Anti-Cancer Drugs 1999, **10**, pp. 55–65

Preclinical paper

Novel artificial endonucleases inhibit base excision repair and potentiate the cytotoxicity of DNA-damaging agents on L1210 cells

J-M Barret, C Étiévant, J Fahy,[1] J Lhomme[2] and BT Hill

Division de Cancérologie Expérimentale and [1]Division de Chimie Médicinale 5, Institut de Recherches Pierre Fabre, 81106 Castres Cedex, France. [2]LEDSS, Chimie Biorganique, UMR CNRS 5616, Université Joseph Fourier, 38041 Grenoble Cedex 9, France.

A series of molecules with apurinic/apyrimidic (AP) endonuclease activity targeted to abasic sites in DNA, which incorporate an intercalating moiety linked to a purine base by a polyamino chain and recognize and cleave abasic sites in DNA with high efficiency, has been studied. The aim was first to establish whether these compounds were inhibitors of base excision DNA repair, since abasic sites are generated during this process. Using an extension of a recently established methodology, two members of this series have been identified as definite repair inhibitors. Secondly, the potential of using such compounds as sensitizers of alkylating agents has been investigated by determining the cytotoxic activity of these compounds on L1210 cells in culture. A concentration-dependent potentiation of nitrosoureas has been demonstrated, but interpretation is complicated by the inherent cytotoxic properties of the test compounds themselves. Such molecules, however, provide interesting lead compounds for new strategies aimed at enhancing the cytotoxic potential of clinically useful DNA-damaging agents. [© 1999 Lippincott Williams & Wilkins.]

Key words: Abasic site, alkylating agents, artificial endonucleases, excision repair, potentiation.

Introduction

Resistance is one of the major factors which limits the long-term efficacy of anticancer drugs.[1] The emergence of resistance is known to be multifactorial, and the mechanisms involved vary depending on the drugs used and on the type of tumor cells being treated.[1,2] Amongst these mechanisms, certain DNA repair processes have now been implicated in cases of resistance to DNA damaging agents.[3] Base excision repair (BER) is one of the cellular processes used to repair damaged bases in DNA.[4] This process has been elucidated mechanistically and can be summarized in three steps (Figure 1): (i) recognition of damage and excision of the damaged base by a DNA glycosylase, creating an abasic site, (ii) breaking of the DNA strand at the abasic site by, essentially, the HAP1 enzyme, and (iii) neosynthesis by two possible mechanisms: the short-patch or the long-patch repair pathway. The short-patch repair pathway results in the replacement of one or two nucleotides and involves DNA polymerase β (pol-β), XRCC1 enzymes and a ligase (I or III). The long-patch repair, which results in the replacement of up to 10 nucleotides, involves FEN1, PCNA, a polymerase (β, δ or ε) and a ligase.[4] BER is known to repair certain damage induced by mono-alkylating agents.[5] However, the reaction of bis-alkylating agents with DNA produces intermediate mono-adducts, some of which may be repaired by the BER process.[5] Thus, a deficit in BER could result in cellular hypersensitivity to bis-alkylating agents, as observed by Engelward *et al.* with *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and mitomycin C.[6]

BER has not been as fully investigated in terms of its involvement in resistance to anticancer drugs, in comparison with other factors such as glutathione levels, the drug resistance associated P-glycoprotein, etc.[1] However, a recent study has shown that cells from mice deficient in 3-methyl DNA glycosylase, a DNA glycosylase used in BER, have increased sensitivity to alkylating drugs like BCNU and mitomycin C.[6] However, the involvement of DNA glycosylase in the resistance phenotype is controversial, depending of the approaches which have been used for studying its

Correspondence to J-M Barret, Division of Cancerologie Expérimentale, Institut de Recherches Pierre Fabre, 17 avenue Jean Moulin, 81106 Castres Cedex, France.
Tel: (+33) 5 63 71 42 11; Fax: (+33) 5 63 71 42 99;
E-mail: jean_marc.barret@pierre-fabre.com

association.[7-10] Results obtained with pol-β, the polymerase principally implicated in BER, have been more definitive, since it was found that pol-β was overexpressed in response to DNA-damaging agents,[11]

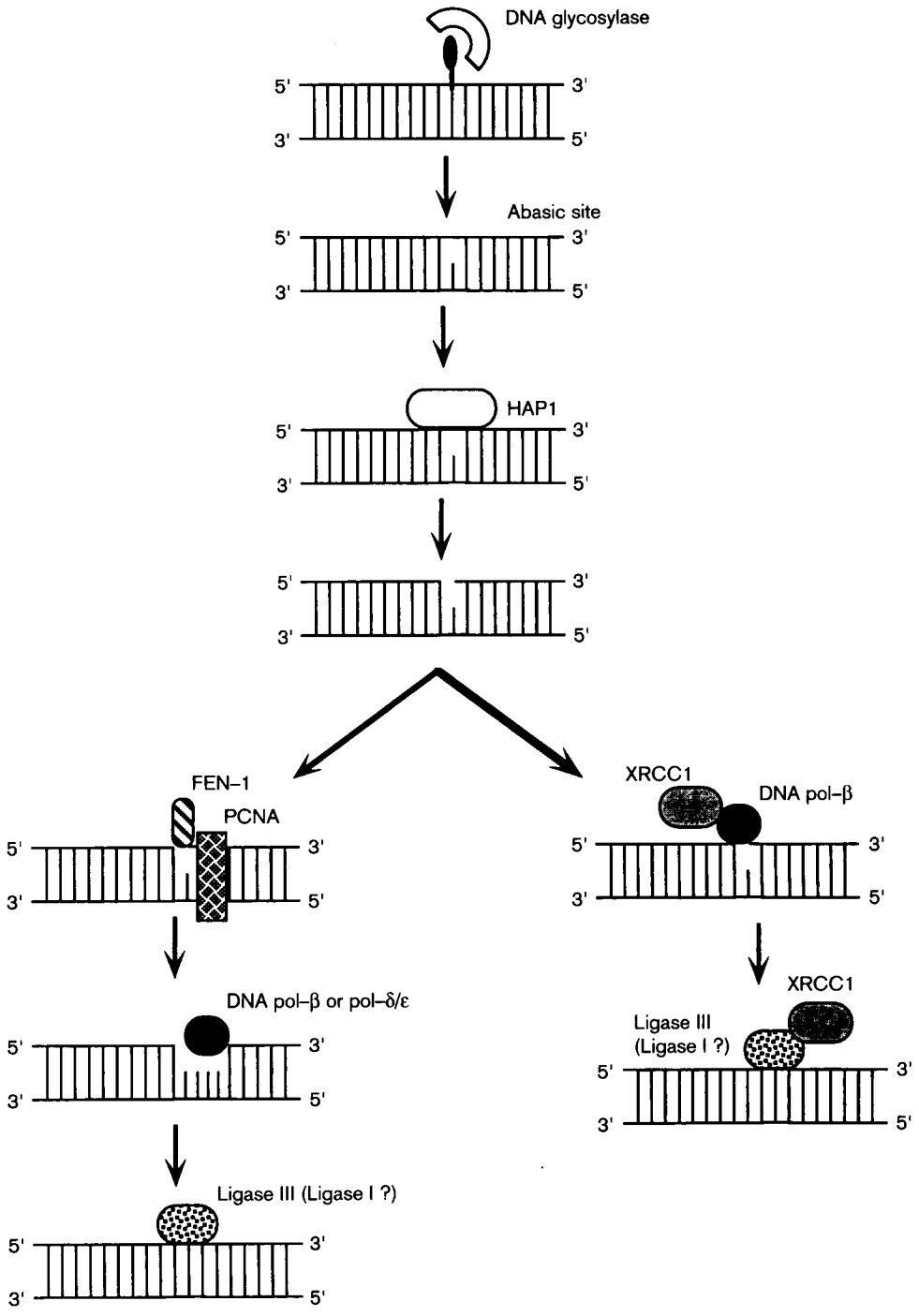


Figure 1. Schematic representation of BER. This process can be summarized in three steps: (i) recognition of damage and excision of the damaged base by a DNA glycosylase, creating an abasic site, (ii) breaking of the DNA strand at the abasic site by, essentially, the HAP1 enzyme, and (iii) neosynthesis by two possible mechanisms: the short-patch or the long-patch repair pathway. The short-patch repair pathway results in the replacement of one or two nucleotides and involves the pol-β, XRCC1 enzymes and a ligase (I or III). The long-patch-repair, which results in the replacement of up to 10 nucleotides, involves FEN1, PCNA, a polymerase (β, δ or ε) and a ligase.

and its overexpression has been observed in cells resistant to both mono- and bis-alkylating agents.[12-14] It has also been suggested that drug resistance may be associated with elevated levels of AP endonucleases, enzymes interacting at a crucial step of BER.[7,15]

With this background, therefore, studies have been initiated with the aim of exploiting inhibitors of enzymes involved in BER, with the long-term goal of reducing or overcoming the resistance phenomena associated with BER and/or potentiating the cytotoxicity of alkylating agents against tumor cells. Several proteins involved in BER have been identified as possible targets. Thus, pyrrolidine-based inhibitors have been successfully tested *in vitro* on bacterial (AlkA) and human (ANPG) DNA glycosylases.[16] Nucleotide analogs, such as 2',3'-dideoxycytidine (ddC) and 3'-azido-3'-deoxythymidine (AZT), have also been evaluated and found to inhibit DNA pol-β.[14,17] To date, no BER inhibitors which interact and inhibit apurinic/apyrimidic (AP) endonucleases have yet been described. However, a new concept of inhibition in relation to the AP site has emerged in the last few years: artificial endonucleases.[18-21] These compounds are constituted by three parts: a nucleic base for abasic site recognition, an intercalating agent (9-amino-acridine) to reinforce and stabilize the interaction with DNA, and a polyamino-chain linking the two moieties and which can cleave DNA by a β-elimination process.[22,23] These compounds could mask the abasic site to AP endonucleases and thus result in its inhibition. Moreover, the DNA cleavage produced by these compounds should drastically increase the cytotoxicity of DNA-damaging agents whose adducts are repaired by BER, since DNA cleavage from artificial endonucleases occurs irrespective of the co-regulation of proteins involved in the BER mechanism.

For these reasons, we have proposed that the compounds presented here might prove effective sensitizers of alkylating agents. First, therefore, we have verified the inhibitory activity of this series of artificial nucleases on BER using a recently described *in vitro* methodology[24] and then we have studied their effects in combination with various alkylating agents on the growth of L1210 cells in culture.

Materials and methods

Cells and chemicals

The murine lymphocytic leukaemia L1210 line was obtained from the ATCC (Rockville, MD). These cells were cultured in RPMI 1640 medium (Seromed, Polylabo, Strasbourg, France) supplemented with 10% heat-inactivated horse serum (Gibco, Cergy Pontoise, France), penicillin (2.0×10^{-5} U/l) and streptomycin (50 μg/ml), at 37°C in an humidified atmosphere of 5% CO_2 in air. Cells were regularly tested and found to be free of mycoplasma (Mycoplasma detection PCR kit; Stratagène, Montigny le Bretonneux, France). BCNU was purchased from Bristol-Meyers Squibb, thiotepa from Lederle (Peteaux, France), actinomycin D from Jansen (Noisy le Grand, France), cycloheximide from Aldrich (L'Isles d'Abau, France), aphidicolin, methoxyamine, streptozocin and mitomycin C from Sigma (L'Isles d'Abau, France). The artificial endonuclease series tested in this study (Table 1) were synthesized as described in previous publications.[20,22,25] All test compounds were solubilized in water prior to use, except for aphidicolin and actinomycin D, which were solubilized in 1% dimethyl sulfoxide.

Evaluations of the inhibition of BER using the 3-D assay kit (SFRI, St-Jean d'Illac, France)

The protocol of this assay was derived from a recently presented method of quantifying inhibition of DNA damage[26] and NER.[24] It can be summarized by the following four steps:

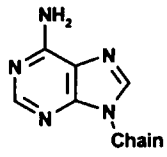
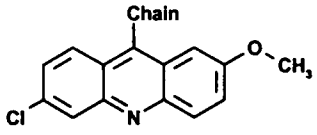
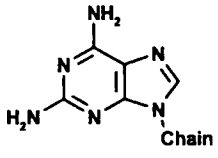
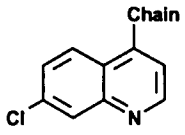
(i) *Plasmid DNA adsorption.* Fifty microliters of a solution of 1 μg/ml of undamaged pBS plasmids was distributed in each sensitized well and incubated for 30 min at 30°C in a microplate incubator (IEMS Labsystems, Cergy Pontoise, France) to permit adsorption. Any non-adsorbed DNA was then eliminated by three washes with PBS containing 0.1% Tween 20.

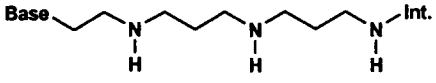
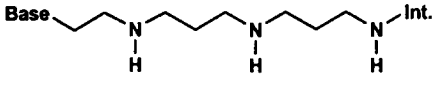
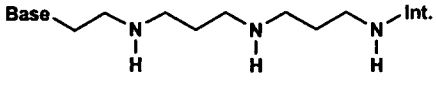
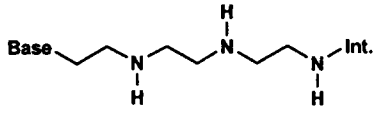
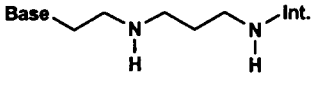
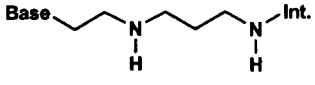
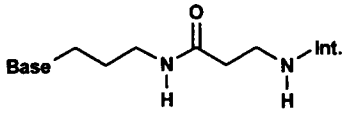
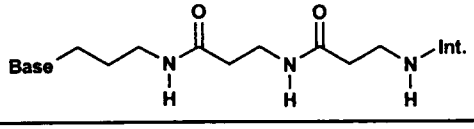
(ii) *Methyl methane sulfonate (MMS)-damaged plasmid preparation.* Fifty percent of wells containing undamaged plasmid were used as control and were not treated, whilst the other 50% was treated with MMS (26 mM) for a 30 min incubation. Any free molecules of MMS were then eliminated by three washes with PBS containing 0.1% Tween 20.

(iii) *DNA repair reaction.* The test compounds were incubated for 3 h at 30°C with a mixture containing all the constituents necessary for the *in vitro* DNA repair reaction.[24] During this reaction, damage on DNA was recognized and the excised long or short patches were replaced by neo-synthesized DNA fragments where digoxygenylated dUTPs were incorporated. The DNA repair reaction was stopped by three washes with PBS containing 0.1% Tween 20.

(iv) *Quantitation of DNA repair activity.* A solution containing an anti-digoxygenin antibody conjugated with alkaline phosphatase (diluted 1/10 000 in PBS

Table 1. Chemical structures of the test compounds

Base		Intercalator	
	**A**		**Ac**
	**D**		**Qu**

Compound	Base	Chain	Intercalator
D-Tn_1-Ac	**D**		**Ac**
A-Tn_1-Ac	**A**		**Ac**
A-Tn_1-Qu	**A**		**Qu**
D-Tn_2-Ac	**D**		**Ac**
D-Dn-Ac	**D**		**Ac**
A-Dn-Ac	**A**		**Ac**
D-Dd-Ac	**D**		**Ac**
D-Td-Ac	**D**		**Ac**

The nucleic bases, adenine (**A**) or 2,6-diaminopurine (**D**), are linked to the intercalating moiety, 3-chloro-7-methoxyacridine (**Ac**) or 7-chloroquinoline (**Qu**), by a polyamino (Tn_1, Tn_2 or Dn) or an amido (Dd or Td) chain.

plus 0.025% acetylated bovine serum albumin and 0.1% Nonidet P-40) was distributed in each well so that during a 30 min incubation at 30°C the digoxygenylated dUMP incorporated during the DNA repair reaction could be recognized. After five washes with PBS containing 0.1% Tween 20, a solution of Lumi-Phos 530 (lumigen), a substrate for alkaline phosphatase, was added for a 15 min incubation at 30°C. The light emitted by the dephosphorylated Lumi-Phos 530 was measured using a luminometer (Luminoskan; Labsystem) and expressed in relative light units (RLU). Under these experimental conditions, the luminometric signal was proportional to the digoxygenylated dUMP incorporated and thus to the DNA repair activity.

The experiments carried out with undamaged pBS plasmids permitted an evaluation of the background signal. These values were systematically subtracted from the corresponding values obtained using MMS-damaged plasmids. The results are defined as the *in vitro* DNA repair activity.

Evaluations of cytotoxicity using L1210 cells

L1210 cells were distributed into 24-well plates (Nunc, Polylabo) at 1.0×10^5 cells/ml and incubated with test compound or solvent (water) used as control. Forty-eight hours later, the surviving cells were counted using a cell counter (Coultronics, Mergency, France) and the quantification of cells expressed as a percentage of the mean control proliferation value.

Evaluation of the inhibition of DNA, RNA and protein synthesis in L1210 cells

Logarithmically growing L1210 cells were seeded at 1.0×10^5 cells/well in 24-well plates containing 1.35 ml of culture medium, detailed above. Various concentrations of test compound (0.15 ml/well) and 1-3 μCi of the respective tritiated marker of synthesis were added and incubated for 1 h at 37°C. Tritiated thymidine (Isotopchim, Le Peyruis, France), tritiated uridine (Isotopchim) and a mixture of tritiated amino acids (Amersham, Les Ulis, France) were used as markers of DNA, RNA and protein synthesis, respectively. After removal of medium, cells were transferred to glass fiber filters (GF/C Millipore, Molsheim, France). Acid-precipitable radioactivity was collected after 5 washes with 3×2 ml of 10% trichloroacetic acid and 2×2 ml of 95% ethanol. Radioactivity was assayed using Emulsifier-Safe (Packard, Rungis, France) in a scintillation spectrometer (250TR; Packard).

Results

The inhibitory activities of the artificial nucleases on BER were evaluated by the 3-D assay. This assay, recently used with UV-damaged DNA to identify NER inhibitors,[24] was performed in this study with MMS-damaged DNA. Damage introduced into DNA by MMS, a mono-alkylating agent, was principally repaired by the BER process.[27] Inhibition with this assay was apparent at higher drug concentrations than with the assay previously used to identify NER inhibitors.[24] This might be due to the shorter patch of nucleotides resynthesized with BER (2-10 nucleotide) compared to that of NER (more than 20 nucleotides). Thus, the probability of incorporating digoxygenylated dUTP was clearly lower with BER than with NER. Moreover, the efficacy of *in vitro* BER could be higher than that of *in vitro* NER, as suggested by Salles *et al.*,[26] which would implicate a systematic need for more drugs to inhibit *in vitro* BER than to inhibit *in vitro* NER so as to result in similar inhibitory activity.

However, this modified methodology rendered possible the confirmation of the hypothesis that certain compounds from this series may be inhibitors of BER. The results indicate that D-Tn_1-Ac and A-Tn_1-Ac (Table 2) were the most potent inhibitors of BER with IC_{50} values of 70 and 62 μM, respectively. These compounds were clearly more potent than methoxyamine, a known inhibitor of BER used as a reference,[28]

Table 2. Evaluation of *in vitro* DNA repair inhibition of test compounds or metoxyamine used as reference

Compound	Inhibition of DNA repair of MMS-damaged DNA [IC_{50} (μM)][a]
D-Tn_1-Ac	70
A-Tn_1-Ac	62
A-Tn_1-Qu	138
D-Tn_2-Ac	>500
D-Dn-Ac	100
A-Dn-Ac	156
D-Dd-Ac	>500
D-Td-Ac	>500
Metoxyamine	480

[a] *In vitro* BER inhibition was evaluated as described in Materials and methods with plasmids damaged by MMS. IC_{50} values correspond to the concentration (μM) of the compounds necessary to reduce DNA repair activity to 50% of that obtained with the solvent only. These values were derived from three independent experiments in triplicate.

Table 3. Effects of test compounds from the series of artificial endonucleases on the proliferation of L1210 cells, and on their overall synthesis of DNA, RNA and protein

Compound	Cytotoxicity on L1210 cells[a] [IC_{50} (μM)][b]	Inhibition of[a]		
		DNA synthesis [IC_{50} (μM)][c]	RNA synthesis [IC_{50} (μM)][c]	Protein synthesis [IC_{50} (μM)][c]
D-Tn_1-Ac	2.2	17	19	18
A-Tn_1-Ac	1.6	100	79	76
A-Tn_1-Qu	80	>100	>100	>100
D-Tn_2-Ac	38	>100	>100	83
D-Dn-Ac	28	68	78	94
A-Dn-Ac	14	45	55	49
D-Dd-Ac	70	>100	>100	>100
D-Td-Ac	>100	>100	>100	>100
Aphidicolin	ND	3.0	>100	>100
Actinomycin D	ND	7.0	0.006	>100
Cycloheximide	ND	13	19	0.38

[a]Note that the IC_{50} values reflecting cytotoxicity or inhibition of macromolecular synthesis should not be directly compared, since the durations of exposure to test compounds are dissimilar.
[b]IC_{50} values correspond to the drug concentration required for a 50% decrease of cellular proliferation measured in the presence of solvent only, following a 48 h *in vitro* incubation with the test compound. Values result from at least three experiments involving duplicate assays. ND, not determined.
[c]IC_{50} values correspond to the drug concentration required for a 50% decrease of the baseline rates of DNA, RNA or protein synthesis, following a 1 h *in vitro* incubation with test compound. Each compound was evaluated 2–3 times using duplicate estimation.

which had an IC_{50} value of 480 μM. The other compounds from this series though were found to be either less active (D-Dn-Ac and A-Dn-Ac) or inactive (D-Tn_2-Ac, D-Dd-Ac and D-Td-Ac), in terms of their inhibition of *in vitro* BER.

Before considering the use of D-Tn_1-Ac and A-Tn_1-Ac as inhibitors of DNA repair in combination with alkylating agents, their overall effects on mammalian cells were characterized and compared with those obtained with the other compounds of these series. As shown in Table 3, most of the compounds tested showed little (A-Tn_1-Qu, D-Tn_2-Ac and D-Dd-Ac) or no cytotoxicity (D-Td-Ac). The most cytotoxic compounds were D-Tn_1-Ac and A-Tn_1-Ac, with IC_{50} values of 2.2 and 1.6 μM, respectively. None of these compounds resulted in any specific inhibition of either DNA, RNA or protein synthesis. Indeed when inhibitory activity for one synthetic pathway was noted, the other pathways were found also to be inhibited to similar extents at comparable concentrations. On the other hand, aphidicolin, actinomycin D and cycloheximide exhibited clear specificity against, respectively, DNA, RNA and protein synthesis. Thus, results obtained with these artificial endonucleases suggest that the cytotoxicity observed with L1210 cells was not related to the endonuclease activity of these compounds, since such an activity would have led to a preferential inhibition of DNA and, eventually, of RNA synthesis. Such selectivity vis-à-vis DNA and/or RNA synthesis was classically representative of cytotoxic compounds which interact with, or damage, DNA.[29]

To confirm that D-Tn_1-Ac and A-Tn_1-Ac could sensitize cells to certain alkylating agents, the cytotoxicity of MMS in combination with these artificial nucleases has been evaluated against L1210 cultured cells. The second objective of such an evaluation was to confirm in cultured cells the repair inhibition of MMS-damaged DNA already observed *in vitro* with certain compounds. Initially, a non-cytotoxic concentration of the artificial nuclease was used and only the concentration of MMS was varied (Figure 2A). This non-cytotoxic concentration was defined in terms of the IC_{50} values identified, which were reduced by 1 log, e.g. 0.2 and 0.15 μM with D-Tn_1-Ac and A-Tn_1-Ac, respectively. At these low concentrations, no potentiation of the cytotoxicity of MMS was obtained with either D-Tn_1-Ac or A-Tn_1-Ac. Similar inactivity was noted with the other compounds of this series as shown, e.g. in Figure 2(A) with A-Tn_1-Qu. Nevertheless, definite potentiation of MMS cytotoxicity was identified with D-Tn_1-Ac and A-Tn_1-Ac when they were used at concentrations corresponding to their IC_{50} values reduced by a factor of 50%, i.e. 1.0 and 0.7 μM, respectively. Under these conditions, these concentrations of D-Tn_1-Ac and A-Tn_1-Ac, when used alone, resulted in around 10% cytotoxicity against these L1210 cells (Figure 2B). Thus, in the presence of MMS the IC_{50} value shifted from 350 μM with MMS alone to

around 10 μM with a combination of MMS and either A-Tn_1-Ac or D-Tn_1-Ac. The extent of potentiation can also be appreciated by comparing the data curves obtained with A-Tn_1-Qu used as an example of an additive effects, when incubation with the two drugs provided in IC_{50} value of 220 μM.

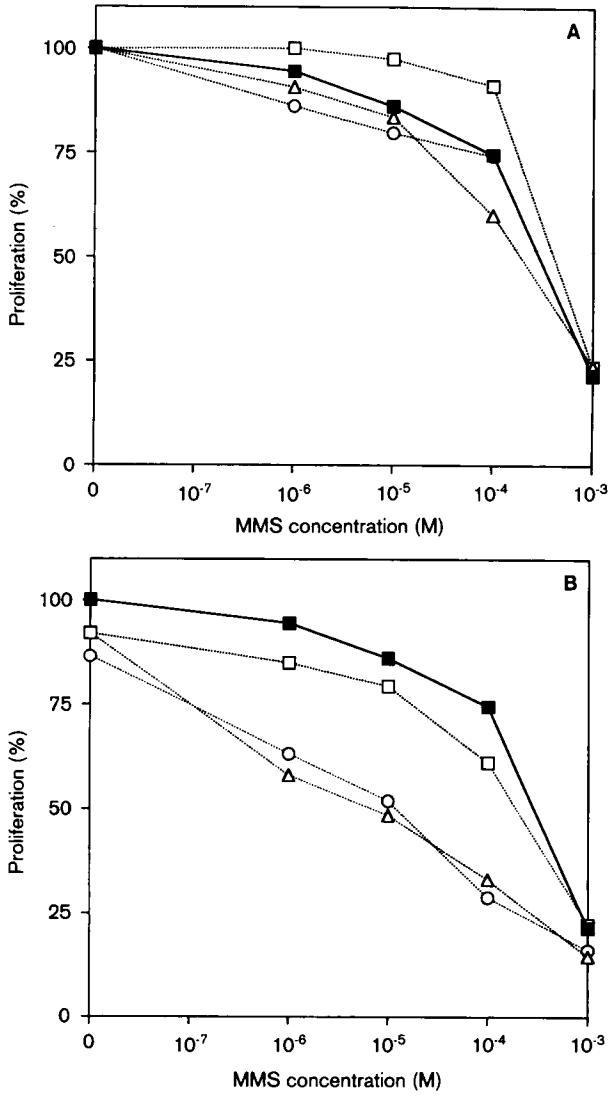


Figure 2. Cytotoxic activity on L1210 cells of a combination of MMS with a test compound from the artificial endonuclease series. Cytotoxicity was evaluated using non-toxic concentrations of the artificial endonucleases (A) or using a concentration of artificial endonucleases which alone resulted in 10% cytotoxicity (B). L1210 cells were incubated with D-Tn_1-Ac (○), A-Tn_1-Ac (△), A-Tn_1-Qu (□) or with water as control (■) in the presence of different concentrations of MMS. Results were analyzed as described in Materials and methods, and values are presented as percentage of proliferation and were obtained from three experiments using duplicate evaluations (SEM < 10%).

Further experiments have shown that such potentiation was also possible with certain other alkylating agents, like thiotepa in the presence of D-Tn_1-Ac (Figure 3), and yet this observation was not invariable, since no potentiation of mitomycin C was observed (data not shown). Moreover, when a compound showed no cytotoxicity against L1210 cells, such as streptozocin, the addition of D-Tn_1-Ac at 1 μM, a concentration corresponding to 10% cytotoxicity, failed to result in any sensitization (Figure 3). Interestingly, sensitization to BCNU, a clinically useful anticancer drug, was also observed (Figure 4). Thus, in the presence of BCNU the IC_{50} value shifted from 5.6 μM with BCNU alone to 0.43 or 0.85 μM with a combination of BCNU and A-Tn_1-Ac or D-Tn_1-Ac, respectively, when the artificial endonucleases were used at a concentration corresponding to 10% cytotoxicity. The extent of potentiation can also be appreciated by comparing the data curves obtained with A-Tn_1-Qu used as an example of additive effects of the two incubated drugs which provide in an IC_{50} value of 3.3 μM. Moreover, as observed with MMS (Figure 2A) no potentiation was found when a non-cytotoxic concentration of the artificial endonuclease

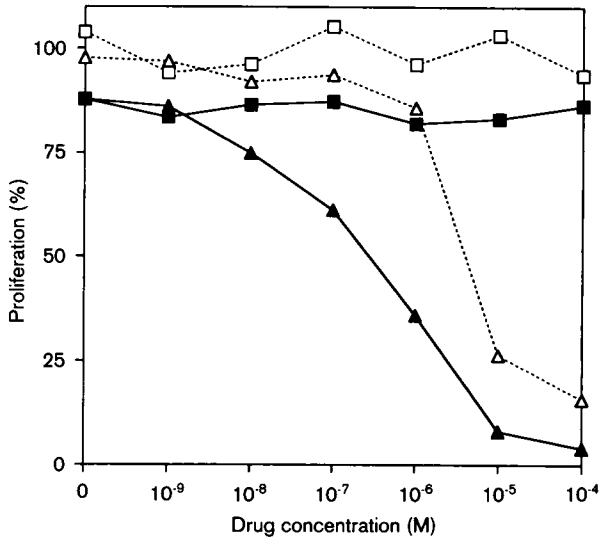


Figure 3. Cytotoxic activity on L1210 cells of a combination of D-Tn_1-Ac with either thiotepa or streptozocin. L1210 cells were incubated with 0.9 μM of D-Tn_1-Ac (open symbols) or with water as a control (filled symbols) in the presence of increasing concentrations of thiotepa (triangles) or streptozocin (squares). The concentration of D-Tn_1-Ac used in these experiments corresponded to that inducing 10% cytotoxicity by itself. Results were analyzed as described in Materials and methods, and values are presented as percentage of proliferation and were obtained from two independent experiments using duplicate evaluations (SEM < 10%).

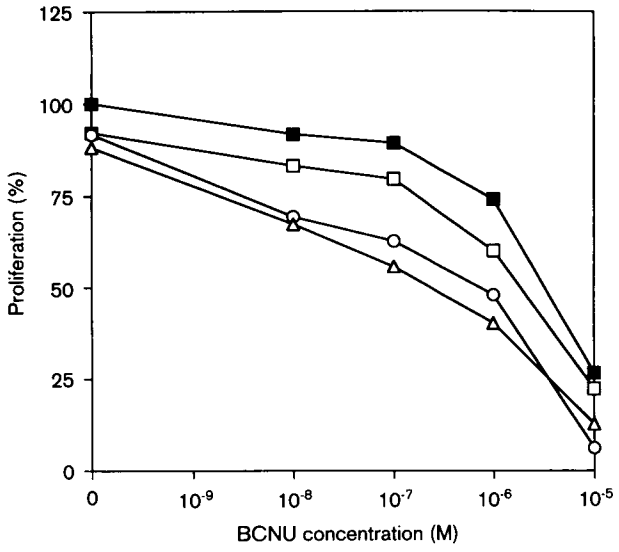


Figure 4. Cytotoxic activity on L1210 cells of a combination of BCNU with a test compound from the artificial endonuclease series. Cytotoxicity was evaluated using a concentration of artificial endonucleases which alone resulted in 10% cytotoxicity. L1210 cells were incubated with D-Tn_1-Ac (○), A-Tn_1-Ac (△), A-Tn_1-Qu (□) or with water as control (■) in the presence of different concentrations of BCNU. Results were analyzed as described in Materials and Methods, and values are presented as percentage of proliferation and were obtained from three experiments using duplicate evaluations (SEM < 10%).

was used. Nevertheless, the potentiations observed with BCNU were limited to factors of around 8 or 4 with A-Tn_1-Ac or D-Tn_1-Ac, respectively (Figure 4), which is clearly lower that those observed in the presence of MMS where a sensitization of more than 20-fold was noted (Figure 2B).

Discussion

The pharmacological inhibition of BER has not been extensively studied, in part at least because this pathway remains incompletely understood, but also because BER is known to repair only certain damage induced by mono-alkylating agents, therefore limiting the benefits of such pharmacological intervention. However, the reaction of bis-alkylating agents with DNA produces intermediate mono-adducts, certain of which may be repaired by the BER process.[5,6] For this reason, we decided to investigate the potential of inhibiting BER with the aim of sensitizing tumor cells to alkylating agents.

One promising target should be HAP1 which is a crucial enzyme in BER, since it is nearly the sole AP endonuclease involved in this process (Figure 1).[4] Moreover, the fact that its endonuclease activity and its regulatory function of proto-oncogenes by redox activity are not coupled[30] should exclude any unexpected effects. To date, no BER inhibitors which interact and inhibit HAP1 have been described. However, the artificial endonucleases may achieve this goal indirectly since they could mask the abasic site to HAP1, cleave the DNA strand at or near the AP site and therefore result in an inhibition of AP endonuclease activity.[21,23] Moreover, the DNA cleavage produced by these compounds should drastically increase the cytotoxicity of DNA-damaging agents whose adducts are repaired by BER. In this study, we have verified the concept that artificial endonucleases could be used to inhibit BER and thus potentiate the cytotoxic effects on cells of certain alkylating agents.

Initially, the inhibitory activities of artificial nucleases on BER were evaluated using the 3-D assay. The results indicated that D-Tn_1-Ac and A-Tn_1-Ac were the most potent inhibitors of BER (Table 2), suggesting that the choice of the base (adenine or diaminopurine) constituting these compounds modestly influenced their inhibitory activity. On the other hand, compounds in which the chain had been modified were found to be either less active (D-Dn-Ac and A-Dn-Ac) or inactive (D-Tn_2-Ac, D-Dd-Ac and D-Td-Ac). Previous studies have shown that these types of chains (Dn, Tn_2, Dd and Td) led to weaker endonuclease activities compared to those found with compounds including the Tn_1 chain.[22,25] Moreover, replacement of the acridine (Ac) by a 7-chloroquinoline (Qu), a less potent intercalator, resulted in a lesser inhibitory activity against BER, as was found here with A-Tn_1-Ac and A-Tn_1-Qu with their IC_{50} values of 62 and 138, respectively. Thus, all these results suggest that both a high affinity for DNA and a potent nuclease activity are necessary for these compounds to strongly inhibit BER. In addition, in a preliminary study, we have verified that the strand breaks due to the nuclease activity of these compounds did not appear without DNA repair activity. Thus, with the 3-D assay used as described by Salles *et al.* to quantify DNA damage,[26] no damage was detected after a co-incubation of undamaged DNA with any of these compounds, without the addition of cellular extracts (data not shown). Thus, these compounds did not react *in vitro* with DNA in the absence of cellular extract. This result confirms that artificial endonucleases need the presence of abasic sites on the DNA to cleave it and therefore to inhibit BER.

Secondly, the most potent inhibitors of BER (D-Tn_1-Ac and A-Tn_1-Ac) identified in this study were tested

for their abilities to potentiate the cytotoxicity of MMS, so as to confirm that the inhibition observed *in vitro* could be also observed in cultured cells. The easiest conditions to use in such evaluations is to treat cells with non-cytotoxic concentrations of the sensitizers. For this reason, the cytotoxicity of the artificial endonucleases themselves was first determined (Table 3) and it was established that a concentration 10-fold lower than that corresponding to the IC_{50} values was totally non-cytotoxic. However, none of these compounds revealed any significant potentiation of MMS when added at such concentrations (Figure 2A). Nevertheless, potentiation of MMS was found with both D-Tn_1-Ac and A-Tn_1-Ac when these compounds were used at 5-fold higher concentrations, and under these conditions they were then shown to exert around 10% cytotoxicity when added to L1210 cells alone (Figure 2B). Indeed, it is interesting to note that those two compounds, D-Tn_1-Ac and A-Tn_1-Ac, which were found to be the most potent BER inhibitors, were also found previously to be the most specific compounds for reacting with the abasic sites and had the most DNA cleavage activity of this series.[21,23] This concentration exerting 10% cytotoxicity had been determined previously with the curves corresponding to the cytotoxicity on L1210 cells of D-Tn_1-Ac or A-Tn_1-Ac incubated alone (data not shown).

Such potentiating effects of artificial endonucleases can be extended to other alkylating agents since, under similar conditions, sensitization was found with certain other drugs, like thiotepa (Figure 3) or BCNU (Figure 4). However, similar effects have not been obtained with all the alkylating agents tested, e.g. with streptozotocin (Figure 3), suggesting that certain other parameters remain to be defined in order to clearly understand the precise mechanism of the sensitization demonstrated in this study. The potentiation of BCNU is interesting, since this anticancer drug is used clinically. Moreover, it is generally considered that such bis-alkylation is repaired by NER process. However, an evaluation of *in vitro* NER using UVC-damaged plasmids, as previously described,[24] has shown that D-Tn_1-Ac or A-Tn_1-Ac were at least 50-fold less potent than aphidicolin, a classical NER inhibitor. It is difficult to establish a strict parallel between *in vitro* and cellular experiments, but such NER inhibition was probably not sufficient to potentiate BCNU cytotoxicity. However, several studies have also suggested an influence of BER on the cytotoxicity of bis-alkylating drugs like BCNU.[6,12-14] This influence might be direct, via repair of monoalkylated intermediates, or indirect by some unspecified regulationing event. Moreover, the fact that potentiation appeared only at cytotoxic concentrations of these artificial endonucleases cannot exclude the possibility that potentiation may result from a mechanism other than BER, since this cytotoxicity appeared not to be linked to the endonuclease activity of these test compounds.

Clearly, this apparent concentration requirement for potentiation complicates the use of such compounds and interpretation of the results obtained. Indeed, using this L1210 model system and estimating cytotoxicity in terms of growth inhibition quantitated by cell counting revealed that in their own right these artificial endonucleases were cytotoxic, with only an approximate 2-fold difference in their concentrations which resulted in 50% versus 10% growth inhibition. Furthermore, such cytotoxicity should essentially be considered as non-specific in view of their general inhibitory effects on overall DNA, RNA and protein synthesis, and clearly should not be related directly to their BER inhibitory activities. Our current efforts are aimed at identifying derivatives of these compounds with similar or superior BER inhibitory activities, yet without any associated cytotoxicity themselves, which should demonstrate marked potentiation of the cytotoxicity of clinically useful alkylating agents.

An artificial endonuclease activity has already been described for various compounds with simpler structures, including 9-amino-ellipticine,[31] 3-aminocarbazole[32] and a tripeptide Lys-Trp-Lys.[33] It has also been suggested for DMP-840, a bis-naphtalimide anticancer agent, currently in phase II clinical trials.[34] To date, however, these compounds have not been tested either as inhibitors of BER or as sensitizers of cells to alkylating drugs. The only exception being DMP-840 which has been tested as a sensitizer using similar procedures to those described in this paper, but no potentiation was identified at the concentrations tested.[35] Nevertheless, such compounds may provide novel leads for chemical synthesis of derivatives, which may provide future generations of new sensitizers of DNA-damaging agents.

Overall, we propose that the findings presented here, together with certain recent literature reports, provide the basis for a rational new approach aimed at improving the clinical efficacy of currently available DNA-damaging agents by identifying new sensitizers with unique mechanism(s) of action, to use in combination therapies.

Acknowledgments

The authors are pleased to acknowledge the contributions of Drs J-F Constant and M Demeunynck in synthesizing the artificial endonucleases for this study

and for helpful discussions. The technical assistance of A Limouzy, S Rigaud and S Gras is also gratefully acknowledged. Finally, the constructive comments, criticisms and additions made to the manuscript in preparation by Drs A Kruczynski, D Perrin and B van Hille were much appreciated.

References

1. Lehnert M. Clinical multidrug resistance in cancer: a multifactorial problem. *Eur J Cancer* 1996; **32A**: 912-20.
2. Hill BT. Drug resistance: an overview of the current state of the art. *Int J Oncol* 1996; **9**: 197-203.
3. Chaney SG, Sancar A. DNA repair: enzymatic mechanisms and relevance to drug response. *J Natl Cancer Inst* 1996; **88**: 1346-60.
4. Lindahl T, Karran P, Wood RD. DNA excision repair pathways. *Curr Opin Genet Dev* 1997; **7**: 158-69.
5. Ludlum DB. DNA alkylation by haloethylnitrosoureas: nature of modifications produced and their enzymatic repair of removal. *Mutat Res* 1990; **233**: 117-26.
6. Engelward BP, Dreslin A, Christensen J, Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. *EMBO J* 1996; **15**: 945-52.
7. Matijasevic Z, Boosalis M, Mackay W, Samson L, Ludlum DB. Protection against chloroethylnitrosourea cytotoxicity by eukaryotic 3-methyladenine DNA glycosylase. *Proc Natl Acad Sci USA* 1993; **90**: 11855-9.
8. Lefebvre P, Zak P, Laval F. Induction of O^6-methylguanine-DNA-methyltransferase and N^3-methyladenine-DNA-glycosylase in human cells exposed to DNA damaging agents. *DNA Cell Biol* 1993; **12**: 233-41.
9. Ibeanu G, Hartenstein B, Dunn WC, *et al.* Overexpression of human DNA repair protein *N*-methylpurine-DNA glycosylase results in the increased removal of *N*-methylpurines in DNA without a concomitant increase in resistance to alkylating agents in Chinese hamster ovary cells. *Carcinogenesis* 1992; **13**: 1989-95.
10. Tano K, Dunn WC, Darroudi F, *et al.* Amplification of the DNA repair gene O^6-methylguanine-DNA methyltransferase associated with resistance to alkylating drugs in a mammalian cell line. *J Biol Chem* 1997; **272**: 13250-4.
11. Fornace AJ, Jr, Zmudzka B, Hollander C, Wilson SH. Induction of β-polymerase mRNA by DNA-damaging agents in Chinese hamster ovary cells. *Mol Cell Biol* 1989; **9**: 851-3.
12. Friedman HS, Dolan ME, Kaufmann SH, *et al.* Elevated DNA polymerase α, DNA polymerase β, and topoisomerase II in a melphalan-resistant rhabdomyosarcoma xenograft that is cross-resistant to nitrosoureas and topotecan. *Cancer Res* 1994; **54**: 3487-93.
13. Gomi A, Shinoda S, Sakai R, *et al.* Elevated expression of DNA polymerase β gene in glioma cell lines with acquired resistance to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea. *Biochem Biophys Res Commun* 1996; **227**: 558-63.
14. Moynihan K, Elion GB, Ali-Osman F, *et al.* Enhancement of melphalan activity by inhibition of DNA polymerase-α and DNA polymerase-β. *Cancer Chemother Pharmacol* 1996; **38**: 349-54.
15. Xu Y, Moore DH, Broshears J, *et al.* The apurinic/apyrimidic endonuclease (APE/ref-1) DNA repair enzyme is elevated in premalignant and malignant cervical cancer. *Anticancer Res* 1997; **17**: 3713-20.
16. Schärer OD, Nash HM, Jiricny J, Laval J, Verdine L. Specific binding of designed pyrrolidine abasic site analog to multiple DNA glycosylases. *J Biol Chem* 1998; **273**: 8592-7.
17. Bouayadi K, Hoffman J-S, Fons P, *et al.* Overexpression of DNA polymerase β sensitizes mammalian cells to 2',3'-deoxycytidine and 3'-azido-3'-deoxythymidine. *Cancer Res* 1997; **57**: 110-6.
18. Constant J-F, O'Connor TR, Lhomme J, Laval J. 9-[(10-(aden-9-yl)-4,8-diazadecyl)amino)-6-chloro-2-methoxy-acridine incises DNA at apurinic sites. *Nucleic Acids Res* 1988; **16**: 2691-703.
19. Constant J-F, Fkyerat A, Demeunynck M, *et al.* Design of molecules which specifically cleave abasic sites in DNA. *Anti-Cancer Drug Des* 1990; **5**: 59-62.
20. Belmont P, Boudali A, Constant J-F, *et al.* Efficient and versatile chemical tools for cleavage of abasic sites in DNA. *New J Chem* 1997; **21**: 47-54.
21. Berthet N, Constant J-F, Demeunynck M, Michon P, Lhomme J. Search for DNA repair inhibitors: selective binding of nucleic bases-acridine conjugates to a DNA duplex containing an abasic site. *J Med Chem* 1997; **40**: 3346-52.
22. Fkyerat A, Demeunynck M, Constant J-F, Michon P, Lhomme J. A new class of artificial nucleases that recognize and cleave apurinic sites in DNA with great selectivity and efficiency. *J Am Chem Soc* 1993; **115**: 9952-9.
23. Coppel Y, Constant J-F, Coulombeau C, *et al.* NMR and molecular modeling studies of the interaction of artificial AP lyases with a DNA duplex containing an apurinic abasic site model. *Biochemistry* 1997; **36**: 4831-43.
24. Barret J-M, Salles B, Provot C, Hill B. Evaluation of DNA repair inhibition by antitumor or antibiotic drugs using a chemiluminescence microplate assay. *Carcinogenesis* 1997; **18**: 2441-5.
25. Fkyerat A, Demeunynck M, Constant J-F, Lhomme J. Synthesis of purine-acridine hybrid molecules related to artificial endonucleases. *Tetrahedron* 1993; **49**: 11237-52.
26. Salles B, Provot C, Calsou P, *et al.* A chemiluminescent microplate assay to detect DNA damage induced by genotoxic treatments. *Anal Biochem* 1995; **232**: 37-42.
27. Sancar A. DNA repair in humans. *Annu Rev Genet* 1995; **29**: 69-105.
28. Liuzzi M, Talbaert-Borle MA. A new aproach to study of the base-excision repair pathway using methoxyamine. *J Biol Chem* 1985; **260**: 5252-8.
29. Chen SF, Behrens DL, Czerniak PM, *et al.* XB596, a promising bis-naphtalimide anti-cancer agent. *Anti-Cancer Drugs* 1993; **4**: 447-57.
30. Barzilay G, Walker LJ, Rothwell DG, Hickson ID. Role of the HAP1 protein in repair of oxidative DNA damage and regulation of transcription factors. *Br J Cancer* 1996; **74**: S145-50.
31. Malvy C, Prévost P, Gansser C, Viel C, Paoletti C. Efficient breakage of DNA apurinic sites by indole-amine related 9-amino-ellipticine. *Chem-Biol Interact* 1986; **57**: 41-53.

32. Vasseur JJ, Rayner B, Imbach JL, *et al.* Structure of the adduct formed between 3-amino-carbazole and the apurinic site oligonucleotide model [Tp(Ap)pT]. *J Org Chem* 1987; **52**: 4994-8.
33. Behmoaras T, Toulmé J-J, Hélène C. A tryptophan-containing peptide recognizes and cleaves DNA at apurinic sites. *Nature* 1981; **292**: 858-9.
34. Grafström RH, Sun T, Doleniak D. AP endonuclease: a possible target for a novel tumoricidal compound. In: *Repair and processing of DNA damage* 1995: 279.
35. Bobola MS, Berger MS, Stevens B, Silber JR. Variation of DMP840 resistance and relationship to apurinic/apyrimidic endonuclease activity and alkylating agent sensitivity in 12 brain tumor cell lines. *Proc Am Ass Cancer Res* 1997; **38**: 314.

(Received 18 July 1998; revised form accepted 11 August 1998)