Abasic Site Recognition in DNA as a New Strategy To Potentiate the Action of Anticancer Alkylating Drugs?

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Inhibition of abasic site repair in the cell seems an attractive strategy to potentiate the action of antitumor DNA alkylating drugs. Molecules that bind specifically and strongly to the abasic site are possible candidates to achieve such inhibition. We explored this strategy by preparing molecule **4** that incorporates (1) an aminoacridine intercalator for DNA binding, (2) an adenine moiety for abasic site recognition, and (3) a linker containing two guanidinium functions to increase binding to DNA without inducing cleavage at the base-sensitive abasic site. Compound **4** was compared to analogues containing secondary amines, i.e., **1**. We report on synthesis of the new heterodimer **4**. We show by physicochemical studies—including determination of association constants with calf-thymus DNA, T_m measurements, and high-field NMR examination of the complexes formed with abasic DNA duplexes—that **4** binds specifically and more strongly to the abasic site than the analogues. Compound **4** does not cleave abasic plasmid DNA. Compound **4** shows apparent synergy with the anticancer bischloroethylnitrosourea (BCNU) in L1210 and A549 cell lines in vitro. It potentiates BCNU in the in vivo tests. The results favor the pertinence of the strategy.

Introduction

Many antitumor drugs display their toxic effects through covalent binding to DNA.[1] Such modifications which are usually repaired by specific enzymes can become mutagenic or lethal if DNA integrity is not fully restored, e.g., in the case of therapeutic exposition to relatively high doses, thus producing multiple lesions and saturating the DNA repair machinery.[2] However, inducible DNA repair enzymes, such as O-6 methyl guanine transferase, have been identified as major sources of resistance to alkylating drugs.[3] For this reason, inhibition of DNA repair comes out as an attractive strategy to potentiate chemotherapeutic alkylating drugs.[4]

Different enzymes involved in DNA repair have been targeted. O^6-Alkylguanine-DNA transferase inhibitors were shown to potentiate alkylnitrosoureas and alkyltriazenes known to produce O^6-alkylguanine cytotoxic lesions.[5] Inhibitors of DNA polymerase significantly enhanced cell killing by L-phenylalanine mustard (L-PAM) or a combination of DNA-damaging agents ThioTEPA and Cisplatin.[6] Inhibition of poly(ADP-ribose)polymerase, a key enzyme in the repair process of DNA strand breaks, also gave encouraging results.[7]

Abasic sites which result from the loss of a base in DNA, either spontaneously[8] or enzymatically as intermediates in the repair of the modified bases,[9,10] appear as the most common damages induced by chemotherapeutic DNA alkylating agents. AP-endonucleases which recognize and cleave DNA at these sites in the first stages of the repair process, are interesting target candidates for potentiation of such drugs. So far, methoxyamine,[11] 9-aminoellipticine (9-AE) and isopropyloxazolocarbazole (Ipr-OPC)[12,13] have been proposed as AP-endonuclease inhibitors. Tested on *Escherichia coli*, 9-AE and Ipr-OPC displayed synergistic effects with toxic doses of dimethyl sulfate (DMS). Abasic sites are involved, but the precise role of the drugs remains unknown. Recently, DMP-840, a bisnaphthalimide derivative, was reported as an AP-endonuclease inhibitor and went in phase II anticancer clinical trials.[14] To date, no potentiation effect of this drug has been identified.[15] In addition, very recently, Pommier[16] and Osheroff[17−20] reported that the activities of topoisomerases I and II were dramatically influenced by genomic damages such as abasic sites.[21] When located within the cleavage overhang for topoisomerase I or immediately adjacent to the point of scission for topoisomerase II, abasic sites enhance enzyme-mediated DNA cleavage. It has been suggested that increasing topoisomerase-generated DNA breaks could trigger mutagenic effects and ultimately initiate apoptosis pathways.

Inhibition of abasic site repair thus seems a rational strategy to be explored in the search for new anticancer drugs active per se or in synergy with alkylating drugs. In this paper we disclose our first results along this strategy. Molecule **4** includes the structural moieties that proved highly successful in the design of the AP-endonuclease mimics **1** and **2** that have been shown to selectively recognize the AP-site and cleave quite efficiently (cleavage at nanomolar concentrations).[22−24] High-field NMR studies[25] of the complex formed by the two drugs **1** and **2** with a duplex undecamer containing a stable analogue of the abasic site have revealed that

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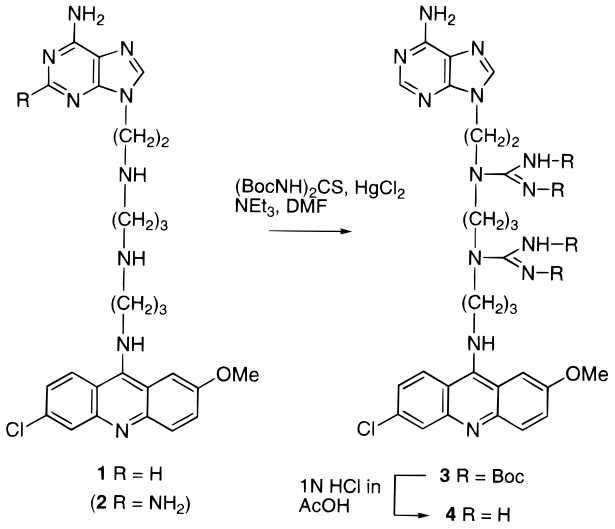
Scheme 1



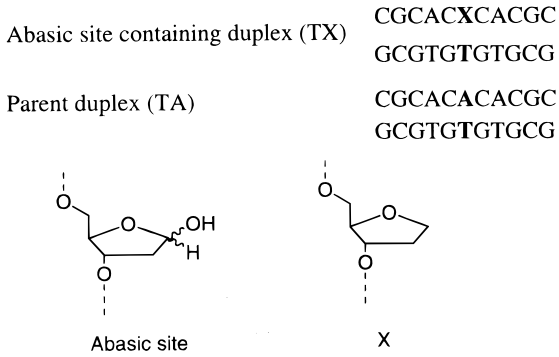
the drugs perfectly fit the abasic site (the acridine intercalates at a two base pairs distance 5′ to the abasic site, the base docks inside the abasic pocket, and the chain lies in the minor groove). The binding was confirmed by an EPR study.[26] The ability of **1** and **2** to inhibit the base excision repair system was demonstrated.[27] Tested on L1210, compounds **1** and **2** potentiate the cytotoxicity of a nitrosourea (*N*,*N*′-bis-(2-chloroethyl)nitrosourea, BCNU) in a concentration dependent fashion.[27] These first biological data being promising, we extended the synthesis of abasic site selective molecules to compound **4**. In molecule **4**, two guanidinium functions have been introduced to increase ionic interaction with DNA and eliminate the cleavage activity of the secondary amines in the nuclease mimic **1**. We report on the properties of molecule **4** designed to bind specifically and strongly to the abasic site thereby blocking the access of the lesion to the repair enzymes and consequently inhibiting the repair activity. We report on its binding to abasic DNA. We show that **4** promotes the activity of the anticancer drug BCNU in L1210 and A549 cancer cells.

Results and Discussion

Synthesis. Compound **4** was prepared in two steps by "post-functionalization" of the hybrid molecule **1** already described (Scheme 1).[22] The two aliphatic amines of the linker in **1** reacted with the reagent developed by Iwanowicz et al.,[28] the bis-*tert*-butoxycarbonythiourea. The protected bis-guanidine derivative **3** was thus obtained after purification in 52% yield. Deprotection was achieved by acidic treatment (1 N hydrochloric acid in acetic acid), and **4** was isolated as a tetrahydrochloride in 94% yield. This "post-functionalization" strategy appeared remarkably selective and efficient.

Interaction with Calf-Thymus DNA. Binding to DNA is a prerequisite condition for molecules targeting abasic sites. The affinity constants for native calf-thymus DNA of **4** was measured using competition experiments with ethidium bromide (EB). Studies on depurinated DNA are precluded because of inherent instability of the abasic sites that easily undergo strand scission. As expected, the new compound **4** interacts

Scheme 2



strongly with native DNA ($K = 2 \times 10^6$ M^{-1}). The number of covered sites is two, in agreement with the rule of the exclusion site for monointercalators. A significant result is the 10-fold increase of the association constant observed for **4** ($K = 2 \times 10^6$ M^{-1}) as compared to **1** ($K = 2 \times 10^5$ M^{-1}) when the two secondary amines of the linker in **1** are substituted for guanidines in **4**. This can be explained by the higher pK_a's of the guanidine functions (pK_a > 11) which are fully protonated at pH 7 as compared to the secondary amines in **1** that were shown to be only partly protonated at pH 7 (pK_a = 9.8 and 6.7),[24] thus contributing more significantly to the binding by ionic interactions with the negatively charged phosphates of DNA.

Interaction with AP-Site Containing Oligonucleotide. I. Thermal Denaturation Study (T_m Measurements). In a preceding paper,[29] we suggested that denaturation study (T_m measurement) constitutes a method of choice to assay binding of drugs at abasic sites. In these experiments, we used synthetic oligonucleotides containing a stable analogue of the lesion, i.e., the deoxyribose moiety was replaced by the chemically stable 3-hydroxy-2-(hydroxymethyl)-tetrahydrofuran (designated as X).[30,31] The sequence of the abasic site containing duplex (TX duplex) and that of the corresponding natural duplex in which X is replaced by adenine (TA duplex) are shown in Scheme 2. The TX duplex contains an apurinic site in which the base opposite the lesion, i.e., a thymine, is susceptible to pair with the adenine base of the synthetic drugs.

The thermal denaturation studies were investigated for the TA and TX duplexes in the presence of increasing concentrations of compounds **1** and **4**. To compare the relative binding affinities of the drugs, we used ΔT_m measurements, where $\Delta T_m = T_m$ (duplex in the presence of drug) $- T_m$ (duplex alone). Results are given in Figure 1. As reported previously,[29] in the absence of drug, the abasic lesion leads to a 19 °C decrease of the duplex stability (TX duplex T_m = 37 °C, TA duplex T_m = 56 °C).

1. Influence of the Drugs on the Stability of the TA Duplex. The two compounds significantly stabilize the TA duplex. The ΔT_m values increase regularly when the drug to DNA ratio *r* is increased. The curves (Figure 1) observed for the two compounds are comparable in terms of shapes and magnitudes, showing no important variations in the slopes. This suggests the absence of specific stoichiometries for the drug−DNA complexes. This corresponds to the type of curve expected for compounds that include in their structure a strong nonsequence specific intercalator.

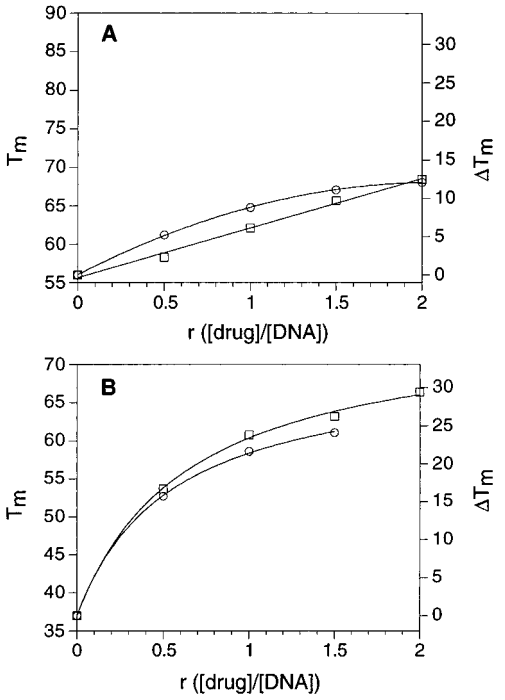


Figure 1. Influence of the drugs on the T_m and ΔT_m of the TA (panel A) and TX (panel B) duplexes. Experiments were conducted at pH 7.0 in a buffer containing 10 mM sodium phosphate/1 mM EDTA/20 mM NaCl. The drugs tested were: (○) **1** and (□) **4**. ΔT_m is defined as the difference between the T_m of the duplex in the presence of the drug and the T_m of the duplex alone. T_m values were measured for various ratios r of drugs (r = [drug]/[Duplex]).

2. Influence of the Drugs on the Stability of the TX Duplex. Again the two molecules show comparable behavior that differs considerably from what was observed in the interaction with the parent TA duplex.The magnitude of the stabilization as reflected by ΔT_m is much higher at all drug to DNA ratios r. The shapes of the curves (Figure 1) that relate the increase of the melting temperatures to the amount of added drugs also are different. At low drug to DNA ratios ($r < 1$) the slopes of the curves are steep. These slopes decrease at ratios superior to 1 ($r > 1$), suggesting preferred formation of 1:1 complexes between the drugs and the abasic duplex at low drug to DNA ratios. This indeed had been definitively established for molecule **1** by high-field NMR study of the interaction.[25]

It thus appears that molecule **4** may be well-suited for association to the abasic site. We thus undertook a high-field NMR study of the complexation with the abasic DNA duplex to determine the binding mode, as replacement of the two amines in the linking chain in **1** by guanidines in **4** may considerably modify the geometry of the complex.

II. High-Field NMR Study of the Complexes Formed between Drug 4 and the Abasic TX Duplex. The interaction of compounds **1** and **2** with the TX duplex has been previously examined by high-field NMR and molecular modeling.[25] The same methodology was applied to the study of the interaction of **4** with the same TX duplex. Known amounts of **4** were progressively added to the sample containing the duplex until a 1:1 ratio was reached. ^{1}H NMR spectra indicated that 1:1 complexes were formed. Inspection of the two-dimensional NMR spectra (TOCSY and NOESY) revealed that the solution contains several complexes in equilibrium. As evaluated from the peak intensities, one major complex accounted for 50% of the total, accompanied by several minor complexes. The presence of competing complexes resulted in dramatic line broadening and cross-peaks overlap which precluded precise proton assignment. However, by comparison with the **1**−DNA complex previously described[25] general features can be drawn for the major **4**−DNA complex. We noticed that the acridine ring protons move upfield by 1 ppm as compared to the free drug (Table 1), suggesting an intercalative binding mode. As described earlier for the **1**−DNA complex, the methoxy group of the acridine part displays two strong intermolecular NOEs in the 250 ms NOESY spectrum at 5.87 and 5.94 ppm, corresponding to C3-H1′ and A4-H1′, respectively. These correlations allow positioning of the acridine residue between C3.G20 and A4.T19, the methoxy group pointing toward the minor groove.

Table 1. ^{1}H Chemical Shift Assignment of Compound **4** in the Major **4**−DNA Complex[a]

proton	free	bound	$\Delta\delta$[b]
H1	7.68	6.97	−0.71
H3	7.75	6.98	−0.77
H4	7.77	6.12	−1.65
H5	7,88	6,96	−0,86
H7	7.60	6.36	−1.24
H8	8.37	7.11	−1.26
O−CH_3	4.14	3.63	−0.51

[a] H_2O is referenced at 4.97 ppm. [b] $\Delta\delta = \delta$(complex) − δ(DNA).

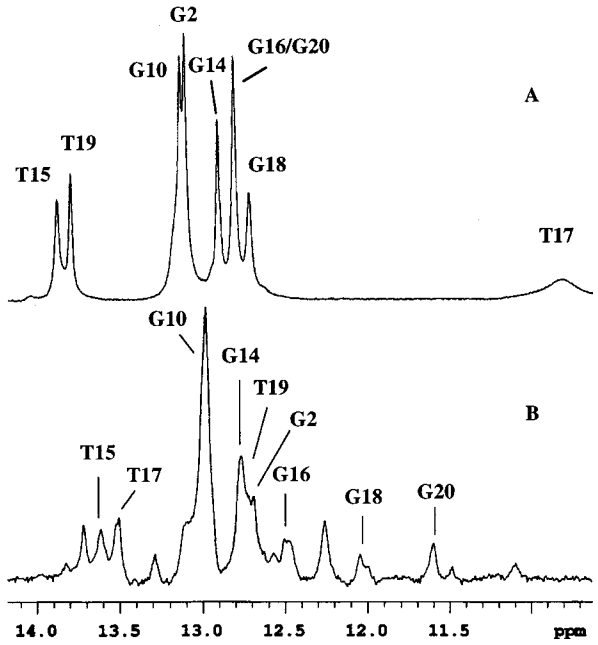


Figure 2. Imino proton spectra at 600 MHz of (A) abasic TX duplex and (B) 1/1 **4**−TX duplex in H_2O at 10 °C.

Analysis of the exchangeable protons allowed us to gain more insight into the characterization of the complexes. The NOESY-JR spectrum and particularly the region corresponding to the imino protons are well-resolved (Figure 2). The chemical shift values are given in Table 2. The cross-peaks characteristic of normal Watson−Crick base pairing are observed, i.e., correlations between the guanine iminos with the hydrogen-bonded and solvent-exposed cytosine amino protons and correlations between the thymine iminos and the ad-

Table 2. Chemical Shift Assignment of Imino Protons for the **4**−DNA Complexes[a]

base pair	major complex	minor complex
G2.C21	12.81 (−0.26)	13.20
C3.G20	11.78 (−1.1)	12.82
A4.T19	12.81 (−1)	13.82
C5.G18	12.07	12.38
X6.T17	13.62	13.62
C7.G16	12.59	12.59
A8.T15	13.72	13.72
C9.G14	12.82	12.82
G10.C13	13.20	13.20

[a] H_2O is referenced at 4.97 ppm. Values in parentheses represent chemical shift changes from free undecamer ≥ 0.10 ppm [$\Delta\delta = \delta$(complex) − δ(DNA)].

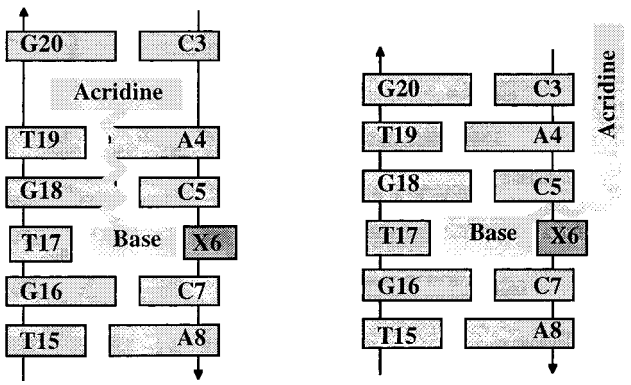


Figure 3. Schematic representation of the major (left) and minor (right) **4**−DNA complexes. In both complexes, the adenine of the drug is stabilized by Watson−Crick pairing with T-17.

enine H2 protons. Additional imino−imino NOEs between flanking base pairs are also noticed throughout the duplex. Sequential assignment could be performed for two complexes: one of them corresponding to the major complex, the other corresponding to one of the minor forms.

For the major complex, sequential connectivities could be followed from G2.C21 to G10.C13 with an interruption between C3.G20 and A4.T19. Moreover we noticed that the G20-H1 and T19-H3 are shielded by 1 ppm, indicating intercalation of the acridine chromophore between the two corresponding base pairs, which is in good agreement with the NOEs detected for nonexchangeable protons. Quite remarkably NOEs were observed between T17-H3 and the H2 proton of the adenine part of **4**, indicating Watson−Crick base pairing between the adenine moiety of the drug and the thymine residue that faces the abasic site in the opposite strand. We can thus conclude that compound **4** binds to the abasic site containing oligonucleotide in the same way as **1** (Figure 3). However, in the **1**−DNA complex, base pairing between T17 and the adenine of the drug could not be evidenced. This can be explained by a slower exchange process for compound **4** compared to **1**. Since the binding mode is similar for all these compounds, we can reasonably suppose that the lifetime of the complex is greater for compound **4** than for **1** or **2**.

For the minor complex, sequential connectivities were followed from G2.C21 to G10.C13 and revealed that no intercalation occurs in this form. However, as observed for the major complex described above, we detected NOE correlations between the adenine moiety of the drug (Ade-H2) and the unpaired thymine (T17-H3) of the

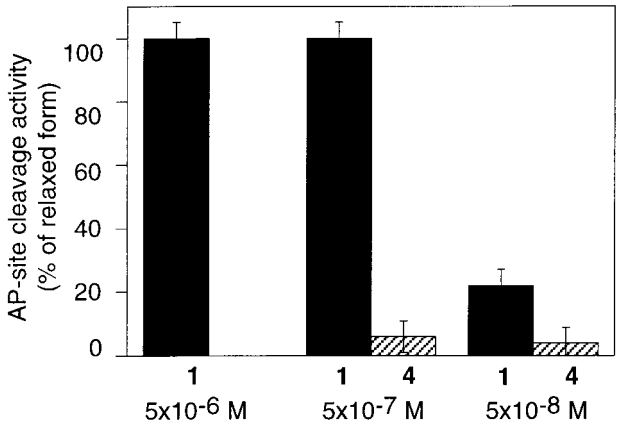


Figure 4. Cleavage of AP-pBR322 plasmid DNA at pH 7 and 4 °C in the presence of **1** (solid bar) and **4** (gray bar) tested at 5×10^{-6}, 5×10^{-7}, and 5×10^{-8} M concentrations. Plasmid DNA precipitates in the presence of **4** tested at 5×10^{-6} M concentration.

DNA duplex, suggesting again Watson−Crick base pairing. Therefore, the acridine moiety lies in a groove but its precise location could not be defined. Meanwhile, Watson−Crick base pairing indicates that the guanidine containing linker lies inside the minor groove.

Absence of Abasic Site Cleavage Activity for Molecule 4. Compound **1** was designed to act as "artificial nuclease", as the secondary amines in the linker, in their nonprotonated form, can induce base-catalyzed cleavage at the abasic site. On the contrary, **4** should be devoid of significant AP lyase activity as the two guanidines in the linker should be totally protonated at pH 7. We tested the guanidine analogue **4** on pBR322 plasmid containing an average of 2 AP-sites per plasmid.[22] The depurinated plasmid was incubated at 4 °C with various concentrations of drug. The drug was tested at three concentrations and compared to **1** tested at the same concentrations. As indicated in Figure 4, **4** does not cleave abasic sites significantly even at concentrations leading to 100% cleavage by **1**. These results are in agreement with our starting hypothesis that at pH 7 the guanidine functions, being protonated, are not able to catalyze the β-elimination reaction that leads to DNA strand breaks.

Pharmacological Studies. Cytostatic/cytotoxic activities of compounds **1** and **4** have been determined on the murine leukemia L1210 (ATCC CCL 219) and on the human pulmonary adenocarcinoma A549 (ATCC CCL185) cell lines. Concentrations inhibiting the growth (L1210) or the survival (A549) of the cells (IC_{50}) were measured. The molecules appeared marginally potent on L1210 cells (IC_{50} of 1.6 μM and 33 μM, respectively). Compound **1** displayed relatively high cytotoxicity on A549 cells (IC_{50} of 0.1 μM), while the bis-guanidinium compound **4** remained moderately active with an IC_{50} around 10 μM. Combination of the two compounds with bis-chloroethyl nitrosourea (BCNU), an alkylating antitumor drug which induces abasic sites in DNA, was investigated by measuring the plating efficiency of A549 cells under various conditions, e.g., simultaneously, sequentially with BCNU followed by the tested substances, or the reverse. The best results were obtained by simultaneously exposing the cells to BCNU and the tested compounds for 24 h. Compounds **1** and **4** led to an apparent synergy. The effect is much pronouced with

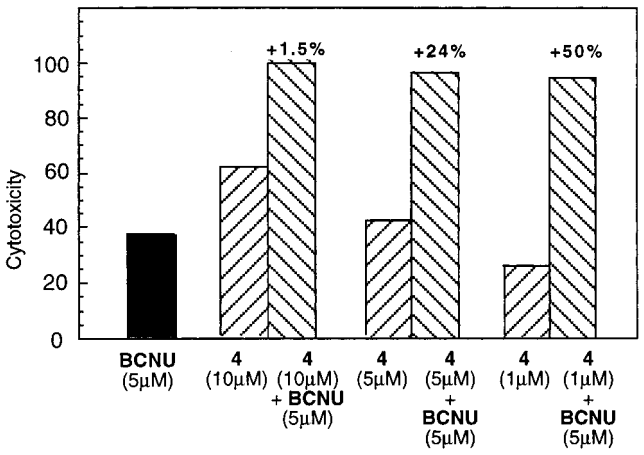


Figure 5. Cytotoxicity on A549 cells of BCNU (5 μM, solid bar) and of **4** (10 μM, 5 μM and 1 μM) alone (bar with lines slanted down to the left) or in association with 5 μM BCNU (bar with lines slanted down to the right). Cytotoxicity of **4**, associated with BCNU, appears significantly higher than the addition of the toxic effect of both drugs alone for 5 μM and 1 μM of **4** (24% and 50%), respectively.

Table 3. Activity of **4** Alone or in Association with BCNU on the P388 Murine Leukaemia

treatment	average survival (days ± sd)	T/C (survival of treated animals/ survival of controls) × 100	number of surviving animals at 60 days
controls	14.9 ± 8		0/7
4 (2 mg/kg)	16.2 ± 3	109	0/6
BCNU (6 mg/kg)	46 ± 17	309	3/6
4 (2 mg/kg) + BCNU (6 mg/kg)			6/6

4, for which the cytotoxicity at 10 μM, 5 μM, and 1 μM, in association with BCNU (5 μM), was significantly higher than the addition of the toxic effect of both drugs alone (increase of, respectively, 1.5%, 24%, and 50%) (Figure 5).

These results prompted us to test molecule **4** in vivo, on the murine leukaemia P388 system. This compound, which ellicits a curare-like acute toxicity with a maximal tolerated dose (MTD) around 5 mg/kg, was tested at half the MTD (2.5 mg/kg) in association with BCNU (6 mg/kg) and compared to BCNU alone. As shown in Table 3, **4** has no antitumor property; treatment with BCNU alone led to 50% of 60 days survival while association with **4** resulted in a 100% survival.

These results clearly demonstrated the potentiating effect of the tested compound **4**, confirming the pertinence of the strategy.

Conclusion

On the basis of a similar modular approach, two generations of abasic site specific drugs were successively designed. In previous work, we reported first-generation molecules **1** and **2** that act as AP-endonuclease mimics. They form specific complexes with abasic DNA model substrates as shown notably by NMR studies using a stable analogue of the abasic site inserted in a DNA duplex. They incise DNA at the abasic site at nanomolar concentrations by β-elimination reaction catalyzed by an unprotonated nitrogen of the diamino linker. The second-generation molecule **4** reported here has been designed as a potential inhibitor of abasic damage repair assuming that the presence of the bis-guanidinium linker between the adenine recognition unit and the acridine intercalator should increase the binding to abasic DNA, eliminate the cleaving activity, and hypothetically mask the access of the abasic lesion to the repair enzymes. The association constants and T_m measurements as described in the present work suggest a specific interaction of **4** at abasic sites. This is unambiguously confirmed by the NMR data, 1:1 complexes are formed involving docking of the adenine part of **4** inside the abasic site. In particular, the Watson−Crick base pairing between the adenine moiety of the drug and the thymine opposite the abasic site as observed both in the major and the minor complexes is quite significant. This was not observed previously in the specific complexes formed with **1** and **2**. This is good indication for stronger binding of **4**. As expected, molecule **4** does not cleave plasmid abasic DNA. It turns out that this same molecule **4** exhibits the most interesting in vitro and in vivo activities. It shows apparent synergy with BCNU in the murine leukaemia L1210 and human pulmonary adenocarcinoma A549 cell lines. It potentiates the effect of BCNU in the in vivo experiments. Those data cannot be interpreted in terms of mechanism of action due to the extreme complexity of the system. They do not prove the validity of the hypothesis; however, we can conclude that targeting the abasic site by specific drugs may be a pertinent and useful strategy to potentiate the activity of anticancer drugs.

Experimental Section

Melting points were determined using a Reicher Thermovar apparatus and were uncorrected. Except when otherwise mentioned, NMR spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer or at 400 MHz on a Varian 400 Unity using solvent as the internal reference (dimethylsulfoxide-d_6 at 2.49 ppm); the chemical shifts are reported in ppm, in δ units. The mass spectra were recorded on a Varian Mat 311 and AET MS 30 instruments. High-resolution mass spectra were obtained from "Centre Regional de Mesures Physiques de l'Ouest", Université de Rennes. Absorption spectra were obtained on a Perkin-Elmer Lambda UV/vis spectrometer. Microanalyses were performed by the "Service Central de Microanalyses du CNRS", Lyon.

Compound **1** has already been described.[22,32]

6-Amino-9-[3,7-bis(*N*,*N*′-bis-*tert*-butoxycarbonyldiaminomethylene)-11-(6-chloro-2-methoxyacridin-9-yl)-3,7,-11-triaza-undecyl]-9*H*-purine 3. To a solution of compound **1** (0.206 g, 0.38 mmol) in DMF (250 mL) cooled to 0 °C and kept under nitrogen were successively added *N*,*N*′-bis-*tert*-butoxycarbonylthiourea (0.267 g, 0.96 mmol), mercuric chloride (0.262 g, 0.96 mmol), and triethylamine (0.117 g, 1.16 mmol). The mixture was stirred for 4 h, and a large excess of triethylamine was added (2 mL). After the mixture was stirred for 4 h, the solvent was evaporated under reduced pressure and the residue chromatographed on silica gel (elution dichloromethane/methanol, 9/1 then 8/2). The desired compound **3** was isolated in 52% yield (0.205 g, 0.2 mmol) as a hygroscopic solid: mp 220−225 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 9.62 (2H, s, 2 NHBoc), 9.23 (1H, s, Acr-NH), 8.28 (1H, s, AdeH-2 or H-8), 8.09 (1H, d, J = 9.35 Hz), 8.01 (1H, d, J = 1.9 Hz), 7.94 (1H, s, AdeH-8 or H-2), 7.82 (1H, d, J = 9.35 Hz), 7.50 (1H, d, J = 2.15 Hz), 7.17 (1H, dd, J = 9.35 and 1.9 Hz), 7.08 (1H, dd, J = 9.35 and 2.15 Hz), 6.01 (2H, s, NH_2), 4.49 (2H, m, Ade-CH_2), 4.10 (2H, m, AcrNH-CH_2), 3.90 (5H, m, OCH_3 and CH_2), 3.61 (2H, m), 3.29 (4H, m), 2.28 (2H, m), 1.80 (2H, m), 1.45 (18 H, s, 2 *tert*-butyl), 1.39 (18 H, s, 2 *tert*-butyl); HRMS (positive FAB, NBA) calcd for $C_{49}H_{69}N_{13}O_9Cl$ 1018.5030, found 1018.5021.

6-Amino-9-[3,7-bis(diaminomethylene)-11-(6-chloro-2-methoxyacridin-9-yl)-3,7,11-triaza-undecyl]-9*H*-purine Tetrahydrochloride 4. Compound **3** (0.5 g, 0.49 mmol) was dissolved in a 1 N solution of hydrochloric acid in acetic acid (11 mL). After being stirred for 30 h at room temperature, the solution was poured into diethyl ether (100 mL). The yellow solid thus formed was filtered off and washed twice with diethyl ether. Compound **4** was crystallized from acetone/water and obtained in 94% yield (0.35 g, 0.46 mmol): mp 225−228 °C; ^{1}H NMR (200 MHz, D_2O) δ ppm 8.24−8.18 (3H, m, AdeH-2, AdeH-8 and Acr-H), 7.60−7.44 (5H, m), 4.38 (2H, m Ade-CH_2), 4.17 (2H, m, AcrNH-CH_2), 3.98 (3H, s, OCH_3), 3.71 (2H, m), 3.48 (2H, m), 3.21 (4H, m), 2.27 (2H, m), 1.67 (2H, m); ^{13}C (50 MHz, D_2O) δ ppm 159.3, 159, 158.9, 158.5, 153.8, 151.7, 149.2 (CH), 146.9 (CH), 143.7, 142, 136.7, 130.2 (2CH), 130.1, 127.3 (CH), 123.1 (CH), 120.8, 120.1 (CH), 116.3, 112.1, 105.5 (CH), 59.2 (OCH_3), 51.2 (CH_2), 49.5 (CH_2), 49.0 ($2CH_2$), 48.5 (CH_2), 44.4 (CH_2), 29.8 (CH_2), 27.0 (CH_2); HRMS (positive FAB) calcd for $C_{29}H_{37}N_{13}OCl$ 618.2932, found m/z = 618.2855; UV/vis (H_2O) λ_{max} (ϵ) 266.4 (45860), 277.3 (46020), 343.4 (4320), 422.6 (8240), 443.7 (7910) nm. Anal. Calcd for $C_{29}H_{36}N_{13}OCl\cdot 4HCl\cdot 5H_2O$: C, 40.78; H, 5.9; N, 21.32. Found C, 40.94; H, 5.74; N, 21.13.

DNA Binding Studies. DNA binding experiments were performed with Perkin-Elmer MPF-44A and LS50 spectrofluorometers in a thermostated quartz cell (25 °C). Excitation and emission were monitored at the ethidium bromide bands (520 and 600 nm). All solutions were made in 25 mM Tris-HCl, pH 7.0, 0.1 M NaCl, and 0.2 mM EDTA buffer. DNA concentration was 1.6×10^{-5} M (in base pairs), and that of tested drug was 2×10^{-5} M.

Preparation of DNA Containing Apurinic Sites (AP-pBR322 DNA). pBR322 DNA was dissolved in 25 mM sterilized acetate buffer (pH 4.9) at the concentration of 1 mg/mL and heated at 70 °C for 20 min.[33] This treatment introduced approximately 1.8 apurinic sites per DNA molecules.

Incision of AP-pBR322 DNA. AP-pBR322 DNA (0.25 μg) was incubated 4 °C for 20 min in the presence of the drug in 20 μL of reaction buffer (8.5×10^{-6} M in nucleotide phosphate units). The reaction was stopped by extracting the drugs with butanol saturated with water (100 μL), and the DNA was precipitated by ethanol (75% v/v) in the presence of sodium chloride (0.2 M final concentration). The nicked (form II) and supercoiled (form I) DNA molecules were separated by agarose gel electrophoresis (0.8%) using TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) as a migrating buffer and a constant voltage of 100 V for 2 h. After migration, the gel was stained by soaking in TBE containing ethidium bromide (0.5 μg/mL) and examined on a UV transilluminating table (312 nm). The gels were photographed with Polaroid MP4 camera (orange filter) and Polaroid 55-type films. The relative amount of the two forms of the plasmid were estimated from the negatives of the films which were densitized on a StudioScan IIsi (Agfa). Analysis was performed on a Macintosh computer using the public domain NIH Image program. The percentage of cleavage was calculated as follows: % cleavage = [amount of form II/(amount of form I + amount of form II)] × 100. The experiments were triplicated.

Thermal Denaturation Studies. Oligonucleotide Synthesis. Parent or modified (containing the stable abasic site analogue, tetrahydrofuran) oligodeoxyribonucleotides were synthesized using standard solid phase cyanoethyl phosphoramidite chemistry on a Milligen/Biosearch 8700 DNA synthesizer. The detritylated oligodeoxyribonucleotides were purified by anion exchange chromatography on a Pharmacia Mono Q HR 5/5 column and desalted on Waters Sepack cartridges. Oligonucleotide concentrations were checked spectrophotometrically by measuring the absorbance at 260 nm.

Preparation of the Solutions. Standard solutions of the DNA oligomers were prepared in pH 7 buffer (10 mM sodium phosphate, 1 mM EDTA, 20 mM NaCl).

UV Absorption Spectrophotometry. The absorbance versus temperature melting profiles of the Watson−Crick TA duplex and the modified TX duplex were measured at 260 nm on a Lambda 5 UV/visible spectrophotometer equipped with a Perkin-Elmer C570-070 temperature controller and interfaced with a Epson AX2e computer. All duplexes melting curves were measured at a strand concentration of 7.69 μM. Before each experiment, samples were heated for 5 min at 80 °C and cooled slowly to make sure that the starting oligonucleotides were in the duplex state. Samples were heated at a rate of 1 °C/min (from 2 to 80 °C) while absorbance at 260 nm was recorded every 1 min. Melting curves were analyzed with a two-state model. Melting points (T_m) were determined by using one of the procedures described by Breslauer,[34] in which α is defined as the fraction of single strands in the duplex. From the curve α vs temperature, the melting point T_m is defined as the temperature at which $\alpha = 50\%$.

NMR Study. Sample Preparation. The oligonucleotide duplex was chromatographed over chelex 100 resin to remove dicationic metal ions. The NMR sample was lyophilized once from water, twice from 99.96% D_2O, and finally dissolved in 600 μL of 99.99% D_2O containing 20 mM sodium phosphate buffer at pH 7.0. For NMR experiments involving exchangeable protons, the oligomer was dissolved in 90% H_2O /10% D_2O. The final duplex concentration was about 1.5 mM.

A dilute stock solution of **4** was prepared by dissolving 11 mg of the drug in 1 mL of 99.96% D_2O. Known amounts of **4** were added to the sample containing duplex DNA in 0.25 molar equivalent per increment, and the complete complex formation was monitored by 1D NMR.

NMR Experiments. NMR data sets were recorded on Varian Unity Plus 500 and Varian Unity 600 spectrometers at 10 °C. Chemical shifts were referenced to the chemical shift of water, which had been calibrated relative to the 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP-d_4). All 2D NMR experiments in D_2O were performed using the standard pulse sequences with presaturation of the residual HOD signal during the relaxation delay and during the mixing time for the NOESY. The NOESY spectra with mixing times of 250 ms were obtained using 2048 complex points in t_2, and 300 t_1 increments with 128 scans for each t_1 value. The TOCSY experiment with a mixing time of 60 ms was acquired with 2048 complex points in t_2, and 300 increments in t_1, with 64 transients for each FID. The NOESY in 90% H_2O (NOESY-JR) was carried out by replacing the first pulse of the standard sequence with a 270° composite pulse[35] and the observation pulse with a 1−1 jump and return pulse to suppress the solvent signal.[36] A total of 4092 complex points were collected in t_2, 300 points in t_1 and 128 scans for each FID. All the data were processed with a Gaussian window function prior to Fourier transformation and were zero-filled in t_1.

Biological Studies. In Vitro Assays. L1210 (mouse leukaemia, ATCC CCL 219) and A549 (human pulmonary adenocarcinoma, ATCC CCL 185) cells were cultivated in Dulbecco's MEM supplemented with 10% fetal calf serum at 37 °C in a water-jacketed CO_2 incubator (5% CO_2). L1210 cells were seeded at 10^5 cells/mL in 1 mL microwell plates. After 24 h (usually (3−4) × 10^5 cells/mL) tested compounds were added in duplicate at various concentrations and incubated for 24 h. Cells were counted with a Coulter-Counter ZM (Coultronics Inc.). The dose inhibiting the growth by 50% (IC_{50}) was extrapolated from regression curves obtained with experimental points without significant toxicity.

A549 cells were plated in 3 cm diameter multiwell plates (200 cells/well). After 24 h, tested compounds were added in duplicate at various concentrations, and cells were incubated for another 24 h. Cells were washed with phosphate buffered saline (PBS) and then reincubated for 14 days with fresh medium free of drug. Colonies were numbered after washing with PBS and staining with Giemsa. Survival was expressed as the percentage of untreated controls, and IC_{50} was extrapolated from regression curves obtained with experimental points.

In Vivo Testing. P-388 leukaemia was maintained in vivo by serial passage on female DBA/2 mice; ascitic P-388 cells were directly used for the tests. Female BDF_1 mice were

inoculated intraperitoneously (IP) with 10^6 cells (J_0) (seven controls and six animals in each test group). Test compounds were injected by the same way, as a single injection, 24 h later. T/C are expressed as the ratio of the mean survival time of treated animals to the mean survival time of the controls multiplied by 100. Surviving animals were sacrified at J_{60}.

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