SUPPLEMENTARY INFORMATION

Efficient inhibition of human AP endonuclease 1 (APE1) via substrate masking by abasic site-binding macrocyclic ligands

Naoko Kotera, Florent Poyer, Anton Granzhan, and Marie-Paule Teulade-Fichou

“CNRS UMR9187 / INSERM U1196 “Chemistry, Modelling and Imaging for Biology”, Centre de Recherche, Institut Curie, 91405 Orsay, France
E-mail: anton.granzhan@curie.fr
Experimental Section

Materials: Recombinant, polyhistidine-tagged APE1 enzyme was purchased from Life Technologies, reconstituted in 10 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 0.05 mM Na2EDTA, 200 µg mL⁻¹ BSA, 50% glycerol, pH 8.0 at a concentration of 10 nM, aliquoted and stored at −20 °C. DNA oligonucleotides (HPLC purity grade) were purchased from Eurogentec, reconstituted in deionized water at a concentration of 100 µM and hybridized at a concentration of 10 µM in the following buffer: 50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl₂, pH 8.0. Calf thymus DNA solution used in competition experiments (10 mg mL⁻¹, Invitrogen UltraPure™) was diluted in the same buffer to a nucleotide concentration of ≈ 5 mM; the actual concentration was determined using the extinction coefficient of 6600 cm⁻¹ M⁻¹. 7-Nitroindole-2-carboxylic acid (CRT0044876) and aurintricarboxylic acid (ATCA) were purchased from Acros and Alfa Aesar, respectively. The macrocycles 2,7-BisNP-O × 4 HCl and 2,7-BisNP-NH × 6 HCl were synthesized as described elsewhere.¹ Stock solutions of the macrocycles were prepared in deionized water and stored at +4 °C in darkness.

Thermal denaturation experiments: The experiments were performed with 17-mer oligonucleotides (5′-CCAGTTCGTAGTAACCC-3′/5′-GGGTTACTNCGAACTGG-3′; N = dSpacer, 17-TΦ: N = A), in 50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl₂, pH 8.0 buffer solution. Thermal denaturation profiles were recorded with a Cary 300 Bio spectrophotometer (Agilent) equipped with a thermoelectric temperature controller. The samples, containing oligonucleotides 17-TΦ or 17-TA (duplex concentration of 6 µM) in the absence or in the presence of ligands (12 µM) were heated from 20 to 80 °C at a rate of 0.2 deg min⁻¹ while the absorbance was monitored at 260 nm. The temperatures of DNA melting transitions were determined from the first-derivative plots of absorbance versus temperature.

Solution-based assay for APE1 kinetics and inhibition: A fluorescence-based assay for the APE1 activity was set up by an adaptation of published procedures.²³ The DNA substrate was formed by annealing of a fluorophore-labelled 17-mer oligonucleotide, 5′-(6-FAM)-CCAGTTCGTAGTAACC-3′/5′-GGGTTACTNCGAACTGG-3′ (Φ = dSpacer). For kinetic studies, the reaction samples contained APE1 (0.1 nM), varied concentrations of DNA substrate, and inhibitor in a reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl₂ and 2 mM DTT, pH 8.0), total volume: 1.00 mL. Samples were thermostated at 37 °C, and the fluorescence intensity F was monitored in real time during 90 s with a Cary Eclipse spectrofluorimeter (Agilent), using excitation and emission wavelengths of 488 and 515 nm, respectively, and integration time of 2 s. The data from the initial linear region of the kinetic curve were used to calculate the initial reaction rate, \( v_0 = \frac{dF}{dt} \). The kinetic parameters of the enzymatic reaction (\( K_M, V_{max}, \) and \( k_{cat} = \frac{V_{max}}{c_{APE1}} \)) were determined from the non-linear fitting of \( v_0 \) as a function of substrate concentration (\( c_{DNA} = [S]_0 = 10–100 \text{ nM} \)) with the Michaelis–Menten equation (Equation S17 below), in the absence or in the presence of inhibitor (0–1 µM). For the determination of IC₅₀ values, the concentration of DNA substrate was fixed at 25 nM, and the concentration of
inhibitor \((c_I)\) was varied from 0.1 nM to 0.1 mM. The enzymatic activity was calculated as a ratio of \(v_0\) in the presence and in the absence of inhibitor \((\% \text{ activity} = \frac{v_{0\text{inh}}}{v_0})\) and plotted as a function of the logarithm of concentration of inhibitor. The IC\(_{50}\) values were obtained by fitting the data with a dose-response function (Equation S1).

\[
\% \text{ activity} = \frac{100}{1 + 10^{(\log c_I - \log IC_{50})p}}
\]  
(S1)

**Gel-based assays for APE1 inhibition:** A gel-based assay for the DNA-cleavage activity of APE1 with a fluororescently labelled DNA substrate was performed following an adaptation of a published protocol.\(^3\) The DNA substrate bearing a THF abasic site (FAM-30-T\(\Phi\)) was formed by annealing of the abasic-site-containing, fluorophore-labelled 30-mer DNA oligonucleotide 5\(^\prime\)-\((6\text{-FAM})\text{-CGATCA-TCACT}\Phi\text{TTGAGACTGACACTGACC-3}^\prime\) with its complementary sequence, 5\(^\prime\)-\(\text{GGTCAGTGT-CAGTCTCAATAGTGATGATCG-3}^\prime\). The reaction vials containing DNA substrate (final concentration: 0.1 \(\mu\)M), APE1 (0.2 nM), and varied concentration of the inhibitor (10 nM to 50 \(\mu\)M) in a reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl\(_2\) and 2 mM DTT, pH 8.0), total volume: 20 \(\mu\)L, were incubated at 37 °C for 15 min, and the reactions were quenched by addition of formamide (10 \(\mu\)L). Sample aliquots (7.5 \(\mu\)L) were mixed with a gel loading buffer (6X, 1.5 \(\mu\)L), loaded onto a denaturing (7 M urea) 20% polyacrylamide gel (16 × 14 cm), and separated at ambient temperature during 2 h using a constant voltage setting of 320 V (running buffer: TBE). The bands corresponding to the uncleaved duplex substrate and the labelled single-stranded 11-mer cleavage product were visualized with a Typhoon Trio imager (GE Healthcare) using excitation and emission wavelengths of 488 and 526 nm, respectively, and quantified with ImageQuant TL software (GE Healthcare). The assays included a positive control (substrate and enzyme in the absence of the inhibitor) as well as negative control (substrate in the absence of APE1), corresponding to 100% and 0% activity, respectively. The enzymatic activity (% activity) was calculated as a ratio of the amount of the 11-mer cleavage product obtained in the presence of inhibitor over its amount obtained in the positive control, and the data from three independent experiments were plotted as a function of the concentration of inhibitor.

The DNA substrate bearing a natural abasic site (FAM-30-TX) was obtained from the uracil-containing precursor [FAM-30-TU, 5\(^\prime\)-(6-FAM)-CGATCATCCTUTTGAGACTGACACTGACC-3\(^\prime\) / 5\(^\prime\)-GGTCAGTGT-CAGTCTCAATAGTGATGATCG-3\(^\prime\)]. FAM-30-TU (100 pmol) was incubated with UDG (New England BioLabs, 5000 U mL\(^{-1}\), 1 \(\mu\)L) in 1X UDG Reaction Buffer (20 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, pH 8, total volume: 400 \(\mu\)L) for 20 min at 37 °C. The reaction mixture was extracted with phenol–chloroform–isoamyl alcohol (25:24:1 v/v, 400 \(\mu\)L) followed by chloroform (2 × 400 \(\mu\)L). DNA was precipitated by addition of absolute EtOH (1000 \(\mu\)L), kept at −20 °C overnight, collected by centrifugation (12,000 g, 15 min, 4 °C), vacuum-dried and dissolved in buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl\(_2\), 2 mM DTT, pH 8.0, 50 \(\mu\)L). The DNA was quantified via single-wavelength fluorescence measurements (excitation: 495 nm, emission: 520 nm, slits: 5/5 nm) using a calibration curve obtained with FAM-30-TU. Gel assay was performed
as described above; the 12-mer band corresponding to the product of β-elimination was assigned using a control experiment involving NaOH treatment of FAM-30-TX (cf. Fig. S9).

**Differential scanning fluorimetry:** The thermal denaturation profiles of APE1 were recorded with an Agilent Cary Eclipse spectrofluorimeter equipped with a thermoelectric temperature controller. The samples were prepared by dissolving APE1 to a final concentration of 0.34 µM in a buffer (50 mM HEPES, 50 mM NaCl, 1 mM MgCl₂, 2 mM DTT, pH 8.0), adding SYPRO Orange dye (Life Technologies, final concentration: 5X) in the absence or in the presence of inhibitors (20 µM). The samples were gently stirred with a pipette and were cooled from ambient temperature to 10 °C, kept at 10 °C for 10 min, and finally heated to 70 °C at a rate of 0.5 deg min⁻¹. During the temperature ramp, the fluorescence intensity was monitored using the following experimental parameters: excitation wavelength, 492 nm; emission wavelength, 610 nm; slit width, 5 nm; PMT voltage, 700 V. The temperatures of protein denaturation were determined from the first-derivative plots of absorbance versus temperature. In the absence of inhibitors, the protein denatured at $T_{m}^{\text{APE1}} = 48.9$ °C.

**Cell line and cell culture conditions:** U-87 MG (ATCC HTB-14) human glioblastoma cell line was purchased from American Type Culture Collection. Adherent U-87 MG cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) Glutamax™ (Life Technologies) with 1 mM sodium pyruvate supplemented with 10% fetal calf serum (FCS, Eurobio), 100 units mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Life Technologies) in humidified atmosphere under 5% CO₂ in air at 37 °C.

**Cytosolic, nuclear and whole cell extracts:** U-87 MG cells were harvested by trypsin–EDTA treatment, washed with PBS, half of which were using for the whole cells extract and the other half for the cytosolic and nuclear extracts. For the whole cell extract, cells were re-suspended in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, one complete protease inhibitor cocktail tablet). The suspension was frozen at −80 °C for at least 30 min and then thawed at 4 °C. KCl was added to cell suspension to a final concentration of 222 mM and incubated on ice for 30 min. The cell suspension was clarified by centrifugation at 12,000 g for 15 min at 4 °C, the supernatant (whole cell extract) was retained, the protein concentration determined using the Thermo Scientific Pierce BCA Protein Assay Kit Reducing Agent Compatible and aliquots stored at −80°C. For the cytosolic and nuclear cell extracts, cells were resuspended in the cytosolic extraction buffer (20 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1mM DTT, 20% (v/v) glycerol, one complete protease inhibitor cocktail tablet) and incubated on ice for 10 min. The cell suspension was clarified by centrifugation at 800 g for 5 min at 4 °C, the supernatant (cytosolic extract) was retained and aliquots stored at −80 °C. The pellet was re-suspended with the nuclear extraction buffer (20 mM HEPES, pH 7.6, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 20% (v/v) glycerol, one complete protease inhibitor cocktail tablet) and incubated on ice for 30 min, while vortexing in 10 min intervals for 10 seconds at the highest setting. The cell suspension was clarified by centrifugation at 14,000 g for 15 min at 4 °C, the supernatant (nuclear extract) was retained and aliquots stored at −80 °C. The protein concentration of each extract was determined using the Thermo Scientific Pierce BCA Protein Assay Kit Reducing Agent Compatible.
Western blot analysis: Proteins (30 µg) from the different cellular extracts (cytosolic, nuclear and whole cell extracts) of U-87 MG cells and 100 ng of the recombinant APE1 (Life Technologies) were separated using a Mini Protean TGX Precast Gel 4–15% 12-wells (Bio-Rad) using a Tris/Glycine/SDS Buffer. The samples including the molecular weight marker (Precision Plus Protein™ Dual Color Standard, Bio-Rad) were loaded in duplicate into the gel. One part was used for the Coomassie Blue staining and the other part was used for the transfer. After the migration, proteins were transferred onto a 0.2 µm PVDF membrane using Trans-Blot Turbo mini PVDF transfer pack (Bio-Rad) and the Trans-Blot Turbo rapid western blotting transfer system (Bio-Rad). The membrane was incubated with blocking buffer (Tris-buffered saline 0.2% (v/v) Tween 20 containing 5% (w/v) of powdered non-fat milk) for 1 h at room temperature. Then, the membrane was incubated with primary antibody (mouse anti human APE1, Novus Biologicals, 1/500 dilution) at 4 °C overnight. After washing in Tris-buffered saline 0.2% (v/v) Tween 20, membrane was incubated with secondary antibody conjugated with horseradish peroxidase (goat anti-mouse, 1/10 000 dilution, Pierce – Thermo Fischer) for 1 h at room temperature. The immunoblot was revealed using Amersham ECL Western Blotting Detection Reagent (GE Healthcare). The immunoblot and the staining Coomassie Blue SDS PAGE were analyzed using G:BOX Chemi XL (Syngene).

Enzymatic kinetics with substrate masking (indirect inhibition)

In our model (Scheme S1), we use the following hypotheses:

(1) the inhibitor (or ligand) I reversibly binds the substrate S with a dissociation constant $K_d$;
(2) the inhibitor does not bind to the enzyme E (an indirect proof of that is provided by the DSF experiment);
(3) the substrate–inhibitor complex SI is not recognized by the enzyme (“masking”);
(4) as a consequence, we do not consider formation of a possible ternary complex (ESI);
(5) non-specific binding of the inhibitor to DNA substrate (i.e. outside the abasic site) is much weaker and does not interfere with the enzymatic activity (an indirect proof is provided by the experiments with competitor DNA, cf. Fig. S5).

\[
\begin{align*}
E + S & \xrightleftharpoons[k_{-1}]{k_1} ES & \xrightarrow{k_{\text{cat}}} P \\
I & \xrightarrow{K_d} SI
\end{align*}
\]

Scheme S1. Model of indirect inhibition by substrate masking.
The reaction rate is described by Equation S2:

\[ v = \frac{d[P]}{dt} = k_{\text{cat}}[ES] \]  

(S2)

From the steady-state approximation:

\[ \frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_{\text{cat}})[ES] = 0; \]  

(S3)

\[ [ES] = [E][S] \frac{k_1}{k_{-1} + k_{\text{cat}}} = \frac{[E][S]}{K_M}, \]  

(S4)

where \( K_M \) is the Michaelis constant:

\[ K_M = \frac{k_{-1} + k_{\text{cat}}}{k_1}. \]  

(S5)

Mass balance on E: 

\[ [E]_t = [E] + [ES]; \]  

(S6)

\[ [E] = [E]_t - [ES]; \]  

(S7)

\[ [ES]K_M = [S]([E]_t - [ES]); \]  

(S8)

\[ [ES] = \frac{[E][S]}{K_M + [S]^2} \]  

(S9)

According to our hypothesis, the inhibitor I has no influence on the formation of the catalytic complex ES, and changes only the equilibrium concentration of the substrate [S]:

\[ K_d = \frac{[S][I]}{[SI]}. \]  

(S10)

Mass balance on S: 

\[ [S]_0 = [S] + [ES] + [SI] \]  

(S11)

We assume that \([ES] \ll [S] + [SI]\) if total concentration of the enzyme is much lower than that of the substrate (in this work, \([E]_t = 0.1 \text{ nM}\) and \([S]_0 = 10 \text{ to } 100 \text{ nM}\)). Then,

\[ [S]_0 \approx [S] + [SI] \]  

(S12)

\[ [S]_0 = [S] + \frac{[S][I]}{K_d} = [S] \left( 1 + \frac{[I]}{K_d} \right); \]  

(S13)

\[ [S] = \frac{[S]_0}{1 + \frac{[I]}{K_d}} \]  

(S14)

Substituting \([S]\) into Equation S9,
\[
[ES] = \frac{[E][S]_0}{1 + \frac{[I]}{K_d}} \left( \frac{K_M + \frac{[S]_0}{1}}{1} \right) = \frac{[E][S]_0}{K_M \left( 1 + \frac{[I]}{K_d} \right) + [S]_0} = \frac{[E][S]_0}{K_{M^{pp}} + [S]_0}.
\] (S15)

where \( K_{M^{pp}} = K_M \left( 1 + \frac{[I]}{K_d} \right) \). (S16)

Thus, the initial rate of the enzymatic reaction \((t \approx 0)\) is governed by the Equation S17:

\[
\nu_0 = \frac{k_{cat}[E][S]_0}{K_{M^{pp}} + [S]_0} = \frac{V_{max} [S]_0}{K_{M^{pp}} + [S]_0}.
\] (S17)

**Table S1**  Stabilization of the abasic site-containing oligonucleotide (17-TΦ) and the fully matched oligonucleotide (17-TA) induced by 2,7-BisNP-O and 2,7-BisNP-NH, from thermal denaturation studies.\(^a\)

<table>
<thead>
<tr>
<th>Duplex</th>
<th>( T_m^0 ) (°C)</th>
<th>Ligand-induced ( \Delta T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,7-BisNP-O</td>
<td>2,7-BisNP-NH</td>
<td></td>
</tr>
<tr>
<td>17-TΦ</td>
<td>50.3 ± 0.1</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>17-TA</td>
<td>62.9 ± 0.3</td>
<td>−1.5 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) Experimental conditions: \( c(DNA) = 6 \mu M \), \( c(ligand) = 12 \mu M \) in 50 mM Tris-HCl, 50 mM NaCl, 1mM MgCl\(_2\) buffer, pH 8. \(^b\) Melting temperature in the absence of the ligand.

**Table S2**  IC\(_{50}\) values (µM) for selected APE1 inhibitors\(^a\)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>purified APE1(^b)</th>
<th>U87 cellular extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nuclear(^c)</td>
</tr>
<tr>
<td>2,7-BisNP-O</td>
<td>0.055</td>
<td>1.3</td>
</tr>
<tr>
<td>2,7-BisNP-NH</td>
<td>0.057</td>
<td>1.8</td>
</tr>
<tr>
<td>CRT0044876</td>
<td>13.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>ATCA</td>
<td>0.011</td>
<td>0.36</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>1.4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(^a\) From the kinetic APE1 assay with THF substrate, \( c(DNA) = 25 \text{ nM} \). \(^b\) \( c(APE1) = 0.1 \text{ nM} \). \(^c\) Total protein concentration \( c(prot) = 15.5 \mu g \text{ mL}^{-1} \). \(^d\) \( c(prot) = 11.3 \mu g \text{ mL}^{-1} \).
Fig. S1 UV-Monitored thermal denaturation profiles of oligonucleotides a) 17-TΦ and b) 17-TA (6 µM) in the absence and in the presence of macrocycles 2,7-BisNP-O and 2,7-BisNP-NH (12 µM).

Fig. S2 Inhibitory profile of 2,7-BisNP-O from the gel-based assay. “% Enzyme activity” is calculated as a ratio of the amount of the 11-mer cleavage product obtained in the presence of inhibitor (2,7-BisNP-O) over its amount obtained in the positive control, and fitted according to Equation S1. Each point was measured in triplicate.
**Fig. S3** Fluorescence-monitored thermal denaturation profiles (DSF) of APE1 ($c = 0.34 \mu\text{M}$) in the presence of inhibitors ATCA, **2,7-BisNP-O**, or **2,7-BisNP-NH** (20 µM) and indicator dye SYPRO Orange (5X). Excitation wavelength: 492 nm, emission wavelength: 610 nm.

**Fig. S4** Inhibition of APE1 activity by **2,7-BisNP-O**, **2,7-BisNP-NH**, CRT0044876, ATCA and ethidium bromide in the solution-based kinetic assay with the purified enzyme. The enzymatic activity is calculated as a ratio of initial reaction rates ($v_0$) in the presence and in the absence of an inhibitor (% enzyme activity = $v_0^{\text{inh}} / v_0$). Each point was measured in triplicate.
Fig. S5 Enzymatic activity of APE1 in the presence of inhibitors (ethidium, 2,7-BisNP-O, and 2,7-BisNP-NH, \(c = 1 \, \mu M\)), in the absence (filled bars) and in the presence (empty bars) of calf thymus DNA competitor (260 \(\mu M\) bases); \(c(\text{substrate}) = 25 \, \text{nM}\); \(c(\text{APE1}) = 0.1 \, \text{nM}\). “% Enzyme activity” is defined as above. Data from two series of measurements.

Fig. S6 Detection of APE1 (35 kDa for the native form, *) in different cellular extracts from U-87 MG glioblastoma cell line. a) Staining SDS PAGE with Coomassie Blue and b) Western blot. Lane 1: molecular weight marker; 2: whole cell extract; 3: nuclear extract; 4: cytosolic extract; 5: purified recombinant APE1 protein (37 kDa for the recombinant form, *). ** corresponds to BSA (66.5 kDa) used in preparation of purified APE1.
Fig. S7 Inhibition of abasic site-specific endonuclease activity of nuclear extracts of U87 cells by a) 2,7-BisNP-O, b) 2,7-BisNP-NH and c) ATCA (data from the solution-based kinetic assay). The enzymatic activity is calculated as a ratio of initial reaction rates ($v_0$) in the presence and in the absence of inhibitor (% activity = $v_{0\text{inh}} / v_0$). Each point was measured in duplicate.
**Fig. S8** Inhibition of abasic site-specific endonuclease activity of cytosolic extracts of U87 cells by a) **2,7-BisNP-O**, b) **2,7-BisNP-NH** and c) **ATCA** (data from the solution-based kinetic assay). The enzymatic activity is calculated as a ratio of initial reaction rates ($v_0$) in the presence and in the absence of inhibitor ($\% \text{ activity} = \frac{v_{0}^{\text{inh}}}{v_{0}}$). Each point was measured in duplicate.
**Fig. S9** DNA cleavage activity of 2,7-BisNP-O assessed by gel electrophoresis with a fluorescently labelled 30-mer substrate bearing a natural abasic site (FAM-30-TX). Lanes 1 and 13: negative control (FAM-30-TX, $c = 0.1 \mu M$), lanes 2 and 12: FAM-30-TX incubated with 0.2 nM APE1, lane 3: FAM-30-TX incubated with NaOH (5 M, 1 µL), lanes 4–11: FAM-30-TX incubated with indicated concentrations of 2,7-BisNP-O. Band assignment: (*) uncleaved substrate, (**) 11-mer product of APE1 cleavage, (***) 12-mer product of $\beta$-elimination.

**References**

