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Potent Inhibition of Human Apurinic/Apyrimidinic Endonuclease 1 by Arylstibonic Acids[S]

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ABSTRACT

Human apurinic/apyrimidinic endonuclease (Ape1) plays an important role by processing the >10,000 highly toxic abasic sites generated in the genome of each cell every day. Ape1 has recently emerged as a target for inhibition, in that its overexpression in tumors has been linked with poor response to both radiation and chemotherapy and lower overall patient survival. Inhibition of Ape1 using siRNA or the expression of a dominant-negative form of the protein has been shown to sensitize cells to DNA-damaging agents, including various chemotherapeutic agents. However, potent small-molecule inhibitors of Ape1 remain to be found. To this end, we screened Ape1 against the NCI Diversity Set of small molecules and discovered aromatic nitroso, carboxylate, sulfonamide, and arylstibonic acid compounds with micromolar affinities for the protein. A further screen of a 37-compound arylstibonic acid sublibrary identified ligands with IC_{50} values in the range of 4 to 300 nM. The negatively charged stibonic acids act by a partial-mixed mode and probably serve as DNA phosphate mimics. These compounds provide a useful scaffold for development of chemotherapeutic agents against Ape1.

Every day, more than 10,000 abasic sites are formed in each cell as a result of the spontaneous depurination of DNA bases (Lindahl and Nyberg, 1972). Without repair, these abasic sites are both mutagenic and cytotoxic (Boiteux and Guillet, 2004). Human apurinic/apyrimidinic endonuclease (Ape1) plays the important role of processing these lesions so that they may be recognized by subsequent enzymes and repaired. Ape1 accounts for more than 95% of the abasic site cleavage activity within the cell (Wilson and Barsky, 2001). This protein cleaves the DNA 5′ to the abasic site, producing a 5′ deoxyribose phosphate group and a 3′ hydroxyl. The 5′ deoxyribose phosphate is a substrate for DNA polymerase β, which removes this blocking group and adds the correct nucleotide. The remaining nick in the DNA is then closed by DNA ligase, consummating repair of the site (Dianov et al., 2003). Ape1 is also a pivotal component of the base excision repair pathway, which is responsible for removing aberrant bases from the genome. This pathway is initiated by enzymatic removal of a damaged or incorrect base by a DNA glycosylase, also producing an abasic site. After cleavage of this abasic site by Ape1, the DNA is repaired through the action of the same enzymes as described above (Dianov et al., 2003). In addition to its role in DNA repair, Ape1 has been shown to be an important facilitator of both redox-dependent and -independent DNA-transcription factor binding, giving the protein the alternative name of redox factor-1 (Ref-1). Many of the transcription factors regulated by Ape1, which include Jun, Fos, nuclear factor-κB, and p53, play a pivotal role in the regulation of cell growth and apoptosis (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992; Jayaraman et al., 1997).

Both the DNA cleavage and transcription factor binding activities of Ape1 result in increased resilience of cells to proapototic stimuli such as radiation, oxidative stress, and chemotherapy (Fishel and Kelley, 2007). Not surprisingly, the overexpression of Ape1 has led to resistance to DNA-damaging agents in several human tumor cell lines (Silber et al., 2002; Yang et al., 2005b). Conversely, decreasing Ape1 expression using small interfering RNA or a dominant-negative form of the protein leads to hypersensitivity to chemically induced DNA damage in both cell culture and tumor

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ABBREVIATIONS: Ape1, apurinic/apyrimidinic endonuclease; FAM, 6-carboxyfluorescein; Ref-1, redox factor-1; DMSO, dimethyl sulfoxide; HOS, human osteosarcoma; THF, tetrahydrofuran; UNG, uracil DNA glycosylase; MMS, methyl methanesulfonate; MX, methoxyamine.

xenograft models (Walker et al., 1994; Silber et al., 2002; Liu et al., 2003; Wang et al., 2004; McNeill and Wilson, 2007). Tumor promotion by Ape1 hasbeen shown not only in the laboratory but also in the clinic, where higher levels of Ape1 expression and altered Ape1 localization have been correlated with tumor progression and poor prognosis for patients with various malignancies including osteosarcomas and breast, lung, cervical, prostate, germ cell, ovarian, and head-and-neck cancers (Xu et al., 1997; Herring et al., 1998; Kelley et al., 2001; Koukourakis et al., 2001; Puglisi et al., 2001, 2002; Robertson et al., 2001; Tanner et al., 2004; Wang et al., 2004; Minisini et al., 2005). Although small molecule inhibitors of the Ape1 endonuclease or redox factor activities have been reported (Luo and Kelley, 2004; Madhusudan et al., 2005; Yang et al., 2005a,b), these inhibitors are either fairly weak or nonspecific or the effects in cell culture have been difficult to reproduce (Fishel and Kelley, 2007). The development of effective small-molecule inhibitors of Ape1 would provide useful research tools to explore its role in DNA repair, cancer, and redox-coupled transcription.

It is difficult to envision the rational design of small molecule inhibitors of Ape1 using the structural information provided by its complex with an abasic DNA substrate (Mol et al., 2000). These structures reveal an active site composed of a small hydrophobic pocket surrounded by residues that form electrostatic interactions with the DNA phosphates flanking the abasic site. Specific interactions with the abasic sugar are lacking (Mol et al., 2000), and, as a result, Ape1 is able to cleave 5′ to any abasic analog that is small, hydrophobic, and unbranched, including a simple ethanediol linkage (Wilson et al., 1995). Although we have previously used substrate-fragment tethering strategies to design small molecule inhibitors of several DNA repair enzymes (Jiang et al., 2005; Jiang et al., 2006; Krosky et al., 2006), this approach requires an initial substrate fragment, which is not obvious in the case of Ape1. Accordingly, we turned to high-throughput screening methods to identify and then characterize potent small molecule inhibitors of Ape1 that specifically bind to the enzyme and inhibit its activity at nanomolar concentrations.

Materials and Methods

Ape1 Purification and DNA substrates. The plasmid HAP1-pET15b was a gift of Dr. J. B. Alexander Ross (University of Montana, Missoula, MT). Recombinant Ape1 was purified as described previously (Erzberger et al., 1998). The DNA substrates used in this study were synthesized using conventional solid phase synthesis and reagents supplied by Glen Research. The sequences of the substrate DNA strands are 5′-FAM-GAG AAΦ ATA GTC GCG-3′ and 3′-dabsyl-CTC TTG TAT CAG CGC-5′ (where Φ is a tetrahydrofuran abasic site analog). After synthesis, the oligonucleotides were HPLC purified over a Zorbax column (Phenomenex, Torrance, CA) and desalted using reversed phase columns (PD-10; Bio-Rad Laboratories, Hercules, CA). The sequence of the oligonucleotides was verified using matrix-assisted laser desorption ionization mass spectrometry. The kinetic parameters for Ape1 cleavage of the hybridized DNA were determined using a Fluoromax-3 fluorimeter (Horiba Jobin Yvon, Edison, NJ).

High-Throughput Screening. To perform the screening assay, 5 μl of each library member in DMSO was first spotted to a well of a black 384-well microtiter plate (Costar 3710; Corning Life Sciences, Acton, MA). The addition of protein and substrate was then carried out using a MAP-C2 liquid handling system (Titertek, Huntsville, AL). First, 40 μl of Ape1 in reaction buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 0.05% Brij-35) was added to each well, and the plate was incubated at room temperature for 20 min. The reaction was then initiated through the addition of 10 μl of substrate, and the plate was incubated at room temperature for an additional 60 min. After 60 min, the final fluorescence was measured using a FLUOstar Optima plate reader (BMG Labtech GmbH, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively. The final concentrations of the reagents used in the assay were 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.05% Brij-35, 20 μM small molecule, 50 nM abasic-containing DNA substrate, and 5 pM Ape1. Hits were defined as those compounds able to inhibit Ape1 activity (measured as final fluorescence) by 40% or greater. Compounds that appeared highly colored, contained disulfide bonds, or had structures suggestive of DNA intercalation were excluded from further analysis. Hits from the initial screen were analyzed further for inhibitory potency using the fluorescence-based assay and decreasing dilutions of inhibitor. The assay was carried out as before, except that the reactions were initiated by the addition of enzyme rather than substrate, and FAM fluorescence was monitored every 5 min for a total of 30 min to acquire initial rate values. The final concentrations of the reagents used in the assay were 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.05% Brij-35, 0.1 to 10 μM small molecule, 50 nM abasic-containing DNA substrate, and 10 pM Ape1. Percentage of inhibition was determined relative to a DMSO-only control.

DNA oligonucleotides for a secondary radioactivity assay were synthesized as described above with the exclusion of the fluorescent groups. The sequences of the DNA strands are 5′-GGG CGC PΦA GTC GCG-3′, where P is 2-aminopurine, and 3′-CCC GCG TAT CAG CGC-5′. The THF-containing strand was labeled on the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) before hybridization. The individual reactions were initiated in the same manner as the fluorescence assay and quenched at 30 or 60 min by adding EDTA and heating for 20 min at 70°C. The final concentration of the reagents used in the assay were 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.05% Brij-35, 100 nM inhibitor, 50 nM abasic-containing DNA substrate, and 5 pM Ape1. Samples from each quenched reaction were run on a 20% denaturing polyacrylamide gel and the substrate and product bands were quantified using a Fuji BAS 2500 filmless autoradiographic analyzer.

IC_{50} Determinations. IC_{50} analyses were carried out on the best hits by first plating 10-fold dilutions (10 pM–10 μM) of each inhibitor onto black 384-well plates. The reactions were set up in the same manner as the initial screen except that FAM fluorescence was measured every 5 min after reaction initiation for a total of 60 min. Using the initial rate values from the assay, percentage activity was calculated for each sample relative to a negative (DMSO only) control. The data were fitted to a sigmoidal dose-response model using Prism software (GraphPad Software, San Diego, CA), and IC_{50} values were determined using the equation % Activity = $100/(1 + 10^{(\log[I] - \log ic_{50})})$.

DNA Intercalation Assay. An ethidium bromide-based DNA binding assay was carried out essentially as described previously (Boger et al., 2001). In brief, 10-fold dilutions of hit compounds in DMSO were spotted onto a 384-well black microtiter plate in 2.5-μl aliquots. To each well was added 47.5 μl of a mixture of ethidium bromide and DNA substrate in Ape1 reaction buffer. Ethidium bromide fluorescence was monitored at excitation and emission wavelengths of 544 and 612 nm, respectively. The final concentrations of the reagents used in the assay were 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.05% Brij-35, 100 pM–100 μM inhibitor, 1 μM ethidium bromide, and 133 nM DNA substrate to maintain a ratio of 1:2 of ethidium bromide to DNA base pairs. Percentage fluorescence was calculated relative to a negative (DMSO only) control.

Mode of Inhibition Analysis. A mode of inhibition analysis was carried out on the best arylstibonate inhibitors by first plating dilu-

tions of each inhibitor onto white nonbinding 96-well microtiter plates (Corning 3600). Dilutions of substrate in Ape1 buffer were added to each well, followed by the addition of Ape1 protein to initiate the reactions. FAM fluorescence was then monitored every 5 min for a total of 30 min. The final concentration of the reagents used in the assay were the same as the screening conditions except that the NaCl concentration was increased to 75 mM to increase K_m and allow rate measurements at substrate concentrations much less than K_m. A range of inhibitor concentrations between 5 nM and 10 μM and substrate concentrations in the range between 62.5 nM and 2 μM were used. The data were analyzed using Prism software, and initial rate values were used for global discrimination fitting by the computer simulation program Dynafit v.3.28 (BioKin, Pullman, WA) (Kuzmic, 1996).

Cell Culture Studies. Modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (Invitrogen, Carlsbad, CA) was used for all tissue culture. Human osteosarcoma (HOS) cells (American Type Culture Collection, Manassas, VA) were plated onto 96-well tissue culture plates at a density of 3000 cells/well and allowed to adhere overnight. The following day, inhibitor (in media) was added to a concentration of 5 μM, and the cells were incubated an additional 24 h. On the third day, the old media was removed, and fresh inhibitor and 0, 25, or 50 μM MMS was added to the cells. After 48 h of additional incubation, the cells were counted, diluted, and plated in triplicate onto six-well tissue culture plates. Colonies were allowed to grow for 1 week before being rinsed with phosphate-buffered saline and fixed and stained with 6% glutaraldehyde and 0.5% crystal violet for 1.5 h. Stained colonies were counted and clonogenic survival was determined relative to untreated cells.

Results

High-Throughput Screening. A robust molecular beacon assay was used to identify inhibitors of Ape1. In brief, a double-stranded DNA substrate containing a tetrahydrofuran (THF) abasic site mimic was synthesized, purified, and annealed to its complementary strand. The strand containing the THF site was labeled on the 5′ end using FAM, whereas the 3′ end of the opposite strand was labeled with a dabsyl quench (Fig. 1A, DAB). Upon DNA backbone cleavage by Ape1, a small seven-base oligonucleotide labeled with the FAM group spontaneously dissociates from the remaining DNA (Fig. 1B), causing fluorophore emission to increase by approximately 6-fold under the assay conditions used (Fig. 1C). The k_{cat} and K_m of Ape1 for this substrate in a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 0.05% Brij-35 are 8 ± 1 s^{-1} and 90 ± 30 nM, respectively (Supplemental Fig. 1). The turnover number k_{cat} was not significantly dependent on the NaCl concentration in the range 50 to 150 mM, but K_m increased to 570 ± 100 nM at = 100 mM NaCl (data not shown). Based on these findings, we chose to carry out the screening studies at 50 mM NaCl, where K_m was fairly low (to conserve substrate), and to use a substrate concentration equal to the K_m, so that both competitive and uncompetitive inhibitors could be detected.

High-throughput screening for inhibitors of Ape1 was carried out using the 2000-compound Diversity Set obtained from the National Cancer Institute. Initial hits were defined as those compounds that inhibited protein activity by 40% or greater. The initial screen identified approximately 140 molecules as inhibitors of Ape1, giving a hit rate of 7%. All but 15 of these compounds reconfirmed as hits in a secondary fluorescence assay. To cull the remaining 125 confirmed hits, 86 molecules with weak inhibition (<75% inhibition at 20 μM compound) were eliminated. For these 125 compounds, those that contained disulfide bonds or with structures suggestive of DNA intercalation were also not investigated further, as were nine weakly inhibitory and intensely colored dye-like compounds. Ten of the remaining 32 hits were found to inhibit Ape1 activity by ≥50% at concentrations in the range of 0.1 to 10 μM (Table 1). The inhibitory activities of four representative compounds (**13778**, **P7810**, **28620**, and **15596**) were then verified using a radioactive assay (Supplemental Fig. 2).

From the initial screen, two of the best Ape1 inhibitors, **15596** and **13778**, were arylstibonic acids, suggesting that such compounds may serve as useful scaffolds for further diversification. Accordingly, we screened an additional 37-member arylstibonic acid library that we obtained from the Developmental Therapeutics Program at the National Cancer Institute. This effort identified two more inhibitors (**13755** and **13793**) with increased potency over the initially screened compounds (Fig. 2). The IC_{50} values for these arylstibonic acids ranged from 4 to 17 nM using the screening buffer that contains 50 mM NaCl (Table 2).

Mode of Inhibition. A detailed mode of inhibition analysis was carried out on the two most potent arylstibonate inhibitors (**13755** and **13793**). Initial rates were measured as a function of substrate concentration at fixed amounts of each inhibitor (Fig. 3A). The observed rates at each substrate concentration were plotted against inhibitor concentration and fitted to various inhibition models using the simulation program Dynafit (Kuzmic, 1996) (Fig. 3B).

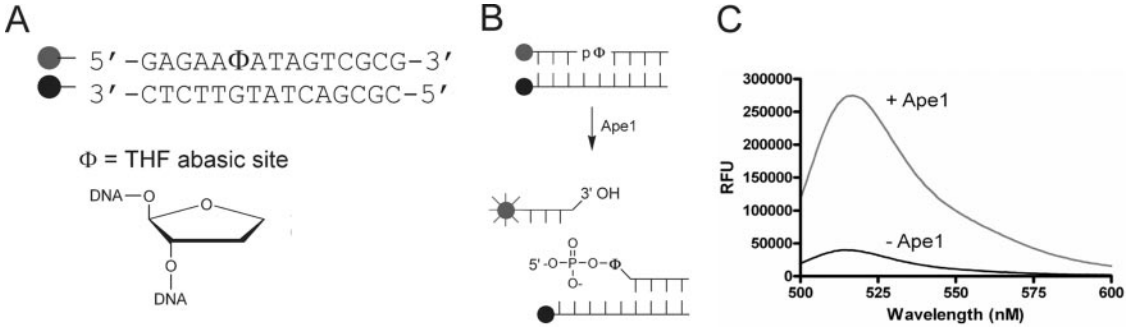


Fig. 1. Molecular beacon assay for Ape1. A, an oligonucleotide containing a THF abasic site mimic was used in the Ape1 molecular beacon substrate. The top strand was labeled on the 5′ end with a FAM fluorophore, the bottom on the 3′end with a dabsyl (DAB) quench. B, upon cleavage by Ape1, the fluorophore dissociates from the dabsyl quench, causing an increase in fluorescence. C, an approximate 6-fold increase in fluorescence is observed upon DNA cleavage by Ape1.

TABLE 1
Inhibitory activity for diversity set hits
Reactions were carried out in the presence of 50 nM substrate and 10 pM Ape1. Numbers represent averaged duplicate measurements

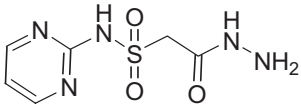
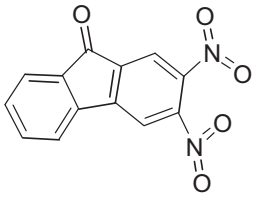
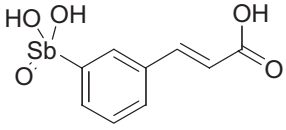
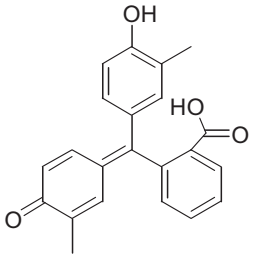
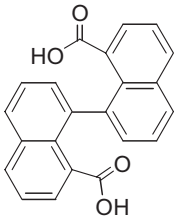
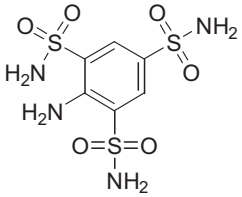
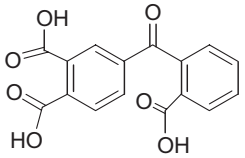
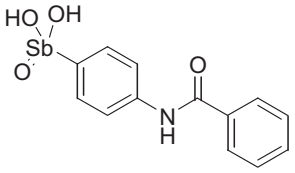
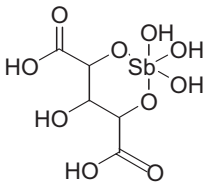
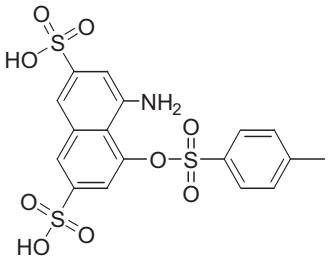
Compound	Chemical Structure	Inhibition			
		10 μM	5 μM	0.5 μM	0.1 μM
		%			
329379		69	55	4	0
133896		50	35	12	15
13778		100	98	87	61
10470		91	84	17	10
P7810		83	69	30	10
25857		100	90	26	8
28620		74	61	20	14

TABLE 1 (Continued)

Compound	Chemical Structure	Inhibition			
		10 μM	5 μM	0.5 μM	0.1 μM
		%			
15596		73	61	19	19
15603		71	63	27	7
16168		63	42	0	0

From this analysis, a partial mixed-type inhibition mode for each compound was ascertained. Other possible mechanisms (competitive, uncompetitive, noncompetitive, partial-noncompetitive, mixed, double-partial-noncompetitive, and double-partial-mixed) were excluded by statistical criteria. Thus, these compounds bind to both the free enzyme and the enzyme-substrate complex, with the ESI complex exhibiting a much lower turnover number (Scheme 1). The kinetic parameters, as determined by computer simulation, are given in Table 3.

Arylstibonates Showed Specificity for Ape1 and Did Not Intercalate DNA. As a test for whether these compounds were general protein poisons, we checked whether **13778** inhibited uracil DNA glycosylase (UNG), the enzyme that acts just before Ape1 in the base excision repair path-

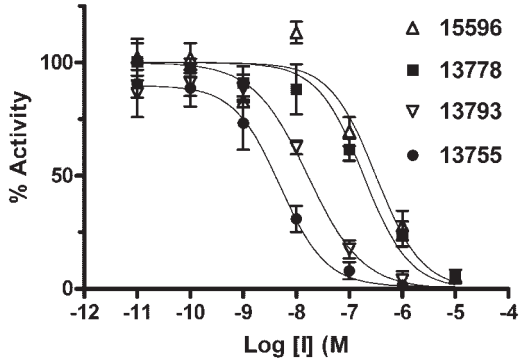
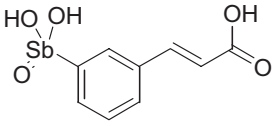
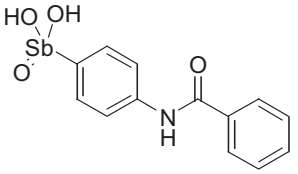
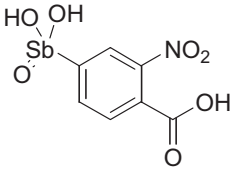
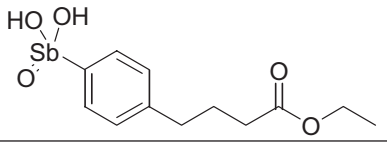


Fig. 2. IC_{50} analyses for the most potent aryl-stibonic acid inhibitors. The five best antimony-containing inhibitors have IC_{50} values in the low to mid-nanomolar range (Table 2).

TABLE 2

Structures of stibonic acid sublibrary hits and IC_{50} values

IC_{50} determinations were carried out in the presence of 50 nM substrate and 50 mM NaCl. The values in parentheses are the K_i values corresponding to the partial mixed-type mechanism using 75 mM NaCl (see Scheme 1).

Compound	Chemical Structure	IC_{50}
		nM
13778		200 ± 100
15596		300 ± 100
13755		4 ± 1 (19 ± 6)
13793		17 ± 2 (36 ± 10)

way. Compared with Ape1, a 333-fold higher concentration of **13778** was needed to reduce UNG activity by 50% [IC_{50} (UNG) ~ 100 μM; Supplemental Fig. 3]. Because **13778** inhibited Ape1 with an IC_{50} of 300 nM, we concluded that **13778,** and its related inhibitors, showed considerable specificity for Ape1 and were not general protein poisons. In addition, an ethidium bromide competition assay was used to examine the possibility that these compounds inhibited Ape1 activity by binding to DNA rather than the enzyme. In this assay, ethidium bromide is prebound to the DNA substrate and increasing concentrations of inhibitor are added. If the inhibitor were able to bind DNA by displacing ethidium bromide, a decrease in ethidium bromide (EtBr) fluorescence would be observed (Boger et al., 2001). As shown in Fig. 4, the five strongest Ape1 inhibitors did not displace bound EtBr at concentrations up to 100 μM. In contrast, the known intercalator distamycin A produced a 50% decrease in ethidium bromide fluorescence in the same concentration range.

Cell Culture Studies. Inhibition of Ape1 in cell culture through the use of small molecules, RNA interference, or the expression of a dominant-negative form of the protein has been shown to increase sensitivity of human cells to the DNA alkylating agent methyl methanesulfonate (MMS) (Luo and Kelley, 2004; Wang et al., 2004; Madhusudan et al., 2005; Fishel and Kelley, 2007; McNeill and Wilson, 2007). In this type of experiment, cells are treated with inhibitor, MMS, or both, and cell survival is determined by counting or clonogenic survival. Using this approach, we examined the activity of the arylstibonate inhibitors in human cell culture. Despite their potent in vitro inhibitory activity, we were unable to detect any decreased survival when cells were treated with 5 μM **25857**, **10470**, **13755**, or **13793** in the presence of 0, 25, or 50 μM MMS (Supplemental Fig. 4). As a positive control, small interfering RNA knockdown of Ape1 expression by 80% produced a 2-fold decrease in cell survival in the presence of 100 μM MMS (Supplemental Fig. 5). It should be noted that none of the tested compounds displayed any cytotoxicity up to concentrations of 5 μM with the exception of **10470**, which was highly toxic as a single agent to HOS, MCF-7 and PC-3 cells at this concentration (Supplemental Fig. 4).

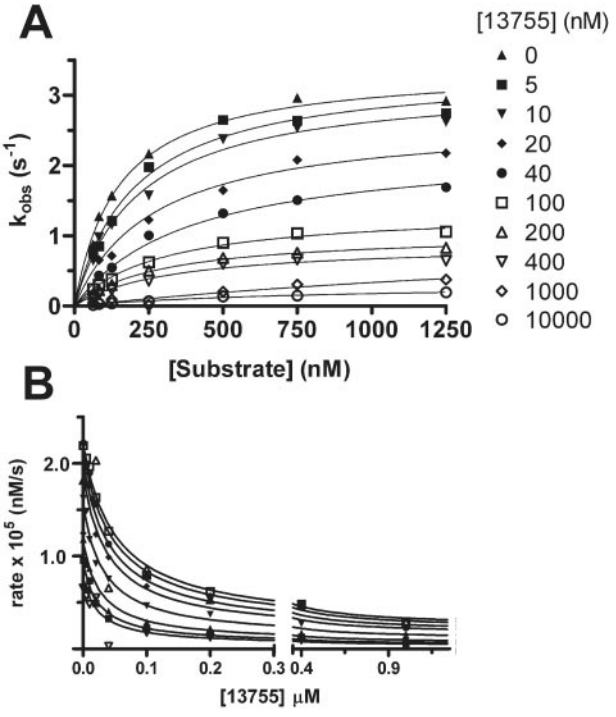


Fig. 3. Representative substrate saturation profiles of Ape1 in the presence of increasing concentrations of **13755** and simulations of the inhibition data. A, Michaelis-Menten curves in the presence of increasing concentrations of **13755**. B, Dynafit simulations for **13755** using a partial mixed-type model for the inhibition.

TABLE 3
Inhibition constants for Ape1 inhibitors
Kinetic parameters correspond to the reaction mechanism shown in Scheme 1. Values were obtained from global fitting simulations using the program Dynafit.

Parameter	13755	13793
K_s (μM)	0.13 ± 0.02	0.17 ± 0.02
k_{cat} (s^{-1})	7.8 ± 0.3	9.6 ± 0.3
K_i (μM)	0.019 ± 0.007	0.04 ± 0.01
K_{is} (μM)	0.06 ± 0.01	0.10 ± 0.03
K_{si} (μM)	0.37 ± 0.15	0.48 ± 0.19
k'_{cat} (s^{-1})	0.9 ± 0.3	2.2 ± 0.45
Inhibition mode	PM	PM

PM, partial mixed-type inhibition (see Scheme 1).

Discussion

Small-Molecule Inhibitors of Ape1. Several classes of inhibitors of Ape1 have been described previously. One unusually simple compound used to inhibit AP endonuclease activity is methoxyamine (MX), which attacks the ring-opened form of abasic sites to form an oxime linkage. Thus, rather than targeting the enzyme directly, MX blocks Ape1 from accessing the lesion site (Liuzzi and Talpaert-Borlé, 1985). Because of this unusual mechanism, MX is not specific for Ape1, and other off-target effects would be expected (Fishel and Kelley, 2007). A second compound, lucanthone, inhibits Ape1 repair in vitro but is also a very potent topoisomerase II poison, which probably accounts for its toxicity with cancer cell lines (Bases and Mendez, 1997; Luo and Kelley, 2004). The small-molecule 7-nitroindole-2-carboxylic acid has recently been shown to inhibit Ape1 in vitro with an IC_{50} of 3 μM and was also reported to sensitize cells to MMS and several other DNA-damaging agents (Madhusudan et al., 2005). However, subsequent studies have not been able to reproduce the sensitizing effects of 7-nitroindole-2-carboxylic acid (Fishel and Kelley, 2007). Similar controversy surrounds the natural product resveratrol, which was originally re-

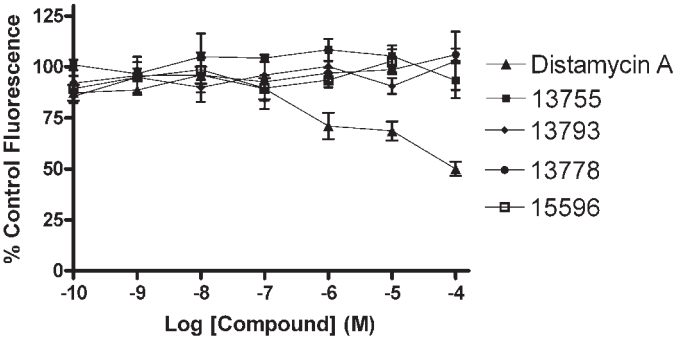


Fig. 4. Ethidium bromide inhibitor-DNA intercalation assay. An ethidium bromide competition assay was used to evaluate the ability of the arylstibonate Ape1 inhibitors to bind DNA. No decrease in ethidium bromide fluorescence was observed upon inhibitor addition, indicating that these compounds do not bind DNA.

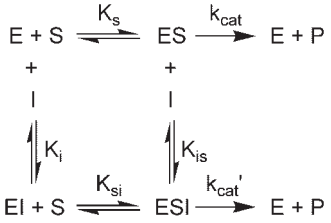
ported to inhibit the redox activity of Ape1 (Yang et al., 2005b); once again, this effect was not reproducible by another group (Fishel and Kelley, 2007). The present compounds inhibit Ape1 at low nanomolar concentrations in vitro (Table 2), making them the most potent Ape1 inhibitors yet reported and promising candidates for further development.

Despite robust activity in vitro, these compounds were not inhibitory in cell culture, as judged by the observation that HOS cells showed no increased sensitivity to the DNA-damaging agent MMS in the presence of compound. Little is known about the cell permeability or intracellular localization of aryl-stibonic acids, but they do appear to be stable in culture medium (Yang et al., 2005a). In contrast, much is known about permeability of the pentavalent antimonial sodium stibogluconate (Pentostam), a first line treatment for *Leishmaniasis*. Pentostam is actively transported into macrophage cells and then reduced by a parasite-specific, thiol-dependent reductase to its active trivalent form upon entering the parasite (Wyllie and Fairlamb, 2006; Mishra et al., 2007). Whether the present compounds require an active entry mechanism is unknown, but reduction to their Sb^{+3} form is unlikely because human macrophage and monocyte cells are unable to reduce Pentostam and thus remain resistant to its toxic effects (Wyllie and Fairlamb, 2006). This is fortunate because the reduced forms of the arylstibonates are not inhibitory (see below). It is reasonable to expect that rather straightforward modifications of these compounds, such as esterification of the carboxylate group, might increase permeability if this is indeed an issue affecting activity in cell culture. Such prodrug forms, which should be substrates for cellular esterases or spontaneous ester hydrolysis, offer attractive avenues for further development.

The three most potent aryl-stibonic acid inhibitors of Ape1 share a common partial mixed-type inhibition pattern (Scheme 1). Thus, these negatively charged compounds can bind to both the free enzyme and the ES complex. The dissociation constants of both the substrate and inhibitor EI and ES complex are approximately 2- to 3-fold greater than their respective constants for binding to the free enzyme, indicating energetic cross-talk between the binding sites (Table 3, Scheme 1). The 10 to 20% residual enzymatic activity in the presence of saturating concentrations of substrate and inhibitor indicates that the ESI complex is in someway perturbed from its optimal state. Plausible physical interpretations for this inhibition pattern are suggested below.

Structure-Activity Relationships for Arylstibonates. The 37-compound arylstibonate library provides some useful structure-activity relationships (Supplemental Table 1). All of the inhibitory compounds have an anionic pentavalent stibonic acid moiety attached to a benzene ring, whereas those containing the reduced (neutral) trivalent antimony show no inhibitory activity. The five most potent inhibitors in Table 2 all have carboxylate or nitro substituents on the benzene ring placed *meta* or *para* to the stibonate group, indicating that negatively charged or polar substituents at these positions are important. Furthermore, several molecules in the library are structurally similar to our most potent inhibitor, **13755** (K_i = 19 nM), yet have no inhibitory activity. Compound **13755** is substituted at the *para* and *meta* positions with a carboxylate and a nitro group, respectively, but compounds **13744**, **13743**, and **13760**, which possess one but not both of these groups, have no inhibitory activity. Thus, the stibonate, nitro and carboxylate functional groups must all be present and in the appropriate positions for maximal inhibition. In addition, **P6966** is structurally similar to **13755**, with a second carboxylate substituting for the nitro group, but it is also inactive. The second most potent inhibitor, **13793** (K_i = 36 nM), is distinct from **13755** because of its esterified butanoic acid moiety attached *para* to the antimony group. The length of this chain seems essential because compounds with shorter carboxylate containing chains attached in the same position are not inhibitory (**P6982**, **P6965**, and **P6971**; Supplemental Table 1). Structure-activity relationships are also apparent for compound **13778**, which possesses a key ethylene linker connecting a carboxylate group to the benzene ring. If this ethylene linker is changed to ethyl (**P6949**) or the length of the linker is reduced by one or two carbon units (**P6970**, **P6953**, or **13759**), inhibition is completely lost.

Did These Arylstibonate Compounds Mimic the Phosphate Esters in DNA? Antimony is a group V-A element of the periodic table with properties similar to phosphorus and arsenic. Compared with phosphorus, antimony has a larger ionic radius and lower charge density, which allows Sb(V) to form an octahedrally coordinated stibonic acid species $H[Sb(OH)_6]$, which dissociates to the stibonate anion $[Sb(OH)_6]^-$ in weak acid or neutral aqueous solution (Filellaa et al., 2002). This property is in contrast with P(V), which is tetrahedrally coordinated with oxygen. The aqueous equilibria of these arylstibonates are not known, but it is possible that they exist in equilibrium between a tetrahedral form (Table 2), or a pentavalent hydrated form in which four hydroxyl groups and one



Scheme 1. Partial mixed-type inhibition mechanism for arylstibonic acids.

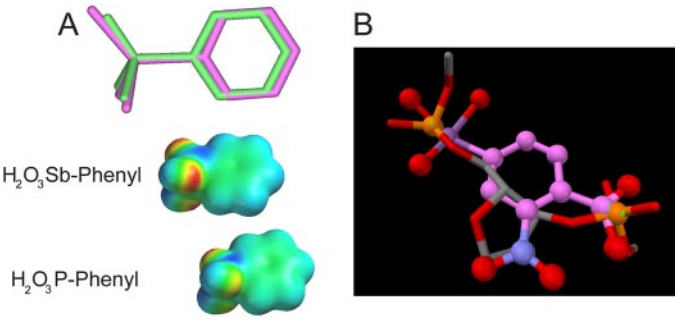


Fig. 5. Structural and electrostatic comparisons of phosphorus and antimony acids. A, models of phenylstibonic acid (pink) and phenylphosphonic acid (green) calculated using the PM3 semiempirical method (Spartan '04; Wavefunction, Inc., Irvine, CA). The Sb-O, Sb-C, and Sb = O bondlengths are all much longer than the corresponding distances in the phosphorus compound (Sb-O = 1.99 Å, Sb-C = 2.10 Å, Sb = O = 1.95 Å; P-O = 1.69 Å, P-C = 1.77 Å, P = O = 1.46 Å.) The electrostatic potential is plotted on the Van der Waals surface of each compound. B, superposition of the antimony and carboxylate atoms of **13755** with the phosphodiester groups of the abasic site extracted from the complex of Ape1 with abasic DNA (Protein Data Bank code 1DEW).

carbon atom are coordinated to the antimony center. In either case, the lower electronegativity of Sb(V) compared with P(V) would be expected to increase the Sb-O bond length compared with P-O, and thus, place a larger negative charge density on the oxygen atoms than the corresponding phosphonate analog. This expectation is confirmed by the electrostatic potential surfaces shown in Fig. 5A, which were calculated using semiempirical (PM3) methods for the model compounds phenyl phosphonic acid and phenyl stibonic acid. Greater charge density on the antimonial oxygens could facilitate stronger coulombic interactions with enzyme groups. Given these properties of arylstibonates, and their similarity with phosphate, we speculate that these anionic molecules may mimic the phosphate backbone groups of DNA. Based on the structure-activity relationships described above, an important requirement of the observed inhibition may be positioning of essential carboxylate and stibonate groups in anion binding pockets originally intended for adjacent 3′ and 5′ DNA phosphates of a DNA strand. The crystal structure of the Ape1-abasic DNA complex shows several such binding interactions with both DNA strands. To support our proposal, we extracted the abasic site from this structure and superimposed the stibonate and carboxylate groups of **13755** onto the phosphates (Fig. 5B). The alignment of the antimony and phosphate centers is excellent, with deviations of only tenths of an angstrom. A mechanism of inhibition involving binding to adjacent phosphate sites on the enzyme would be consistent with the observed partial mixed-type mechanism, because the DNA could still bind and react even if the inhibitor were blocking one or more phosphate binding sites. Consistent with the proposal that these compounds mimic DNA, **13778** has also been shown to inhibit poxvirus type I topoisomerase but not the closely related human type I topoisomerase (Bond et al., 2006). Although much remains to be understood about the selectivity of these compounds, it seems that selectivity for a given target may be provided by the positions of phosphate binding sites on the target and how well the site accommodates the aromatic functional group of the compound. These data indicate that arylstibonates may be especially useful for targeting enzymes that interact with nucleic acids.

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