Recognition of Abasic Sites in DNA by a Cyclobisacridine Molecule

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Abstract: The ability of the cyclobisacridine **CBA** to recognize the abasic lesion in DNA was investigated with modified synthetic oligonucleotide duplexes. **CBA** was shown to cleave a ^{32}P-labeled duplex oligomer (23-mer) containing an apurinic site in the middle of the sequence. The interaction was examined with duplexes which contain a stable tetrahydrofuranyl analogue at the abasic site. Thermal denaturation experiments showed that **CBA** stabilizes an abasic undecamer duplex by a ΔT_m value of 14 °C by forming a 1:1 complex, while no interaction was detected with the unmodified parent duplex. **CBA** displaced a nitroxide abasic site specific probe from the abasic duplex. When irradiated in the presence of a ^{32}P-labeled model abasic site containing duplex (23-mer), **CBA** induced selective photocleavage in the vicinity of the abasic lesion on both strands. All results demonstrate the high specificity of the interaction of **CBA** at the abasic lesion.

Keywords: abasic site recognition · cyclobisacridine · DNA cleavage · DNA recognition · oligonucleotides

Introduction

Loss of a base in DNA by cleavage of the glycosidic bond results in formation of the toxic abasic site lesion.[1] In vivo, abasic sites (apurinic/apyrimidinic: AP-sites) are formed spontaneously,[2] a process that is accelerated by the action of alkylating agents.[3] For example, some anticancer alkylating drugs have been shown to create AP-sites at high frequency in tumor cells. Abasic sites are also formed as intermediates in the protective action of *N*-glycosylases which remove damaged or abnormal bases.[4] The AP-sites thus generated are repaired by specialized enzymes that restore the integrity of the DNA.[5, 6] It is thus of high interest to prepare molecular architectures designed to bind specifically at abasic sites. Such molecules could interfere with the repair machinery in the cell. In previous work[7] we prepared series of molecules such as **ATAc** and **DTAc** possessing a nucleic base linked to an intercalator through a polyamino chain (Figure 1). They recognize abasic sites with high selectivity and incise DNA at nanomolar concentrations, thereby mimicking the action of AP-endonucleases of the lyase family that cleave DNA at the AP-sites by β-elimination of the 3′-phosphate (Scheme 1). These molecules were later shown to act as inhibitors of the abasic site repair and potentiate the action of the anticancer drug *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU).[8] Interestingly, compound **ATAc** was also

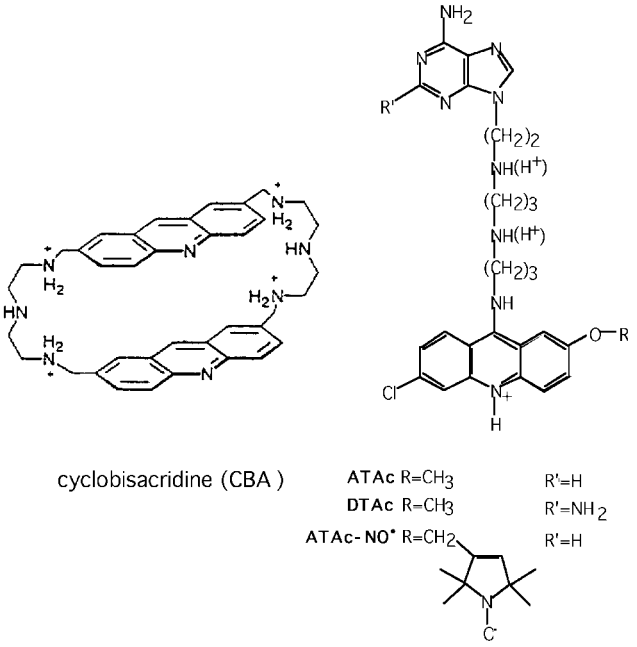

Figure 1. Chemical structures of adenine-triamino-acridine (**ATAc**), diaminopurine-triamino-acridine (**DTAc**), cyclobisacridine (**CBA**), and the abasic site specific EPR probe **ATAc-NO•**.

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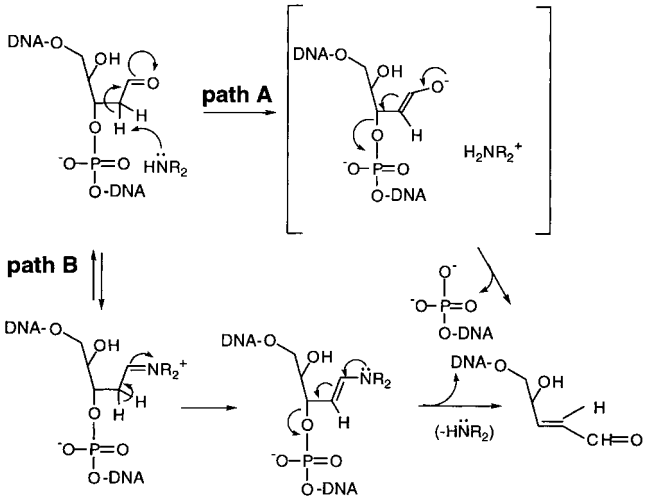

Scheme 1. Proposed mechanisms for cleavage of DNA at the abasic site by class II AP-endonucleases (AP-lyases) and the AP-endonucleases mimics **ATAc** and **DTAc**[7].

shown to bind the unpaired base uracyl in an RNA bulge.[9] Bulges and abasic sites are all characterized by the existence of an unpaired base. The cyclobisacridine (**CBA**)[10] was designed and shown to bind preferentially to single-stranded regions in DNA, especially hairpins.[11] It thus represents a novel structural candidate to bind abasic sites. We report here that **CBA** incises DNA at the abasic site of a synthetic duplex containing an abasic lesion in the middle of the sequence. By using oligonucleotides containing a stable tetrahydrofuranyl analogue of the abasic site (Figure 2), we show by T_m measurements, photocleavage experiments and displacement of a nitroxide abasic site-specific probe[12] that **CBA** binds specifically at the abasic lesion.

Abstract in French: *La reconnaissance de sites abasiques de l'ADN par la cyclobisacridine **CBA** a été étudiée à l'aide d'oligonucléotides de synthèse. Utilisant un duplex marqué au ^{32}P comportant un site apurinique en milieu de séquence, on montre que **CBA** incise le duplex au niveau de la lésion. La spécificité de l'interaction avec des oligonucléotides comportant un analogue stable, tétrahydrofuranyle, du site abasique a été montrée par une série de mesures: accroissement de la température de dénaturation d'un duplex undécamère d'une valeur $\Delta T_m = 14^\circ C$ par formation d'un complexe 1/1, alors qu'aucun effet n'est décelé avec le duplex parent non modifié, déplacement d'une sonde nitroxyde spécifique des sites abasiques, photocoupure d'un duplex (23-mère) au niveau de la lésion et au niveau des deux brins, en présence de **CBA**.*

Results and Discussion

Cleavage of the DNA duplex A containing an abasic site: The ability of **CBA** to cleave DNA at abasic sites was examined with the synthetic ^{32}P-labeled duplex **A** (23-mer) containing an apurinic site in the middle of the sequence. The abasic site at position 12 faces the thymine T_{35} residue in the opposite strand. Duplex **A** was prepared by incubation of the synthetic oligonucleotide precursor **B** that contains an uracil residue at position 12 with uracil-DNA glycosylase. Selective cleavage of duplex **A** at the abasic site by **CBA** was demonstrated (Figure 3) by formation of a labeled fragment identical to that formed in the presence of sodium hydroxide, which is known to cause strand cleavage by β-elimination of the 3′-phosphate (Scheme 1). However, compared with the artificial AP-endonuclease **ATAc**, cleavage by **CBA** is less efficient (29% versus 72% cleavage when operating in identical conditions). This result suggests that **CBA** interacts at the abasic site as was observed previously for **ATAc**.[13]

23-mer

5′ C1 G2 C3 G4 T5 A6 C7 G8 C9 A10 C11 **X12** C13 A14 C15 G16 C17 A18 T19 G20 C21 G22 C23 3′
3′ G46 C45 G44 C43 A42 T41 G40 C39 G38 T37 G36 T35 G34 T33 G32 C31 G30 T29 A28 C27 G26 C25 G24 5′

duplex **A** : X = d-Rib ; duplex **B** : X = U ; duplex **C** : X = Thf

11-mer

5′ C1 G2 C3 A4 C5 **X6** C7 A8 C9 G10 C11 3′
3′ G22 C21 G20 T19 G18 T_{11} G16 T15 G14 C13 G12 5′

duplex **D** : X = Thf; duplex **E** : X = A

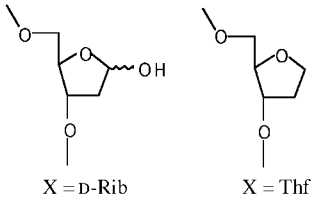

Figure 2. Duplex oligonucleotides **A** – **E** prepared. Note that the 23-mer duplexes contain in their central part the sequences of the 11-mer, flanked by six base pairs on both sides.

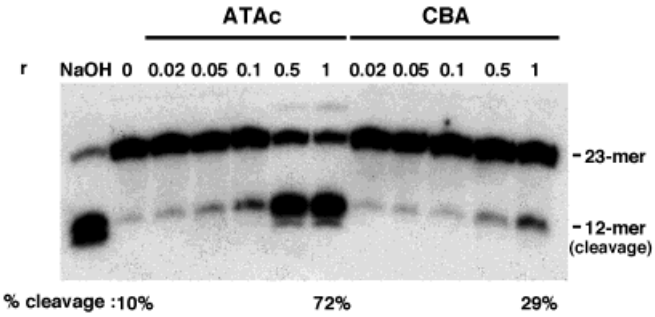

Figure 3. Autoradiograms of 20% denaturing polyacrylamide gel showing cleavage of the 5′-^{32}P-end labeled 23-mer duplex **A** in the presence of increasing amounts of **ATAc** and **CBA**. The reaction mixture contained oligonucleotide duplex (5 pmol), Hepes (pH 8, 66 mM), DTT (1 mM), $MgCl_2$ (5 mM), NaCl (50 mM) in a total volume of 10 μL, *r* is the ratio [drug]/[oligonucleotide]. The cleavage reaction was analyzed after 15 min incubation at 37 °C. Cleavage by NaOH (0.5 N) for 10 min at 90 °C is shown as a reference.

Thermal denaturation of the model abasic duplex D: In order to get information on the possible formation of a noncovalent drug – DNA complex prior to DNA cleavage, we used synthetic DNA fragments containing the stable tetrahydro-

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furanyl (Thf) analogue of the abasic site. This tetrahydrofuranyl moiety is considered to be a good model of the abasic site.[14, 15] It is most commonly used in structural and biological studies as it retains most of the biological properties of the abasic sites. Interaction was first studied by examining the thermal denaturation of the apurinic undecamer **D** in the presence of increasing concentrations of **CBA** (Figure 4). Regular increase of the melting temperature (from 38 °C to 52 °C) was observed as the ratio r = [drug]/[duplex] was increased in the range 0–1. Further addition of the drug ($r > 1$) did not cause any further increase of the melting temperature. This is proof for formation of a 1:1 complex. The T_m value for the parent unmodified duplex **E** remained unchanged in the presence of **CBA** ($T_m = 58$ °C).

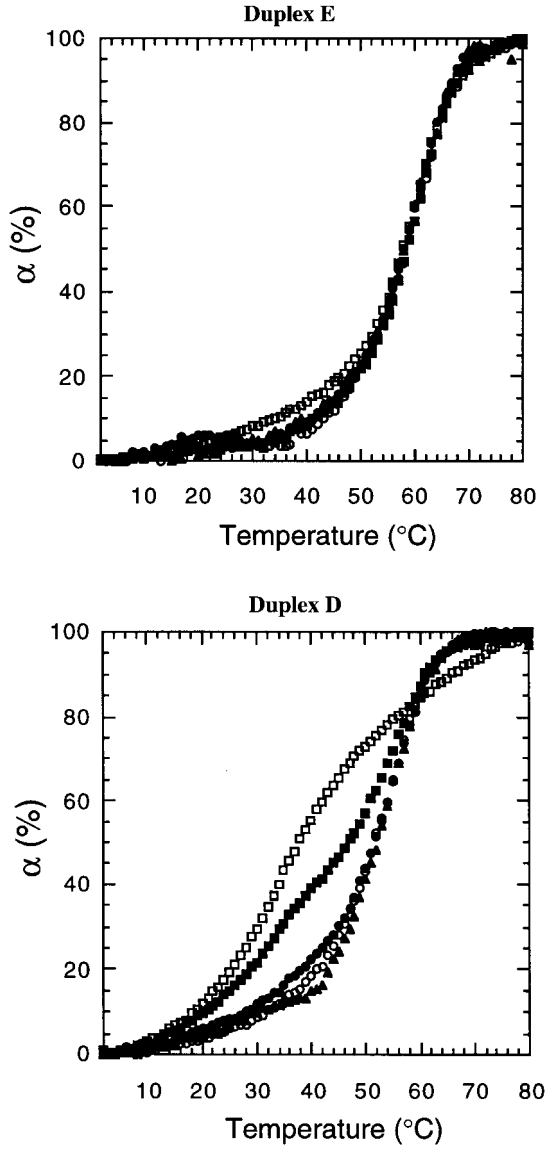

Figure 4. Plots $\alpha = f(T)$ for duplexes **D** and **E** in the absence and in the presence of increasing ratios r (r = [drug]/[oligonucleotide]) of **CBA**, α is defined as the fraction of single strands in the duplex (according to the procedure described by Breslauer[23]). In each experiment, the reaction mixture contained the duplex oligonucleotide (7.69 μM) and various ratios r of drug ($0 \le r \le 2$) in sodium phosphate buffer (pH 7, 10 mM), EDTA (1 mM), and NaCl (20 mM). □, $r = 0$; ■, $r = 0.5$; ●, $r = 1$; ○, $r = 1.5$; ▲, $r = 2$.

Displacement of the nitroxide-labeled abasic site specific probe ATAc-NO•: The nitroxide probe ATAc-NO•[16] is a labeled analogue of the artificial AP-endonuclease **ATAc** that has been shown previously by high-field NMR spectroscopy to bind selectively to the model abasic duplex **D**. In the complex the acridine moiety of the drug intercalates at a two base-pair distance from the abasic site, the adenine residue of **ATAc** docks inside the abasic pocket pairing with the opposite thymine in the complementary strand and the polyamino linker lies in the minor groove.[13] We have equally reported series of EPR measurements indicating that the labeled analogue **ATAc-NO•** forms a similar complex and can thus be used as an abasic site specific EPR probe.[12] Figure 5 illustrates the sets of experiments that confirm binding of

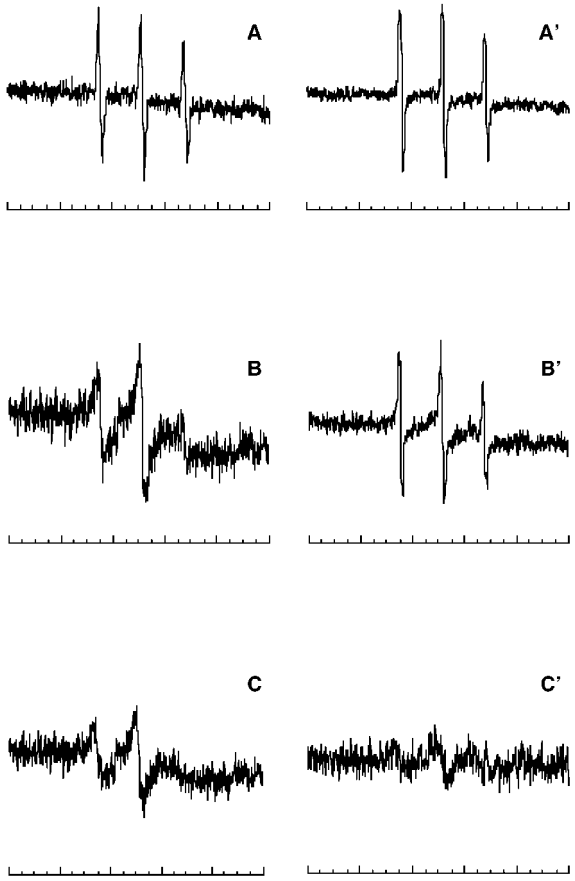

Figure 5. EPR spectra of A) **ATAc-NO•** (1×10^{-5} M) in Tris-HCl (pH 7, 25 mM), NaCl (0.1 M), and EDTA (0.2 mM) with DMSO (2%); B) **ATAc-NO• (1×10^{-5} M) in the presence of the duplex D** (1.8×10^{-5} M); C) subtraction of spectrum A from spectrum B; (A′) **ATAc-NO•** (1×10^{-5} M) + **CBA** (1×10^{-5} M) in Tris-HCl (pH 7, 25 mM), NaCl (0.1 M), and EDTA (0.2 mM) with DMSO (2%); B′) **ATAc-NO•** (1×10^{-5} M) + **CBA** (2×10^{-5} M) in the presence of the duplex **D** (1.8×10^{-5} M); C′) subtraction of spectrum A′ from spectrum B′.

CBA at the abasic site. The spectrum registered for the labeled probe in dilute solution (10^{-5} M) (Figure 5A) is characteristic of a nitroxide radical undergoing relatively rapid motion (correlation time $\tau_c = 1.3 \times 10^{-10}$ s with Kivelson's formula[17]). The spectrum was not modified by addition of equimolar concentration of **CBA** (Figure 5A′) which indicates the absence of interaction between the two mole-

cules at the weak concentrations of the experiments.[18] Addition of a slight excess of the abasic undecamer duplex **D** (1.8×10^{-5} M) to the nitroxide probe (1×10^{-5} M) caused large modification in the observed spectrum (Figure 5B). It corresponds to the sum of the spectra of the two species, the radical **ATAc-NO•** free in solution that accounts for about 10%, and the radical immobilized at the abasic site (≈90%). The spectrum characteristic of the latter species indicating slower movement of the radical (from which a correlation time $\tau_c = 6 \times 10^{-9}$ s was determined with Kuznetsov's formula[19]) was obtained by subtraction of the spectrum corresponding to the radical free in solution from spectrum 5B (Figure 5C). On addition to this probe–DNA solution of a double excess of **CBA** relative to nitroxide, a new spectrum was obtained (Figure 5B′) characterized by considerable increase of the contribution of the radical free in solution (ca. 60%). **CBA** thus shows the ability to displace the labeled **ATAc-NO•** from the abasic duplex. By contrast the EPR spectrum of probe **ATAc-NO•** in the presence of the parent natural duplex **E** was not modified on addition of **CBA**. This result demonstrates that there is neither association nor intercalation between the base pairs.

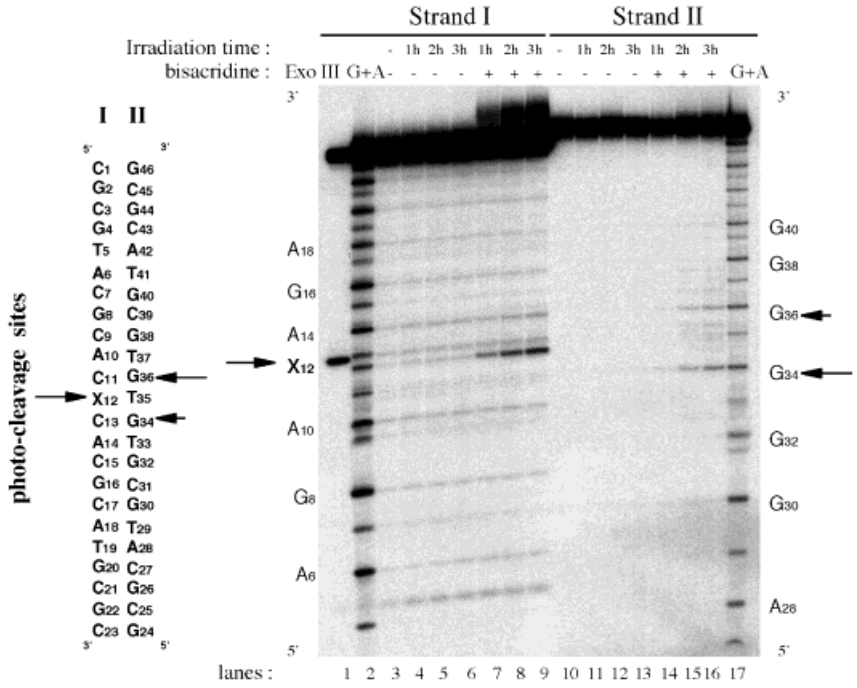

Figure 6. Autoradiograms of 20% denaturing polyacrylamide gel showing cleavage of the ^{32}P-end labeled 23-mer duplex **C** in the absence and in the presence of **CBA**. The reaction mixture contained the duplex **C** (5 pmol) in sodium phosphate buffer (pH 7, 10 mM), EDTA (1 mM), NaCl (20 mM) in a total volume of 10 μL. Lane 1: duplex incubated with exonuclease III to localize the abasic lesion; lanes 2 and 17: G + A sequence markers for strands I and II; lanes 3 and 10: nonirradiated strands I and II in the absence of **CBA**; lanes 4–6 and 11–13: duplex **C** irradiated in the absence of **CBA** for 1, 2 and 3 hours; lanes 7–9 and 14–16: duplex **C** irradiated in the presence of **CBA** (4 μM) for 1, 2, and 3 hours. Following irradiation the samples were treated with piperidine (1M) for 15 min at 90 °C.

Photocleavage of the abasic duplex C by CBA: The acridine subunits of **CBA** are photoactive residues capable of cleaving DNA strands upon irradiation.[20] Additional information relative to the binding specificity of **CBA** could thus be obtained from photocleavage experiments with the 23-mer duplex **C** that contains the stable tetrahydrofuranyl analogue of the abasic site in the middle of the sequence. The two strands of the duplex were successively ^{32}P-labeled at the 5′-ends. Irradiation of the DNA duplex in the presence of four equivalents of **CBA** induced alkali labile modifications revealed by piperidine treatment (Figure 6). One sample was treated by exonuclease III to localize the position of the abasic site in the duplex on the gel (exonuclease III from *E. coli* is a class II endonuclease involved in base excision repair (BER) of damaged bases). It incises DNA at the abasic site by hydrolyzing the phosphodiester bond 5′ to the lesion. It also recognizes and cleaves the tetrahydrofuranyl stable analogue of the abasic site.[15] The autoradiogram of the denaturating polyacrylamide gel (Figure 6) shows specific DNA cleavage on the two strands. The strand that carries the abasic site gives one single major labeled fragment corresponding to cleavage at the abasic position. Two photocleavage sites are observed in the complementary strand, a major one G_{34} and a minor G_{36}, that is the two guanine residues that flank the unpaired thymine T_{35} opposite the lesion. These results provide strong indication for specific binding of the bisacridine drug at the abasic site prior to reaction. However, the selectivity and the efficiency of cleavage suggest close contact between the photoactive subunits of **CBA** and the nucleobases at the abasic site. These observations are in full agreement with the results of a high-field NMR study of the interaction between bisacridine and abasic duplex **D**.[21] In the major complex formed one acridine subunit intercalates between base pairs C_{13} G_{34} and A_{14} T_{33} while the second subunit inserts inside the abasic pocket pushing aside the thymine T_{35} residue in the opposite strand. This scheme could account for major cleavage at guanine G_{34} that is "sandwiched" between the two acridine rings of the drug.

Conclusion

Combination of thermal denaturation, EPR spectroscopy, and photocleavage experiments clearly demonstrate the high specificity of the binding of the cyclobisacridine **CBA** at the abasic site examined, that is an apurinic site opposite a thymine residue flanked by two cytosines. Further studies are going on to determine the generality of the observations and

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evaluate the cyclobisacridine system as potential inhibitor of the AP-endonucleases involved in repair of the abasic site lesions.[8]

Experimental Section

Materials: The **CBA**[10], **ATAc**,[7] and **ATAc-NO•**[16] molecules were prepared as previously reported. T4 polynucleotide kinase was purchased from Pharmacia Biotech (9500 Units mL^{-1}), [γ ^{32}P-] ATP (specific activity 3000 Ci mmol^{-1}) from Isotopchim and Uracil DNA Glycosylase from Gibco BRL Life Technologies. The 11-mer and 25-mer oligonucleotides containing the tetrahydrofuranyl analogue of the abasic site were prepared according to the procedure described by Takeshita et al.[15] The conventional nonmodified oligonucleotides were purchased from Eurogentec. The 23-mer oligonucleotide containing an abasic site at position 12 was generated by treatment of the precursor 23-mer duplex containing an uracyl residue at position 12 by Uracyl DNA Glycosylase (Ura Gly). The duplex precursor (duplex **B**) was incubated with Ura Gly in HEPES (pH 8, 66 mM), containing NaCl (50 mM), $MgCl_2$ (5 mM) and dithiothreitol (DTT) (1 mM). Sufficient enzyme quantity was added to effect almost complete removal of uracil within 1 h at 37 °C. The formation of the abasic site was controlled by quantifying the cleavage product obtained by treating an aliquot of the reaction mixture by NaOH (0.5 M) for 10 min at 90 °C (data not shown). Oligodeoxynucleotides were labeled at the 5′-end by treating with [γ ^{32}P-] ATP and polynucleotide kinase at 37 °C for 30 min. ^{32}P-labeled oligodeoxynucleotides were then annealed to complementary strand by incubation to 70 °C for 5 min and at room temperature for 1 h.

Melting temperature measurements: The absorbance versus temperature for duplexes **E** and **D** was measured at 260 nm on a Lambda 5 UV/Vis spectrophotometer equipped with a Perkin–Elmer C570-070 temperature controller and interfaced with a EPSON AX2e computer. UV absorbance monitored melting experiments were performed at a strand concentration of 7.69 μM in buffer solution containing sodium phosphate (pH 7, 10 mM), EDTA (1 mM), and NaCl (20 mM). Before each melting experiment, samples were heated for 5 min at 80 °C and cooled down slowly to make sure that the starting oligonucleotides were in the duplex state. Samples were heated at a rate of 1.0 °C min^{-1} (starting from 2 °C to 80 °C) while the absorbance at 260 nm was recorded every 1 min. Melting curves were analyzed with a two-state model.[22] Melting temperatures (T_m′s) were determined by using the procedure described by Breslauer,[23] in which α is defined as the fraction of single strands in the duplex. Thus any experimental absorbance versus temperature curve can be converted into an α versus temperature profile. From this curve the melting temperature T_m is defined as the temperature at which $\alpha = 50\%$.

Photocleavage of duplex C: The reaction mixtures contained the 5′-^{32}P-labeled duplex **C** (5 pmol) in sodium phosphate buffer (pH 7, 10 mM), EDTA (1 mM), and NaCl (20 mM) in a total volume of 10 μL. In the experiments performed in the presence of **CBA**, the final drug concentration used was 2 μM. The solutions were irradiated both in the absence and in the presence of **CBA** for 1, 2, and 3 h with a mercury–xenon lamp (Oriel, 200 W) with a KNO_3 solution filter (2 M, 6 cm optical path, $\lambda >$ 340 nm). The samples were successively lyophilized, dissolved in piperidine (1 M, 10 μL) and heated for 30 min at 90 °C. The oligonucleotides were then precipitated in ethanol and the reaction products were dissolved in formamide (95%, 10 μL) dye solution and analyzed by gel electrophoresis (19:1 ratio of acrylamide to bisacrylamide) by using a TBE buffer (Tris-borate (pH 8.3, 89 mM), EDTA (2 mM)). The DNA fragments were visualized by autoradiography with a Kodak X-OMAT AR film.

Cleavage of the abasic site containing duplex A: Cleavage of duplex **A** by **ATAc**, and **CBA** was examined by adding the drugs to the incubation mixture containing the in situ generated abasic duplex. Various quantities of the drugs corresponding to ratios r = [drug]/[duplex] in the range 0–1 in total volumes of 10 μL were added to aliquots containing labeled duplex (5 pmol). After 15 min incubation at 37 °C, the reaction was quenched by addition of a stop solution (20 μL) which contained sodium acetate (pH 4, 70 mM), glycerol (80%), xylene cyanol (0.05%), bromophenol blue (0.05%). Fractions of 4 μL of the final solution were subjected to polyacrylamide gel electrophoresis (20% polyacrylamide gel) in TBE buffer (Tris-borate (pH 8.3, 89 mM) and EDTA (2 mM)). Visualization of the labeled substrate on the gels was achieved by autoradiography with a Kodak X-OMAT AR film and quantitative analysis was performed by using an Agfa studio Scan II Si scanner and the NIH Image 1.52 program.

Electron paramagnetic resonance (EPR) measurements: The EPR spectra were performed on a Bruker ESP 300 E spectrometer operated in the continuous wave (CW) mode. For oligonucleotide binding studies, compounds **CBA** and **ATAc-NO•** were solubilized in DMSO and then diluted in the buffer containing Tris-HCl (pH 7, 25 mM), NaCl (0.1 M), and EDTA (0.2 mM); the final concentration of DMSO was 2%. Substrate concentrations used for measurements were: Duplex oligonucleotide **D** (1.8×10^{-5} M), **CBA** (1×10^{-5} M or 2×10^{-5} M), and **ATAc-NO•** (1×10^{-5} M). Analysis of spectra corresponding to several species was monitored with the Compare Menu of ESP 300E: When the spectrum of a mixture of species possessing different mobilities was recorded, the pure spectra could be obtained by successively subtracting the individual pure species from the spectrum of the mixture.

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