbenzo [b][1,8] napthyridin-5-amine) and AR06 (N-(3-chlorophenyl)-5,6-dihydro-4H-cyclopenta [d] isoxazole-3-carboxamide) (Table 1) were further characterized.

Further validation of AR01, AR02, AR03, and AR06 included inhibition of Ape1 endonuclease activity using a gel-based AP endonuclease assay (see Materials and Methods for details (Kreklau et al., 2001)). The results of the gel-based assay confirm that all four inhibitors effectively inhibit Ape1 AP endonuclease activity. Representative results for AR01 and AR03 are shown in Figure 3A. The inhibitors were also tested for ability to inhibit E. coli endonuclease IV, which is structurally unrelated to Ape1 (Wilson and Barsky, 2001; Garcin et al., 2008). The results of gel-based AP endonuclease assays show that in this assay, AR03 is approximately 8-fold less effective in inhibiting endonuclease IV than Ape1 (40 µM for 50% inhibition vs 5 µM), while AR01 inhibited endonuclease IV and Ape1 at similar concentrations.
(0.6 μM for 50% inhibition vs 1.4 μM) (Figure 3B). AR06 and AR02 inhibited endonuclease IV by 50% at 10 μM and 50 μM, respectively (Supplementary Figure 3).

**Inhibition of Ape1 in SF767 Whole Cell Extracts**

The ability of AR01, AR02, AR03, and AR06 to inhibit AP site cleavage in SF767 glioblastoma whole cell extracts (Kreklau et al., 2001) was examined using the gel-based AP endonuclease assay. AR01 and AR03 inhibited purified Ape1 and AP endonuclease activity in SF767 extracts with similar efficiency (Figure 4A). However, higher concentrations of AR02 and AR06 (~93 μM and 26 μM, respectively, for 50% inhibition) were required to inhibit the AP endonuclease activity in SF767 cell extracts than to inhibit purified Ape1 (Supplementary Figure 4). Importantly, addition of purified Ape1 (0.7-5.6 nM) reversed inhibition of AP site cleavage in SF767 cell extracts treated with 50 μM AR03 (Figure 4B) or 10 μM AR01 in a dose-dependent manner. Immunodepletion of Ape1 from SF767 cell extracts with a polyclonal Ape1 antibody also strongly reduced AP site cleavage activity in the cell extracts (Figure 4C and D). These data suggest that Ape1 is the primary AP endonuclease in SF767 extracts, and that AR01 and AR03 specifically target the endonuclease activity of Ape1, thereby reducing AP site cleavage within the context of glioblastoma whole cell lysate. The higher concentrations of AR02 and AR06 required to inhibit AP endonuclease activity in SF767 cell extracts suggest that they do not selectively target Ape1 in cell extracts; therefore, they were not further characterized.

**Effect of AR03 on SF767 Cells**

Initially, both AR01 and AR03 were tested in an MTT cell proliferation assays using SF767 glioblastoma cells. However, no effect on cell proliferation was observed for AR01 at concentrations as high as 200μM suggesting that it may not be cell permeable, whereas AR03 was found to effectively kill cells at low micromolar concentrations (LD$_{50}$ value of ~1μM)
Therefore, the ability of AR03 to inhibit proliferation and/or reduce survival of SF767 glioblastoma cells was further characterized using the xCELLigence DP system (Roche, Indianapolis IN). This system measures a dimensionless parameter called Cell Index (CI), which evaluates the ionic environment at an electrode/solution interface and integrates information on cell viability, number, morphology, and adhesion (Xing et al., 2005; Kirstein et al., 2006). AR03 reduced CI of SF767 cells efficiently and in a dose-dependent manner, suggesting that it inhibits growth and/or kills SF767 cells (Figure 5A). AR03 also enhanced the cytotoxicity of MMS and TMZ (Figure 5B and C). These data are consistent with the idea that AR03 inhibits AP site cleavage by Ape1 and that MMS and TMZ increase the load of AP sites, leading to increased cytotoxicity.

Quantification of AP Sites in AR03-treated SF767 cells

The above data predict that treatment with AR03 will increase the number of AP sites in MMS-treated SF767 cells. This idea was tested by treating SF767 cells with MMS and AR03 and quantifying AP sites using the Aldehyde Reactive Probe (ARP) assay (Kow and Dare, 2000). As predicted, more AP sites were detected in cells treated with AR03 and MMS than in cells treated with either agent alone (Figure 6). These data further support the conclusion that AR03 inhibits Ape1-catalyzed repair of AP sites in SF767 cells.

Effects of AR inhibitors on Ape1 Redox Activity

The effect of AR01, AR02, AR03 and AR06 on Ape1 redox activity (Tell et al., 2008) was investigated using an electrophoretic mobility shift assay (EMSA) (Georgiadis et al., 2008). In this assay, reduction of c-Jun/c-Fos by Ape1 increases its DNA-binding affinity for DNA containing an AP-1 binding site and is measured by quantitation of the shifted band. AR02, AR03, and AR06 had no effect on the redox activity of Ape1 or the ability of c-Jun/c-Fos to bind
DNA (data not shown). This result confirms the low binding affinity of these compounds for duplex DNA. However, addition of AR01 reduced the ability of c-Jun to bind DNA in this assay (50% inhibition at 15 uM). To determine whether this reduction was dependent on the presence of Ape1, the assay was repeated using reduced c-Jun/c-Fos in the absence of Ape1 protein, and a similar result (50% inhibition at 15 uM) was obtained suggesting that AR01 affects the ability of c-Jun/c-Fos to bind DNA in the absence of Ape1 and is therefore not a redox inhibitor (data not shown).
Discussion

This study reports the results of a high-throughput screen of 60,000 small molecule compounds, leading to the identification and characterization of several small molecule inhibitors of Ape1 AP endonuclease activity. Seven chemically distinct inhibitors were initially selected of which four emerged from initial validation studies as promising inhibitors and were further characterized. AR01 is negatively charged like many of the Ape1 inhibitors reported previously (Madhusudan et al., 2005; Seiple et al., 2007; Simeonov et al., 2009; Zawahir et al., 2009), but is otherwise unrelated to the previously described inhibitors. AR02, AR03, and AR06 are novel inhibitors of Ape1. Having established that AR02 and AR06 may have significant off-target interactions (Supplementary Figure 2), the focus of this study was to characterize the specificity of AR01 and AR03 as Ape1 inhibitors in vitro and in SF767 glioblastoma cells.

The most potent inhibitors identified in this screen have low affinity for duplex DNA (Table 1), a critical characteristic for a specific inhibitor of Ape1. The ability of AR01, AR02, AR03 and AR06 to inhibit AP site cleavage by endonuclease IV was also examined as a measure of specificity, due to functional but not structural similarity to Ape1. AR01 inhibits Ape1 and endonuclease IV at similar concentrations; thus, it is possible that AR01 binds to an active site metal (i.e., through its carboxylate moiety) or another positively-charged protein region, a site that normally binds to the DNA phosphate backbone. The ability of AR01 to inhibit binding of c-Jun/c-Fos to DNA is consistent with the latter mechanism of inhibition. Significantly higher concentrations of AR02, AR03, and AR06 are required to inhibit endonuclease IV than Ape1; these compounds may interact directly with Ape1 or the Ape1-DNA substrate complex, which is structurally distinct from the endonuclease IV-DNA substrate complex.
An important result of this study is that AR01 and AR03 inhibit the predominant AP endonuclease activity in SF767 cell extracts (Figure 4A) and do so as effectively as they inhibit pure Ape1 in vitro. Furthermore, this effect is reversed in a linear- and dose-dependent manner by addition of purified Ape1 to the AR03-treated cell extract (Figure 4B). Immunodepletion of Ape1 from the SF767 cell extract confirms the conclusion, based on other published studies, that Ape1 is the predominant AP endonuclease in SF767 cells (Figure 4C and D). Together, these results suggest that AR03 inhibits the AP endonuclease activity of Ape1 protein in cellular extracts and that Ape1 is the primary target of AR03 in SF767 cells: thus, we conclude that AR03 selectively targets Ape1. Although AR01 also efficiently inhibits AP site cleavage in SF767 cell extracts, it inhibits purified Ape1 and endonuclease IV at similar concentrations and inhibits the binding of c-Jun/c-Fos to DNA. Therefore, the specificity of AR01 for Ape1 is less well defined and warrants further study.

Glioblastoma is a relatively intractable cancer that is often treated with alkylating agents or radiation therapy (Stupp et al., 2007). Here, SF767 glioblastoma cells were used as a representative cancer cell line to explore the potential efficacy of AR03 as an oncotherapeutic agent alone or in combination with other cancer drugs. AR03 inhibits growth and viability of SF767 cells using the xCELLigence and MTT-based proliferation assays (Figure 5A and data not shown). In the present study using SF767 cells, the most likely mechanism for AR03-induced loss of cell viability is the accumulation of cytotoxic AP sites (Evans et al., 2000; Wilson and Barsky, 2001), a mechanism that is strongly supported by direct demonstration of increased accumulation of AP sites in these cells (Figure 6). Further supporting the inhibition of Ape1 by AR03, xCELLigence assays show that AR03 enhances the cytotoxicity of MMS and TMZ (Figure 5B and C). Both of these DNA damaging agents create lesions acted upon by BER and
Ape1, and we expected potentiation of MMS and TMZ under conditions in which Ape1 was not functioning. The combination of AR03 and traditional chemotherapeutic agents could collaboratively block and overload the BER pathway, leading to cancer cell death. In contrast, AR01 is not toxic to SF767 cells (data not shown), which may be due to poor uptake of this negatively-charged compound by these cells.

In conclusion, this study describes several inhibitors of Ape1 AP endonuclease, the most promising of which is AR03. AR03 appears to be a specific inhibitor of Ape1 AP endonuclease activity, with low micromolar efficacy as an inhibitor of purified Ape1 or Ape1 in cell extracts, low affinity for duplex DNA and much lower ability to inhibit E. coli endonuclease IV than human Ape1 protein. AR03 reduces proliferation and viability of cultured SF767 glioblastoma cells and enhances the cytotoxicity of MMS and TMZ, suggesting that it may have potential therapeutic applications. Additional studies are needed to determine the effect of AR03 in combination with other cancer chemotherapeutic agents, in combination with other Ape1 inhibitors or other BER targets such as PARP (Reed et al., 2009) and on other cancer cell lines and in the context of other in vitro or in vivo model systems. Additional analyses will also need to be performed to determine if there are any off-target effects of AR03 such as effects on both mitotic and post-mitotic normal cells. Furthermore, some structure-activity studies and pharmacokinetic studies to determine the mechanism of inhibition of AR03 will need to be performed in order to assess the therapeutic potential of this class of small molecule inhibitor of Ape1 endonuclease activity.

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References


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Figure Legends

Figure 1: High Throughput Screening (HTS) Assay to identify inhibitors of Ape1

(A) Principle of the HTS Assay. This figure represents the principle of the modified fluorescent HTS assay used for our screening purposes. The fluorescence of 6-FAM is diminished by Q because of their proximity to each other. On addition of Ape1, it cleaves the THF residue releasing the short 6-FAM labeled fragment which results in a proportionate increase in fluorescence. This increase in fluorescence can be considered to be an indirect measure of Ape1’s cleavage activity. (B) Results of the initial screen of the 60,000 compound library. After the screen of the entire library, we identified 190 compounds that inhibited Ape1’s activity by 50% or more. (C) Results of a secondary screen of all the hits from the initial screen showing ≥ 50% inhibition of Ape1’s activity. Out of the 190 compounds identified, 174 were re-tested in this same assay to weed out false positives and 41 compounds with ≥ 40% inhibition of Ape1’s DNA repair activity were validated. For both (B) & (C) the graphs are representations of the numbers of compounds plotted with their corresponding % inhibition of Ape1’s activity.

Figure 2: Calculation of the IC₅₀ values of the AR01 and AR03 compounds:

IC₅₀ values of the top hit compounds were determined using the HTS assay. A range of concentrations of each of the compounds were tested with 0.35 nM Ape1. The percent inhibition of Ape1’s activity was calculated using the rates of reaction for each of the concentrations of the compounds as compared against the percent activity of Ape1 alone. The assays for each compound were performed three individual times. 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. (A) IC$_{50}$ value curve for AR01 and (B) IC$_{50}$ value curve for AR03. P values were calculated using the student’s t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); * = $p \leq 0.03$; ** = $p \leq 0.0005$; *** = $p \leq 0.0001$.

**Figure 3: Effect of AR01 and AR03 on the activity of purified Ape1 and endonuclease IV proteins in the gel-based AP endonuclease assay:**

The ability of the top hit compounds to inhibit Ape1’s activity in a distinct gel-based AP endonuclease assay was tested, as was their effect against the *E. coli* endonuclease IV protein. The assay was performed as described in the Materials and Methods. Effect of AR01 and AR03 on purified Ape1 protein (A) and on endonuclease IV (B). Representative gels are shown. Each assay was performed three individual times and is shown here as the average with standard error (B: AR01 and D: AR03). P values were calculated using the student’s t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO); # = $p \leq 0.05$; * = $p \leq 0.005$; ** = $p \leq 0.001$; *** = $p \leq 0.0001$.

**Figure 4: Ability of the top hits to inhibit Ape1’s activity in cell extracts made from SF767 glioblastoma cells:**

(A) The ability of the top hit compounds to inhibit the AP endonuclease activity of SF767 cell extracts was tested. The effect of the compounds on SF767 whole cell extract was determined using the gel-based AP endonuclease assay (as described in the Materials and Methods), and both compounds inhibited Ape1’s repair activity similarly to that of purified Ape1 protein in the gel assay. P values were calculated using the student’s t-test comparing lanes with inhibitor to lane with no inhibitor (DMSO). (B) To further confirm the inhibition of Ape1 in the SF767 cell
extracts by AR03, increasing amounts (0.7-5.6nM) of purified Ape1 protein were added to SF767 cell extracts treated with 10μM AR01 or 50μM AR03 (described in Materials and Methods). Addition of purified Ape1 protein results in a linear rescue of the AP endonuclease activity of the SF767 cell extracts as compared to those without inhibitors. Lane 1: DNA alone (no extract), Lane 2: SF767 cell extract + DMSO, Lane 3: SF767 cell extract + inhibitor, 10 μM AR01 or 50 μM AR03, Lanes 4-7: SF767 cell extract + inhibitor + increasing amounts of purified Ape1 protein (0.7-5.6nM), Lanes 8-11: SF767 cell extract + increasing amounts of purified Ape1 protein (0.7-5.6nM) without inhibitors. To calculate p values, Lanes 4-7 were compared to Lane 3. To confirm that Ape1 is the only protein from the cell extracts that can act in the gel-based AP Endonuclease assay, SF767 cell extracts were immunodepleted of Ape1 using a polyclonal Ape1 antibody (10μg) and the AP endonuclease activity of these extracts was determined. (C) Western blot analysis to show reduced levels of the Ape1 protein in the immunodepleted SF767 cell extracts (0.75 - 3μg of protein loaded). Actin was used as a loading control. (D) The AP Endonuclease activity of the immunodepleted SF767 cell extracts is significantly less than the IgG treated control extracts. Representative gels are shown. Each assay was performed three individual times and is shown here as the average with standard error. For C & D: IgG control lanes were compared to the lanes with the immunodepleted cell extracts. # = p ≤ 0.08, # # = p ≤ 0.01, * = p ≤ 0.005; ** = p ≤ 0.001; *** = p ≤ 0.0001.

**Figure 5: Cell survival analysis of SF767 glioblastoma cells with AR03 alone and in combination with MMS and TMZ:**

The xCELLigence DP system was used to determine cell survival and growth after treatment with AR03 alone and in combination with MMS and TMZ. AR03 can act as a single agent to kill...
SF767 glioblastoma cells (A). Combined treatment of the SF767 cells with AR03 and MMS (B) or TMZ (C) results in greater cell killing as compared to either agent alone. The assay was performed in triplicate, three individual times and shown here is a representative experiment.

**Figure 6: 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. A significant increase in the number of AP sites was seen 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).
Table 1.

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<th>Small Molecule</th>
<th>Mol Wt</th>
<th>cLogP</th>
<th># Rel Cmpds</th>
<th>HTS Assay</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Δ FID DNA-binding</th>
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<td>AR01</td>
<td>245.274</td>
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<td>1</td>
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<td>1.7 ± 0.3</td>
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<tr>
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<tr>
<td>AR03</td>
<td>237.3</td>
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<td>2.1 ± 0.1</td>
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<tr>
<td>AR04</td>
<td>350.414</td>
<td>4.8</td>
<td>1</td>
<td>58 45</td>
<td>14.9 ± 8.4</td>
<td>&lt;15%</td>
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<tr>
<td>AR05</td>
<td>351.379</td>
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<td>75</td>
<td>85</td>
<td>6.6 ± 0.9</td>
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<tr>
<td>AR06</td>
<td>262.692</td>
<td>2.7</td>
<td>1</td>
<td>52 44</td>
<td>1.6 ± 0.1</td>
<td>3.10%</td>
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<tr>
<td>AR07</td>
<td>400.534</td>
<td>5.3</td>
<td>12</td>
<td>80 41</td>
<td>~100</td>
<td>&lt;15%</td>
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Figure 1

A

AP Site (THF)

5' ___________________________ 3'

3' ___________________________ 5'

+ Ape1

Ape1 cleaves the DNA at the THF moiety

5' ___________________________ 3' + 3' ___________________________ 5'

Cleavage of THF results in an increase in fluorescence of 6 – FAM which is indicative of Ape1’s activity
Figure 1

B

Number of Compounds

% Inhibition

No Inhibition

below 0  0 - 10  11 - 20  21 - 30  31 - 40  41 - 50  51 - 60  61 - 70  71 - 80  81 - 90  91 - 100

> 50% = 190

> 60% = 120
Figure 1

C

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<th>% Inhibition</th>
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<tr>
<td>below 0</td>
<td>26</td>
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<tr>
<td>0 - 10</td>
<td>21</td>
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<td>11 - 20</td>
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<td>81 - 90</td>
<td>2</td>
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<tr>
<td>91 - 100</td>
<td>2</td>
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> 40% = 45

> 60% = 8
Figure 2

A

B

% Activity

AR01 (µM)

AR03 (µM)

Downloaded from jpet.aspetjournals.org on April 17, 2021
Figure 4

B

SF767 cell ext
10μM AR01
rApe1 (nM)

Lane: 1 2 3 4 5 6 7 8 9 10 11

SF767 cell ext
50μM AR03
rApe1 (nM)

Lane: 1 2 3 4 5 6 7 8 9 10 11

% Cleaved

hApe1 (nM)

hApe1 (nM)
Figure 5

B

[Graph showing the effect of different treatments on cell index over time. The graph includes lines for DMSO, 0.5 μM AR03, 100 μM MMS, and MMS + AR03. The x-axis represents time in hours, and the y-axis represents cell index.]
Figure 6

![Bar graph showing the number of AP sites per 10^6 base pairs for different treatments.](graph.png)

**Y-axis:** #AP Sites / 10^6 base pairs

**X-axis:** DMSO, 275μM MMS, 3μM AR03, 275μM MMS + 3μM AR03

- DMSO: lowest number of AP sites
- 275μM MMS: moderate increase
- 3μM AR03: further increase
- 275μM MMS + 3μM AR03: highest number of AP sites

Significance levels:
- *: p < 0.05
- **: p < 0.01

Comparison between treatments shows a statistically significant increase in AP sites when MMS is combined with AR03 compared to MMS alone.