Shape-selective recognition of DNA abasic sites by metallohelices: inhibition of human AP endonuclease 1

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ABSTRACT

Loss of a base in DNA leading to creation of an abasic (AP) site leaving a deoxyribose residue in the strand, is a frequent lesion that may occur spontaneously or under the action of various physical and chemical agents. Progress in the understanding of the chemistry and enzymology of abasic DNA largely relies upon the study of AP sites in synthetic duplexes. We report here on interactions of diastereomerically pure metallo–helical ‘flexicate’ complexes, bimetallic triple-stranded ferro-helicates [Fe₂(NN-NN)₃]Cl₄⁺ incorporating the common NN–NN bis(bidentate) helicand, with short DNA duplexes containing AP sites in different sequence contexts. The results show that the flexicates bind to AP sites in DNA duplexes in a shape-selective manner. They preferentially bind to AP sites flanked by purines on both sides and their binding is enhanced when a pyrimidine is placed in opposite orientation to the lesion. Notably, the Δ-enantiomer binds to all tested AP sites with higher affinity than the Δ-enantiomer. In addition, the binding of the flexicates to AP sites inhibits the activity of human AP endonuclease 1, which is as a valid anticancer drug target. Hence, this finding indicates the potential of utilizing well-defined metallo–helical complexes for cancer chemotherapy.

INTRODUCTION

An abasic (apurinic or apyrimidinic, AP) site represents one of the most abundant, although rapidly repaired, DNA lesions in cells. AP sites are generated by the breakage of the α,β-glycosidic bond in DNA, which may occur spontaneously, chemically or enzymatically during base excision repair mechanism (1,2). It is estimated that under physiological conditions, ~10 000 apurinic sites and 500 apyrimidinic are formed per human cell per day (1,3). Therefore, AP site represents an attractive target for diagnostic and therapeutic applications. Selective ligands capable of changing their optical properties upon binding to an AP site could be used for a sensitive detection of these lesions (4–9). Many different structures have been developed to target AP sites; DNA base-intercalator conjugates (10,11), metalloinsertors (12,13), naphthalene derivatives (14), biaryl derivatives (15), an isoquinoline alkaloid berberine (16) or cyclobisacridine (17). It has been suggested that molecules selectively binding to AP sites could assist antitumour drugs by blocking the binding site of the AP endonucleases (10,18). These specialized enzymes are able to cleave DNA strands at AP sites and initialize the first stage of the repair of such lesions by a cascade of specific enzymes. Several molecules have been shown to inhibit AP endonucleases (19,20) and potentiate cytotoxicity of antitumour alkylating agent bis(chloroethyl)nitrosurea (BCNU) in vitro and in vivo (10,21,22).

We have recently reported (23) that [Fe₂L¹amines]Cl₄ helical complexes (Figure 1A) can recognize and stabilize some unusual DNA structures such as Y-shaped three-way junctions, three-way junctions with unpaired nucleotides, the so-called T-shaped three-way junctions and DNA bulges containing one and more unpaired nucleotides. These water stable, optically and diastereochromically pure bimetallic structures with flexible linkers have been created via self-assembly from monometallic complexes containing functionalized pyridine/imine units (24–27). Since the stereoselectivity in these complexes does not rely on the helicate concept of mechanical coupling they are described as flexicates.

In the present study, we explored interactions of [Fe₂L¹amines]Cl₄ flexicates with short DNA duplexes containing an AP site in different sequence contexts. We employed the following techniques: DNA melting temperature (Tm) measurements, fluorescence spectroscopy, gel electrophoresis, DNase I footprinting and isothermal titration calorimetry (ITC). Moreover, we examined if the binding of the flexicates to an AP site can affect the activity of human AP endonuclease 1 (APE1), since APE1 inhibitors have demon-
32P]-ATP was from MP Biomedicals, LLC (Irvine, CA, USA). The synthetic oligodeoxyribonucleotides was 3 × 10⁻⁶ M per strand. The samples were analysed by electrophoresis on 15% polyacrylamide gels in buffered solutions consisting of Tris(hydroxymethyl)amino methane (89 mM), borate (89 mM, pH 8.3) and ethylenediaminetetraacetic acid (1 mM) run at 5°C.

**Gel electrophoresis of DNA duplexes containing an AP site**

Stoichiometric amounts of oligonucleotides at the concentration of 1 × 10⁻⁵ M per strand were mixed together in the buffer to form a duplex. One of the strands was 5'-end labelled using T4 polynucleotide kinase and [γ³²P]ATP. Flexicates were then added to the mixture so that the final concentration of the oligonucleotides in the samples was 5 × 10⁻⁶ M. The samples were analysed by electrophoresis on 15% polyacrylamide gels in buffered solutions consisting of Tris(hydroxymethyl)amino methane (89 mM), borate (89 mM, pH 8.3) and ethylenediaminetetraacetic acid (1 mM) run at 5°C.

**Fluorescence spectroscopy**

A 1 μM solution of the oligonucleotide duplex was prepared in a 1 cm quartz cuvette in a total volume of 2.5 ml. The buffer was composed of sodium phosphate buffer (10 mM, pH 7.0) and NaCl (100 mM). Small volumes (2.5 μl) of flexicates were added to the solution to obtain the desired concentration and thoroughly mixed by pipetting. The mixture was kept undisturbed for 3 min at room temperature. The fluorescence was measured by using Varian Cary Eclipse spectrofluorophotometer. The excitation and emission wavelengths were set to 310 and 365 nm, respectively, the excitation and emission slit widths were 5 nm, and the integration time was set to 5 s.

**Amiloride displacement**

A 1 μM solution of the oligonucleotide duplex and amiloride was prepared in a 1 cm quartz cuvette in a total volume of 2.5 ml. The buffer was composed of sodium phosphate buffer (10 mM, pH 7.0) and NaCl (100 mM). Small volumes (2.5 μl) of flexicates were added to the solution to obtain the desired concentration and thoroughly mixed by pipetting. The mixture was kept undisturbed for 3 min at room temperature. The fluorescence was measured by using Varian Cary Eclipse spectrofluorophotometer. The excitation and emission wavelengths were set to 380 and 415 nm, respectively, the excitation and emission slit widths were 5 nm, and the integration time was set to 5 s.
Isothermal titration calorimetry (ITC)

Heat flow during isothermal titration was measured at 20°C with a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA, USA). The standard titration buffer for these studies contained 100 mM NaCl with 10 mM phosphate buffer (Na$_2$HPO$_4$/NaH$_2$PO$_4$ [pH 7.0]). In a typical experiment, 6 µM DNA duplex solution (1.4 ml) was titrated with 80 µM helicates, using 300 µl syringe rotating at 490 rpm. A titration consisted of 28 injections of 10 µl each, with 20 s duration and 240 s between injections. Samples were equilibrated thermally prior to a titration until the baseline has levelled off. The peaks produced during titration were converted to heat output per injection by integration and correction for the cell volume and sample concentration. Data from individual titrations were analysed by using Origin 5.0 software package (Origin, Northampton, MA, USA) and fitted to a one-site (or n identical sites), two independent sites, or sequential binding or more binding model (29) to extract the relevant thermodynamic parameters.

DNase I footprinting

Top or bottom strands of the oligonucleotide duplexes were 5'-end labelled using T4 polynucleotide kinase and [$\gamma$-32P]ATP and hybridized with the complementary bottom or top strands. A total of 5 µl solutions containing 1.11 × TKMC buffer (10 mM Tris pH 7.9, 10 mM KCl, 10 mM MgCl$_2$, and 5 mM CaCl$_2$), 3 × 10^-4 M DNA (per nucleotide) and various concentrations of the helicates were incubated for 15 min at 25°C. Cleavage was initiated by the addition of 1 µl of DNase I diluted in the precedent experiment to the concentration that was sufficient to achieve partial cleavage of the DNA duplexes. Samples were allowed to react for 10 min at room temperature before quenching with 6 µl of 2× concentrated formamide loading buffer followed by incubation at 90°C for 3 min. A total of 2 µl of the mixture containing DNA cleavage products were then withdrawn and resolved by polyacrylamide (PAA) gel electrophoresis under denaturing conditions (8%/8 M urea PAA gel). The autoradiograms were visualized and quantified by using the bio-imaging analyser.

Inhibition of APE1

Top strands of the oligonucleotide duplexes were 5'-end labelled using T4 polynucleotide kinase and [$\gamma$-32P]ATP and hybridized with the complementary bottom strands. Reaction mixtures (5 µl, total volume) containing DNA duplexes at the concentration of $2 \times 10^{-7}$ M per strand in 50 mM potassium acetate, 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate and 1 mM dithiothreitol (DTT) were preincubated in the absence or in the presence of various concentrations of the helicates for 15 min at 25°C. Then 1 unit of APE1 in 1 µl was added into each reaction mixture and incubated for 10 min at 37°C. A total of 2 µl of the reaction mixture were then withdrawn, mixed with 2× concentrated formamide loading buffer followed by incubation at 90°C for 3 min and resolved by PAA gel electrophoresis under denaturing conditions (24%/8 M urea PAA gel). The autoradiograms were visualized and quantified by using the bio-imaging analyser.

RESULTS AND DISCUSSION

UV melting studies

The melting temperature ($T_m$) was determined to compare binding affinities of the helicates for DNA duplexes containing an AP site flanked by various bases and with various bases placed opposite an AP site. The $T_m$ corresponds to the midpoint of a smooth transition obtained by recording the absorbance at 260 nm as a function of the temperature (Supplementary Figures S1–4). The changed $T_m$ values of the duplexes [$T_m$ of the duplex modified by the helicate—$T_m$ of the control (unmodified duplex) ($\Delta T_m$)] are generally affected by the oligonucleotides’ dissociation constants and the stability of their own secondary structures. The stronger the interaction between DNA and [Fe$_2$L$_{18}$]Cl$_4$, the more the $T_m$ value increases (30,31).

The thermal stabilities of an AP site containing oligonucleotide duplexes flanked by G-C pairs on both sides and with dG, dC, dA and dT in opposite orientation to an AP site ($X_1 = X_2 = G$, $Y_1 = Y_2 = C$, $Z = G$, $C$, $A$ and $T$; Figure 1B) in the presence of the helicates were determined and results are summarized in Table 1. The fully matched duplex GGG/CCC of identical sequence to GFF/CAC duplex in which an AP site was replaced by guanine was used as a control and had a melting temperature of 53.1°C. The presence of an AP site markedly reduced thermal stabilities of the duplexes. The melting temperatures of the duplexes with purines (dG or dA) opposite an AP site were slightly higher than those with pyrimidines (dC or dT): 36.0 and 38.2°C for GFG/CCC and GFF/CAC, respectively, versus 35.4 and 34.1°C for GGG/CAC and GCC/GCC, respectively. The results in Table 1 show that the $T_m$ values of an AP site containing duplexes in the presence of $\Delta$- and $\Delta$-[Fe$_2$L$_{18}$]Cl$_4$ were increased by ~14–18°C and ~5–12°C, respectively. The maximum stabilization effect was observed for the duplexes with pyrimidines in opposite orientation to AP sites. In all cases, the $\Delta$-[Fe$_2$L$_{18}$]Cl$_4$ was more effective in increasing the thermal stability of an AP site containing DNA duplexes. Increasing the flexicate duplex ratio from 1:1 to 2:1 had little impact on the melting temperature of the duplexes (0.1–1.3°C) which is consistent with the presence of a single dominant binding site for the helicates. The value of $T_m$ of the fully matched duplex GGG/CAC was negligibly affected in the presence of the helicates. Table 2 presents data obtained for the duplexes containing an AP site flanked by A-T pairs on both sides and with various nucleotides placed opposite an AP site ($X_1 = X_2 = A$, $Y_1 = Y_2 = T$, $Z = G$, $C$, $A$ and $T$; Figure 1B). Also in this case, the $T_m$ values of an AP site containing duplexes in the absence of the helicates were a little higher if the base situated opposite an AP site was purine: 29.1 and 25.9°C for AFA/TAT, respectively, versus 25.9 and 27.6°C for AFA/TCT and AFA/TTT, respectively. The presence of the helicates had little effect on the $T_m$ of the fully matched duplex AGA/TCT (46.3°C). The $T_m$ values of duplexes containing an AP site flanked by adenines in the presence of $\Delta$- and $\Delta$-[Fe$_2$L$_{18}$]Cl$_4$ were increased by ~11–15°C and ~7–14°C, respectively. The helicates enhanced
the thermal stability of duplexes with pyrimidines opposite an AP site more than those with purines. $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ was a better stabilizer of an AP site containing duplexes than $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$. Doubling the flexicat:duplex ratio from 1:1 to 2:1 had small effect (0.7–1.4 $^\circ$C). The presence of one major binding site for the flexicates.

Finally, results listed in Table 4 show the impact of $\Lambda$- and $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ on the thermal stability of the fully matched duplex TGT/ACA and corresponding duplexes containing an AP site flanked on both sides by T-A pairs ($X_1 = X_2 = T$, $Y_1 = Y_2 = A$, $Z = G$, C, A and T; Figure 1B). In the absence of the flexicates, the melting temperatures of TGT/AGA and TGT/ATA duplexes with a pyrimidine base opposite an AP site in the presence of $\Lambda$- and $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$, were $36.8$ and $35.7$ $^\circ$C for CFC/GGG and CFC/GAG, respectively, versus $31.3$ and $33.0$ $^\circ$C for CFC/GCG and CFC/GTG, respectively. The addition of $\Lambda$- and $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ increased the $T_m$ values of duplexes by $5$–$15$ $^\circ$C. In contrast, the $T_m$ of the fully matched duplex CGC/GGC (54.4 $^\circ$C) was almost unaffected by flexicates. Similarly to the previous results, the duplexes containing pyrimidines (dC or dT) opposite an AP site were stabilized by flexicates more than those with purines (dG or dA). $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ was more efficient than $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ in stabilizing duplexes containing an AP site flanked by cytosines.

**Electrophoretic mobility shift assay**

We employed the electrophoretic mobility shift assay to examine whether the stability of the complex formed between flexicates and an AP site containing duplex is strong enough to withstand migration through a polyacrylamide gel. The autoradiograms of the gels run at 5 $^\circ$C (Figure 2) show interaction of $\Lambda$- and $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ with GFG/CTC and GFG/CAC duplexes. It can be seen that a new slower-migrating band indicating formation of the flexicate–DNA duplex complex appears for GFG/CTC (Figure 2A, lanes 1–6). The autoradiogram of the gel in Figure 2B (lanes 1–6) shows that the interaction of $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ with GFG/CAC duplex was not so strong as in the former case which is consistent with the previous results from the UV melting studies showing that flexicates prefer binding to an AP site with a pyrimidine placed opposite the lesion. We did not observe formation of the complexes between GFG/CTC and GFG/CAC duplexes and $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ (Figure 2A and B, lanes 7–12) which is also in agreement with the previous data demonstrating that $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ is a weaker binder than $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ to AP sites flanked by purines.

| Compound | $\Delta T_m$ ($^\circ$C) at 1:1$^a$ | $\Delta T_m$ ($^\circ$C) at 2:1$^b$ |
|----------|---------------------------------|---------------------------------|
| GGG/CCC ($T_m$ = 53.1 $^\circ$C) | $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 0.2 |
| | $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 0.0 |
| GFG/GCG ($T_m$ = 36.0 $^\circ$C) | $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 15.3 |
| | $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 5.2 |
| GFG/GCC ($T_m$ = 35.5 $^\circ$C) | $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 16.3 |
| | $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 10.5 |
| GFG/GAC ($T_m$ = 38.2 $^\circ$C) | $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 13.9 |
| | $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 6.6 |
| GFG/GTC ($T_m$ = 34.1 $^\circ$C) | $\Lambda$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 17.6 |
| | $\Delta$-[Fe$_2$L$_{1a}$]$\Delta_3$Cl$_4$ | 11.2 |

$^a$Helicate:duplex was 1:1.

$^b$Helicate:duplex was 2:1.
Table 2. Thermal stability of a fully matched duplex AGA/TCT and corresponding duplexes containing an AP site flanked by adenines and with various bases opposite an AP site in the presence of \( \Lambda\) and \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\)

| Compound          | \( \Delta T_m \) (°C) at 1:1\(^{a}\) | \( \Delta T_m \) (°C) at 2:1\(^{b}\) |
|-------------------|--------------------------------------|--------------------------------------|
| AGA/TCT (**T_m** = 46.3°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 0.2                                  | 0.8                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 0.1                                  | 0.3                                  |
| AFA/TGT (**T_m** = 29.1°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 11.1                                 | 11.8                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 7.1                                  | 7.8                                  |
| AFA/TCT (**T_m** = 25.9°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 13.3                                 | 14.7                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 12.7                                 | 13.5                                 |
| AFA/TAT (**T_m** = 28.5°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 9.8                                  | 10.8                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 7.3                                  | 8.5                                  |
| AFA/TTT (**T_m** = 27.6°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 10.6                                 | 11.8                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 10.1                                 | 11.2                                 |

\(^{a}\)Helicate:duplex was 1:1.  
\(^{b}\)Helicate:duplex was 2:1.

Table 3. Thermal stability of a fully matched duplex CGC/GCG and corresponding duplexes containing an AP site flanked by cytosines and with various bases opposite an AP site in the presence of \( \Lambda\) and \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\)

| Compound          | \( \Delta T_m \) (°C) at 1:1\(^{a}\) | \( \Delta T_m \) (°C) at 2:1\(^{b}\) |
|-------------------|--------------------------------------|--------------------------------------|
| CGC/GCG (**T_m** = 54.4°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 0.3                                  | 0.7                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | -0.1                                 | 0.2                                  |
| CFC/GGG (**T_m** = 36.8°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 9.0                                  | 9.3                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 4.8                                  | 6.4                                  |
| CFC/GCG (**T_m** = 31.3°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 14.1                                 | 14.6                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 11.5                                 | 12.0                                 |
| CFC/GAG (**T_m** = 35.7°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 5.8                                  | 8.5                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 3.5                                  | 5.4                                  |
| CFC/GTG (**T_m** = 33.0°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 12.5                                 | 13.2                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 11.2                                 | 11.9                                 |

\(^{a}\)Helicate:duplex was 1:1.  
\(^{b}\)Helicate:duplex was 2:1.

Table 4. Thermal stability of a fully matched duplex TGT/ACA and corresponding duplexes containing an AP site flanked by thymines and with various bases opposite an AP site in the presence of \( \Lambda\) and \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\)

| Compound          | \( \Delta T_m \) (°C) at 1:1\(^{a}\) | \( \Delta T_m \) (°C) at 2:1\(^{b}\) |
|-------------------|--------------------------------------|--------------------------------------|
| TGT/ACA (**T_m** = 48.1°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 0.8                                  | 1.0                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 0.2                                  | 0.4                                  |
| TFT/AGA (**T_m** = 27.9°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 4.7                                  | 6.2                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 5.3                                  | 6.9                                  |
| TFT/ACA (**T_m** = 24.4°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 13.1                                 | 14.1                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 14.6                                 | 15.0                                 |
| TFT/AAA (**T_m** = 28.0°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 4.5                                  | 6.3                                  |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 5.0                                  | 6.6                                  |
| TFT/AFA (**T_m** = 25.6°C) |                                       |                                       |
| \( \Lambda\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 10.2                                 | 11.2                                 |
| \( \Delta\)-[Fe\( _2\)L\( _{1a}^3\)]Cl\( _4\) | 13.4                                 | 14.0                                 |

\(^{a}\)Helicate:duplex was 1:1.  
\(^{b}\)Helicate:duplex was 2:1.
2-aminopurine fluorescence studies

2-aminopurine (2AP), a fluorescent analogue of adenine, has been widely used as a probe of small molecule binding to DNA (32,33) or RNA (34,35). The fluorescence of 2AP is strongly quenched within the structure of double-stranded DNA or RNA, but is enhanced when the base stacking or base pairing is perturbed. Other factors that affect the fluorescence of 2AP are collisions with other bases and biomolecular interactions (36,37). We used AFA/T2APT duplex containing an AP site flanked by A-T pairs on both sides and with 2AP opposite an AP site to monitor the binding of Δ- and Δ-[Fe₂L¹₃]Cl₄ (Figure 3). Titrations of AFA/T2APT by Δ- and Δ-[Fe₂L¹₃]Cl₄ lead to a marked decrease of the fluorescence intensity of 2AP followed by a slight increase of the fluorescence when the concentration of flexicates reached ~2 μM. Plots of the data with linear interpolations superimposed suggest that Δ- and Δ-[Fe₂L¹₃]Cl₄ bind to AFA/T2APT at 1:1 flexicate:duplex ratio. The quenching of the 2AP fluorescence by [Fe₂L¹₃]Cl₄ could be explained by enhanced base stacking interactions between the 2AP and adjacent bases in the presence of [Fe₂L¹₃]Cl₄ or by direct interactions of the 2AP with the flexicates.

We have recently demonstrated that flexicates based on the L¹₄ ligand (Supplementary Figure S6) are weak and nonspecific DNA binders that have no stabilizing effect on unusual DNA structures (23). The experiment was repeated with [Fe₂L²₄]Cl₄ to compare the influence of the L¹₄ and L²₄ based flexicates on the 2AP fluorescence. The plot in Figure 3 shows that titrations of AFA/T2APT by Δ- and Δ-[Fe₂L²₄]Cl₄ resulted in a slight linear decrease of the 2AP fluorescence.

Amiloride displacement

Amiloride has been previously reported to selectively recognize thymine situated opposite an AP site (38). It has been determined by ITC that amiloride preferentially binds to an AP site flanked by G-C pairs with $K_a = 2.7 \times 10^6$ M⁻¹ (7). We titrated GFG/CTC duplex mixed at 1:1 ratio with amiloride by Δ- and Δ-[Fe₂L¹₃]Cl₄ to examine if the flexicates can displace amiloride from its preferential binding site. The plots of the fluorescence intensity of amiloride as a function of Δ- and Δ-[Fe₂L¹₃]Cl₄ concentrations in Figure 4 show that the fluorescence signal of amiloride was growing with increasing concentration of the flexicates up to 1.1–1.2 μM and then it levelled off.

Since flexicates readily displaced amiloride from its binding site it can be concluded that $K_a$ values of Δ- and Δ-[Fe₂L¹₃]Cl₄ are much higher than that of amiloride. The linear interpolations of initial and final segments of the plots in Figure 4 intersect at ~1 μM, which corresponds to 1:1 (flexicate:duplex) ratio. This result is consistent with the presence of one major binding site on GFG/CTC duplex for Δ- and Δ-[Fe₂L¹₃]Cl₄, although the presence of an additional less affinity binding site cannot be excluded as it can be deduced from our ITC data (vide supra).
Table 5. Thermodynamic parameters for the titration of GFG/CTC and GFG/CAC duplexes with \( \Lambda^-\) and \( \Delta^-\)-[Fe\( \text{L}_{1a} \)\text{Cl}_4]$_4$

| Binding parameters | GFG/CTC | GFG/CAC |
|--------------------|---------|---------|
| \( N_1 \) \( (\text{M}^{-1}) \) | 1.03 ± 0.03 | 1.03 ± 0.09 |
| \( K_1 \) \( (\text{M}^{-1}) \) | (6.8 ± 1.8)\( \times10^7 \) | (4.7 ± 2.2)\( \times10^7 \) |
| \( \Delta H_1 \) \( (\text{kJ} \cdot \text{mol}^{-1}) \) | -41.4 ± 0.3 | -21.9 ± 0.4 |
| \( T\Delta S_1 \) \( (\text{kJ} \cdot \text{mol}^{-1}) \) | 2.5 | 21.2 |
| \( N_2 \) \( (\text{M}^{-1}) \) | 0.34 ± 0.05 | 0.26 ± 0.14 |
| \( K_2 \) \( (\text{M}^{-1}) \) | (1.8 ± 0.2)\( \times10^6 \) | (3.2 ± 0.6)\( \times10^6 \) |
| \( \Delta H_2 \) \( (\text{kJ} \cdot \text{mol}^{-1}) \) | -103.2 ± 13.8 | -75.3 ± 35.5 |
| \( T\Delta S_2 \) \( (\text{kJ} \cdot \text{mol}^{-1}) \) | -68.3 | -38.8 |

Figure 5. ITC binding curves for the titration of GFG/CTC (A and B) and GFG/CAC (C and D) duplexes with \( \Lambda^-\)-[Fe\( \text{L}_{1a} \)\text{Cl}_4]$_4$ (A and C) and \( \Delta^-\)-[Fe\( \text{L}_{1a} \)\text{Cl}_4]$_4$ (B and D) at 20°C in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl. The upper panels in each Figure 5A–D show total heat released upon injecting aliquots of the flexicat solution into a reaction cell containing an AP site containing duplex. The lower panels show the resultant binding isotherms (full squares) obtained by integrating the peak areas of each injection; the continuous lines represent the nonlinear least squares fit to a two sets of sites binding model. For other details, see the text.
Isothermal titration calorimetry

In order to characterize the binding of the flexicates to an AP site containing duplexes we used ITC. Figure 5 shows ITC profiles resulting from the injections of Λ- (panels A and C) and Δ-[Fe2L1a]Cl4 (panels B and D) into a solution of GFG/CTC (panels A and B) and GFG/CAC (panels C and D) duplexes. The plots of the heat evolved per mole of the flexicate added against the molar ratio of the flexicate to DNA duplex are shown below each ITC profile. In these plots, the data points correspond to the experimental injection heats, while solid lines represent the calculated fits of the data with the 'two sets of sites' binding model (39); the only one that yielded a reasonable fit of the experimental data. The calculated thermodynamic parameters are listed in Table 5. It can be seen that each ITC profile has two apparent phases suggesting two distinct binding events. The binding stoichiometry values estimated for the first binding event (N1) indicate that the flexicates bind to a higher-affinity site at 1:1 ratio. The steepness of the first of the two phases indicates relatively tight binding interaction. Indeed, fit of the ITC profile for the binding of Λ- and Δ-[Fe2L1a]Cl4 to GFG/CTC duplex gives association constants for the first binding event (K1) of 6.8 × 10^7 M⁻¹ and 4.7 × 10^7 M⁻¹ (Table 5), respectively. These values are higher than the value reported previously for amiloride (2.7 × 10⁶ M⁻¹) which is in agreement with the observation that flexicates readily displaced amiloride from its binding site in GFG/CTC duplex. The binding affinity of Λ- and Δ-[Fe2L1a]Cl4 to GFG/CAC duplex was not so high which also correlates with the results from the UV melting studies and electrophoretic mobility shift assay. The observed enthalpies for Λ- and Δ-[Fe2L1a]Cl4 binding to GFG/CTC duplex, −41.4 and −21.9 kJ·mol⁻¹, respectively, are more exothermic than those associated with binding to GFG/CAC duplex, −25.6 and −4.3 kJ·mol⁻¹, respectively. In other words, the binding of the flexicates to an AP site with thymine placed opposite the lesion is enthalpically favourable. Taken together, the data demonstrate that the Λ-enantiomer binds to GFG/CTC and GFG/CAC duplexes with higher affinity than the Δ-enantiomer and that its binding is also associated with larger enthalpy changes. Unfortunately, we cannot currently provide an explanation and a binding model that would be consistent with the stoichiometry of the second binding event. The stoichiometry values N2 ≈ 0.26–0.37 (Table 5) indicate that the binding of the second flexicate molecule leads to the association of the duplexes. On the other hand, these results demonstrate that the first flexicate molecule binds to a unique high-affinity site on the target duplex.
To further characterize the binding of the flexicates to an AP site, we employed DNase I footprinting. ApaI and Delta1-[Fe2L1a3]Cl4 were mixed with radioactively labelled GFG/CTC duplex at 0.5:1, 1:1, 2:1 and 3:1 (flexicate:duplex) ratios, respectively, followed by partial cleavage by DNase I. The autoradiograms of the DNA cleavage-inhibition patterns for the top and bottom strand labelled GFG/CTC duplex are shown in Figure 6A. As it can be seen, the inhibition of DNase I cleavage activity increases with increasing concentration of the flexicates. The maximum protection was observed when the flexicate:duplex ratio exceeded 1:1. Scheme in Figure 6B summarizing obtained results suggests that ApaI and Delta1-[Fe2L1a3]Cl4 bind directly to an AP site or in its close proximity.

**Discussion**

The results show that [Fe2L1a3]Cl4 flexicates bind to an AP site containing DNA duplexes in a shape-selective manner as in the case of the binding of phenanthrenequinone dimine complexes of rhodium(III) to DNA (40,41). Flexicates preferentially bind to AP sites flanked by purines on both sides and their binding is enhanced when a pyrimidine is placed in opposite orientation to the lesion. This binding preference could be explained by the previously reported observation using nuclear magnetic resonance spectroscopy (42) that pyrimidines opposite an AP site stack poorly and are extrahelical while adenine (A) is stacked in an intrahelical conformation and guanine (G) can exist in both intra- and extrahelical conformations. Apart from an AP site flanked by thymines, the L-enantiomer was binding to all tested AP sites with higher affinity than the Delta1-enantiomer.

Nevertheless, the binding mode of the flexicates to an AP site remains to be determined. The results indicate that the first flexicate molecule binds to a unique high-affinity site on the target duplex. We do not know if the flexicates bind directly to an AP site or in a close proximity to the lesion. The binding preference of the flexicates to AP sites having a poorly stacked pyrimidine base placed opposite the lesion suggests that the flexicate molecule could insert into the hollow cavity formed in the DNA double helix by the AP site and the looped-out base in opposite orientation. Another possibility is that the flexicate sticks to an AP site from the side, probably from the major groove.

In the aggregate, our results show that flexicates constitute a completely new group of ligands capable of selective binding to AP sites. Their structure is totally different from that of the previously reported molecules designed to bind to an AP site. In addition, the ability of the flexicates to inhibit DNA cleavage by human AP endonuclease 1 and that AP endonuclease 1 has been shown to have higher activity in tumour compared to normal tissue (43) suggests that the flexicates might be applied along with antitumour alkylating agents to potentiate their toxic activity in tumour cells (28).

**Supplementary Data**

Supplementary Data are available at NAR Online.

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**Conflict of interest statement**

None declared.

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