Articles

Pyrrolo[1,2-a]benzimidazole-Based Aziridinyl Quinones. A New Class of DNA Cleaving Agent Exhibiting G and A Base Specificity

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Pyrrolo[1,2-a]benzimiazole(PBI)-based aziridinyl quinones cleave DNA under reducing conditions specifically at G + A bases without any significant cleavage at C + T bases. The postulated mechanisms involve phosphate alkylation by the reductively activated aziridine to afford a hydrolytically labile phosphotriester as well as the classic N(7) purine alkylation followed by depurination and backbone cleavage. Evidence is presented that the phosphate alkylation mechanism could contribute. The PBIs possess a unique spectrum of cytotoxicity against cancer cells (inactive against leukemia but active against nonsmall cell lung, colon, CNS, melanoma, ovarian, and renal cancers). Also reported are results of *in vivo* antitumor activity screens.

Introduction

The pyrrolo[1,2-a]benzimidazoles (PBIs) shown in Scheme I represent a new class of antitumor agent exhibiting activity against a variety of cancer cell lines.[1-4] These agents cleave DNA upon reduction of the quinone ring as a result of alkylation reactions by the aziridinylhydroquinone (inset of Scheme I). Questions posed dealt with the sequence specificity and mechanism of PBI-mediated DNA cleavage, and with the spectrum of PBI antitumor activity. In this report evidence is presented for PBI-mediated DNA cleavage at G and A bases. The cleavage process could involve N(7) purine alkylation followed by depurination and backbone cleavage[5] and/or a process involving phosphate alkylation followed by phosphotriester hydrolysis[6] (inset of Scheme I). Some experimental findings suggest that the phosphate alkylation mechanism could be in operation. The spectrum of PBI cytotoxicity observed in 60 cancer cell lines is quite unlike that of any clinically used antitumor agent. Antitumor studies in nude mice models indicate that the PBIs possess *in vivo* activity.

DNA Cleavage Studies

The PBI-mediated cleavage of linear DNA was studied utilizing the 3'-^{32}P-end-labeled 514 bp *Rsa*I/*Eco*RI and the 541 bp *Rsa*I/*Cla*I restriction fragments of pBR322.[7] Shown in Figure 1 is an autoradiogram of the untreated *Rsa*I/*Cla*I fragment (lane A), the *Eco*RI cut of this fragment to afford the 27bp *Eco*RI/*Cla*I fragment (lane B), and the Maxam-Gilbert G + A ladder[8] of the *Rsa*I/*Cla*I fragment (lane C). Lanes A-C of Figure 1 indicate that the DNA used in these studies is pure. Figure 1 also shows the Maxam-Gilbert G + A cleavage of *Rsa*I/*Cla*I (lane D) and the cleavage of this restriction fragment by reduced PBI-A (lane E). Comparisons of Lanes D and E indicate that DNA G + A cleavage occurs upon treatment with reduced PBI-A. Shown in Figure 2 is an autoradiogram of a slab gel obtained from PBI-A cleavage of the RsaI/*Eco*RI fragment with only bases 4310-4332 shown.[9] Lane F shows a Maxam-Gilbert G ladder.[8] Lanes E, D, and B of Figure 1 show DNA which had been treated with reduced PBI-Aat concentrations of 1x, 2x, and 7x (x = 0.69 mM), respectively. The 2x lane shows enhanced cleavage compared to the 1x lane, while the 7x lane is showing signs of over cleavage. When cleavage was attempted in the presence of oxygen (lane C) or in the presence of dithionite without added PBI-A (lane A), no substantial cleavage DNA could be observed. Studies of reactions containing PBI-A and DNA only also did not produce cleavage. A parallel study of PBI-C mediated DNA cleavage (results not shown) provided identical results.

Mechanistic details of PBI-mediated DNA cleavage are now considered. The obvious mechanism for PBI-mediated DNA cleavage is N(7)-alkylation followed by depurination and backbone cleavage. Other aziridinylquinones can in fact cleave at G + A bases by this mechanism.[5b] Consistent with the depurination mechanism and a 5'-phosphate cleavage terminus, the PBI cleavage ladders comigrate with the Maxam-Gilbert G and G + A ladders (Figures 1 and 2). In our attempt to isolate N(7)-purine adducts, we treated sonicated calf thymus DNA with reduced PBI-A and isolated "blue DNA" upon aeration and then precipitation from aqueous sodium acetate/ethanol. Heating the drug-bound DNA at 50 °C in 0.2 M phosphate buffer for several hours afforded small amounts of drug hydrolysis products without any apparent release of N(7)-alkylated purine bases. These findings are analogous to those of Bannon and Verly,[10] who found that N(7)-DNA adducts are more hydrolytically labile than phosphate alkylation products.

In light of the above findings, the phosphate alkylation cleavage mechanism shown in the inset of Scheme I was considered as a possibility for PBI-mediated DNA cleavage. Such a mechanism is actually not out of the ordinary. Indeed, DNA treatment with the nitrosourea ENU[6] results in the formation of hydrolytically labile phosphotriesters. Alkyl methanesulfonates are known to alkylate DNA at both N(7)-positions and phosphate.[10] Furthermore, phosphate oxygens are known to be alkylated by aziridines.[11]

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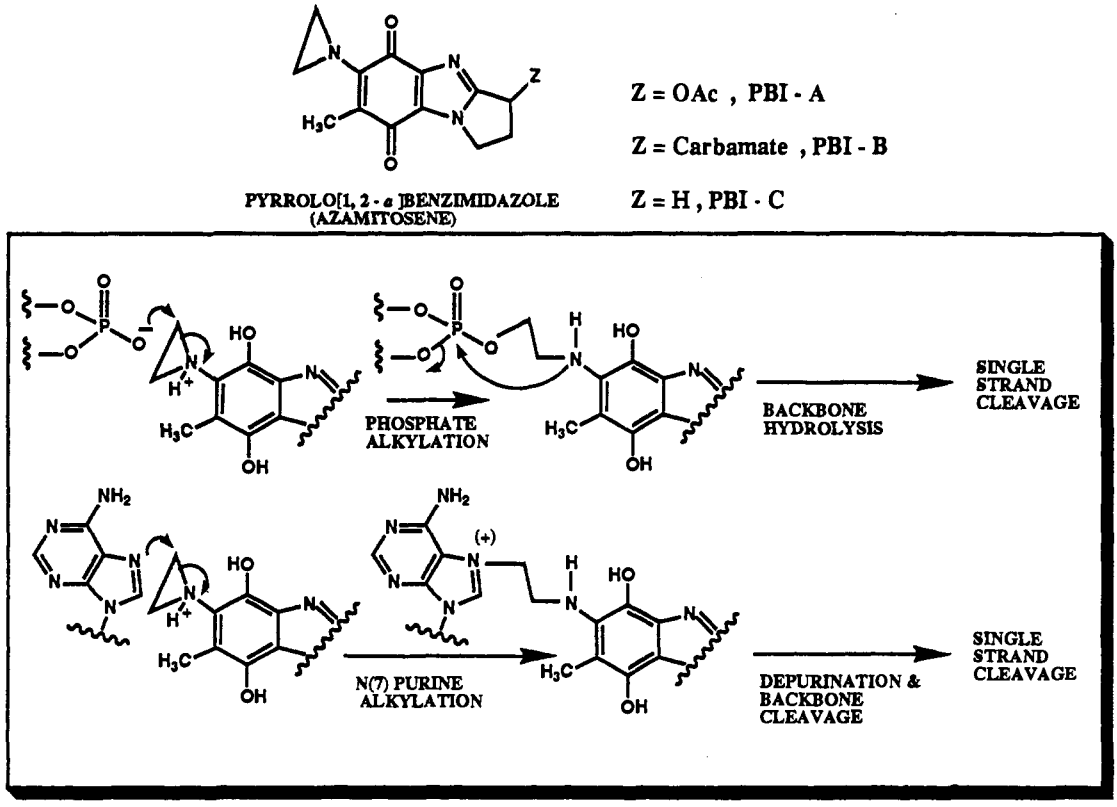
Scheme I



To illustrate the latter point, we treated 5′-dAMP with a reduced PBI (Z-OH) and isolated the quinone adduct shown in Chart I after an aerobic workup.

According to the phosphate alkylation mechanism shown in Scheme I, the G + A specificity of PBI cleavage results from binding of reduced PBI in the major grooveat these bases. Interaction at G requires protonated drug (Chart I), whereas interaction at A requires neutral drug. Molecular modeling (insight II) of reduced PBI-A bound to A in the major groove with hydrogen bonds 1.5–2 Å in length with bond angles between 120 and 180° placed the aziridinyl carbon center ~3 Å from the phosphate oxygen (graphics not shown). The resulting adduct can cause DNA backbone cleavage by a nucleophilic displacement mechanism involving attack of the amino group (formerly the aziridinyl nitrogen center) on the phosphorus center, inset of Scheme I.

Phosphotriester analogues of DNA are usually quite stable to hydrolysis.[10,12] An exception is a β-hydroxyethyl phosphotriester, which undergoes hydrolysis at 37 °C by means of internal oxygen nucleophilic displacement at the phosphorus center.[13] Recently, Browne and Bruice[14] reported that even the phosphodiester, bis(8-hydroxyquinoline) phosphate, is readily hydrolyzed by internal nitrogen nucleophilic displacement at phosphate. Thus, there are precedents for the phosphate backbone cleavage mechanism shown in Scheme I. In fact, reduction of the quinone adduct shown in Chart I and incubation in anaerobic pH 7.4, 0.05 M tris buffer at 37°, results in slow phosphate hydrolysis to afford adenosine and 5′-dAMP (as followed by HPLC). If hydrolysis of the DNA phosphotriester adduct occurs in the 3′ direction only, the observed 5′-phosphate cleavage termini will be formed (see Figures 1 and 2).

In the oxidized (quinone) form, both "blue DNA" and the adduct shown in Chart I are stable in pH 7 aqueous buffer at 37 °C. Indeed, in order to observe cleavage ladders with PBI-treated DNA, it was necessary to heat the DNA in a basic formamide loading solution at 95 °C for 2 min. The observed stability of oxidized PBI adducts is no doubt due to electron withdrawal by the quinone ring from the nitrogen involved in nucleophilic attack.

The conclusion of our mechanistic studies is that the phosphate alkylation mechanism is chemically feasible. We do not reject the N(7) purine alkylation as a possible coexisting cleavage mechanism, however.

Cytotoxicity and Antitumor Studies

Previous studies verified that PBI analogues can cause DNA single strand cleavage in myeloma cells in a dose-dependent fashion.[2] The results cited in this article suggest that cellular DNA cleavage occurs at G + A bases upon reductive activation of the PBI analog. A structural relative of the PBI analogues, mitomycin C,[15] also requires reductive activation but alkylates the guanine amino group. Another structural relative, the aziridinylquinone AZQ,[5] alkylates the guanine N(7) position upon reductive activation. Despite some similarities with these antitumor agents, the PBI analogues have a unique spectrum of cytotoxic activity, which is now discussed in conjunction with the data in Table I.[16]

Found in Table Iis an LC_{50} mean graph obtained from screening PBI-A against a panel of 60 cancer cell lines (LC_{50} is the concentration needed for 50% cell kill).[17] The center line is the log of the mean LC_{50} value; bars to the right of the mean represent activity greater than the mean (lower log LC_{50} values), while bars to the left represent activity lower than the mean (higher log LC_{50} values). Other PBI analogues gave bar graphs similar to that shown in Table I.

The data in Table I show that PBI-A is virtually inactive against leukemia. Other cancers including nonsmall cell

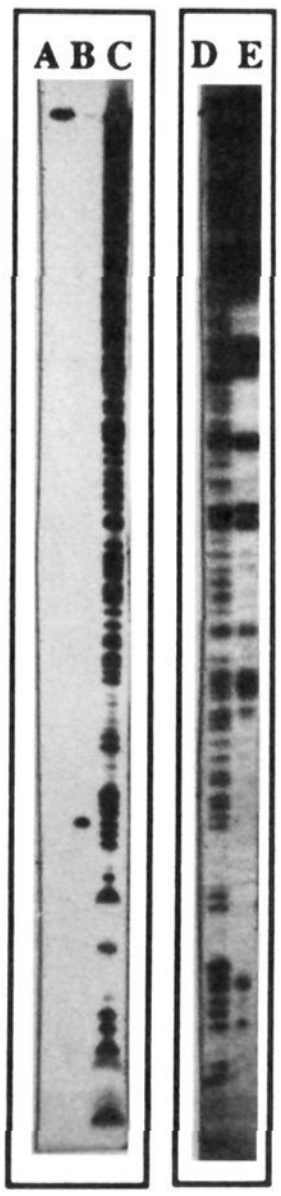


Figure 1. Autoradiogram of 8% polyacrylamide/7 M urea slab gel of the *Rsa*I/*Cla*I fragment: (A) untreated DNA, (B) *Eco*RI cut, (C) Maxam–Gilbert G + A ladder, (D) Maxam–Gilbert G + A ladder, and (E) reduced PBI-A cleavage ladder.

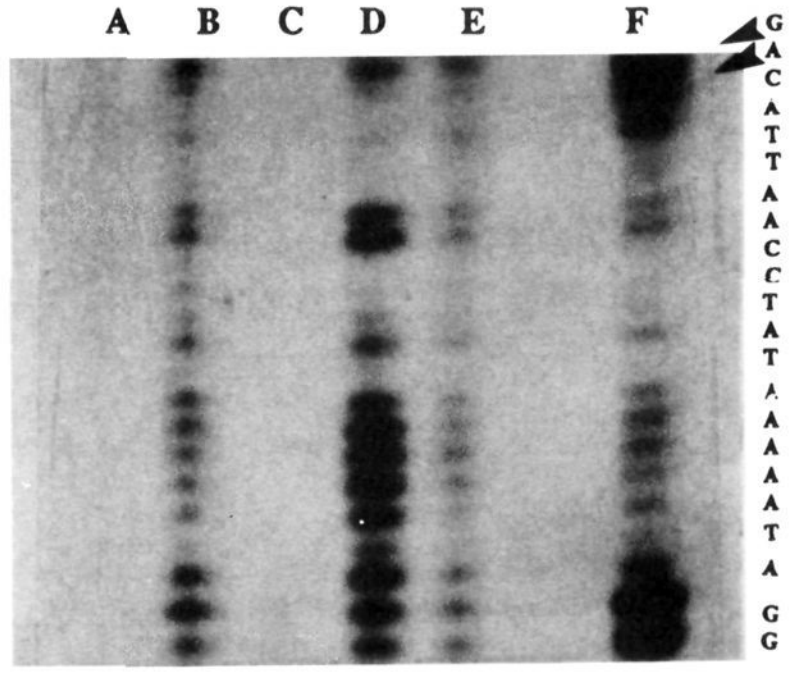
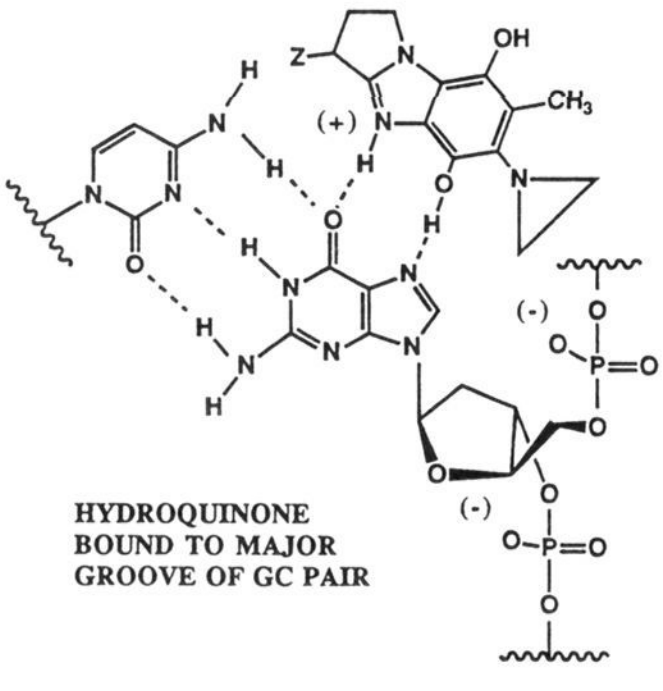


Figure 2. Autoradiogram of 8% polyacrylamide/7 M urea slab gel of the *Rsa*I/*Eco*RI fragment showing bases 4310 (top) to 4332 (bottom): (A) treatment with 2.3 M dithionite, (B) treatment with reduced 4.8 mM PBI-A, (C) treatment with aerobic/reduced 3.48 mM PBI-A, (D) treatment with reduced 1.39 mM PBI-A, (E) treatment with 0.69 mM PBI-A, and (F) Maxam–Gilbert G ladder. All treatments A–E were carried out as described in ref 9 except the PBI-A was left out in (A) and aerobic conditions were employed in (C).

lung, colon, CNS, melanoma, ovarian, and renal show varying degrees of sensitivity to PBI-A—melanoma appears to be the type of cancer most sensitive to this agent. The *in vivo* screening results obtained thus far show some correlation with the *in vitro* results shown in Table I. For example, PBI-A had no activity against ip-implanted P-388 leukemia in female CD2F1 mice.[2] On the other hand, the growth of HCT-116 colon tumor xenographs in athymic nude mice was substantially inhibited by PBI-C. In contrast, PBI-A treatment of athymic nude mice implanted with HCT-13 colon tumor resulted in no inhibition of tumor growth and PBI-C treatment of the same mice implanted with RXF-393 renal tumor likewise resulted in no inhibition of tumor growth. PBI-A was found to increase the life span of mice ip-implanted with LOX IMVI melanoma by 47%, however.

The COMPARE computer program has been developed by the National Cancer Institute to compare mean graphs of cancer drugs.[18] Generally, cancer drugs with similar mechanisms of action have similar mean graphs. Thus the adriamycin IC_{50} mean graph compares well with those of the structurally related deoxydoxorubicin and daunomycin (0.882 and 0.859 correlations coefficients, respectively). Similarly, the alkylating agents chlorambucil, thiotepa, and triethylenemelamine have nearly identical IC_{50} mean graphs.[17] The data in Table I did not compare well with any known antitumor agent. The highest correlations obtained were 0.66 with the anthracycline deoxydoxorubicin, 0.628 with the aziridine derivative triethylenemelamine, 0.591 with the anthracycline daunomycin, and 0.589 with the topoisomerase inhibitor AMSA.

Chart I



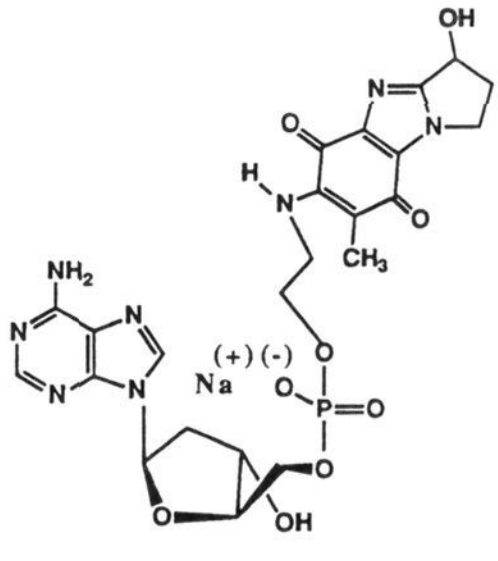
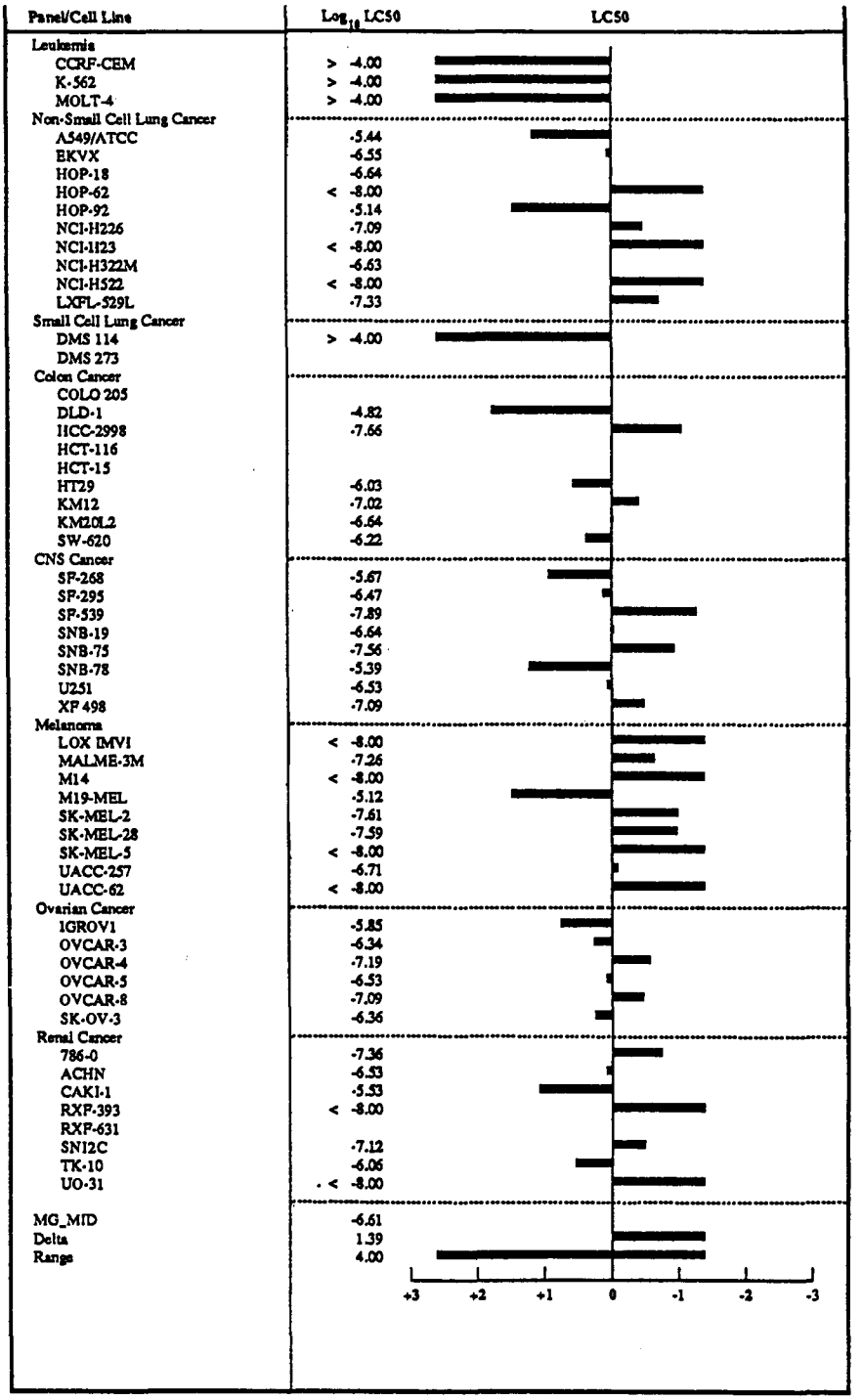


Table I. Mean Graph LC_{50} Data for PBI-A



Significantly, previous comparative studies with a single cell line resulted in the conclusion that PBI-A had anthracycline-like properties.[2] Probably the greatest point of difference between the PBI analogues and other antitumor agents is the complete absence of PBI activity against leukemia.

Conclusion

Some aspects of PBI-mediated DNA cleavage are consistent with a mechanism involving phosphate backbone alkylation followed by hydrolytic cleavage. Thus PBI DNA adducts are stable to hydrolysis and require heating in base in order to see DNA cleavage. In contrast, N(7)-DNA adducts are readily removed by hydrolysis.[10] The results of model studies with a PBI-5′dAMP adduct are consistent with a mechanism involving phosphate alkylation followed by hydrolysis. Other aspects of PBI-mediated DNA cleavage could be interpreted in terms of a mechanism involving N(7) alkylation, however. Thus, PBIs mediate G + A cleavage and produce 5′-phosphate cleavage termini, both of which are observed in aziridinylquinones known to alkylate DNA at the N(7)-position.[5] It is therefore possible that PBIs cleave by N(7) and by phosphate alkylation mechanisms. Indeed, Bannon and Verly[10] found the alkyl methanesulfonates can alkylate DNA at both positions.

The PBIs possess a unique spectrum of cytotoxic activity, which may or may not be related to the mechanism

of DNA cleavage. Previous studies[2] showed that cytotoxicity is due to the presence of DNA cleavage, however. Consistent with PBI cytotoxicity, analogues are shown to possess *in vivo* antitumor activity, which parallels the spectrum of activity observed in cell lines.

Further studies of DNA–PBI interactions and of PBI *in vivo* antitumor activity are in progress: nine PBI are currently in *in vivo* trials at the National Cancer Institute. We will report on the results of these studies in due course.

Experimental Section

Buffer salts, EDTA, and urea were purchased from Sigma and used as is. Acrylamide and methylene bisacrylamide were purchased from BioRad in 99.9% pure form. pBR322 DNA, *Eco*RI, *Rsa*I, and Klenow fragment were purchased from New England Biolabs. ^{32}P Labeled CTP and ATP were purchased from Dupont. Buffers and other solutions were prepared in doubly distilled water. DNA restriction fragments were purified and end labeled using previously reported procedures.[8]

PBI cleavage of DNA was carried out as follows: In a 1.5-mL microfuge tube were combined 1 μL labeled DNA (10 000 cpm), 1 μL of 10 μg/mL cold DNA (pBR322), 1 μL of PBI-A stock (1.3 mM in 0.05 M pH 7.4 Tris buffer), and 6 μL of pH 7.4 Tris buffer. This solution was degassed for 15 min with argon and followed by addition of 1 μL of sodium dithionite (34 mM). We found that dithionite itself will react with reduced PBI-A, and therefore excessive amounts of PBI-A were used in the cleavage study. Nevertheless, dithionite is a convenient reducing agent for limited cleavage experiments. The reaction was incubated for 30 min at 37 °C under an argon atmosphere, and then to the reaction mixture was added 1 μL of 3 M sodium acetate pH 5.2 (0 °C) followed by 30 μL of ethanol (−20 °C). The solution was chilled for 15 min at −70 °C and then centrifuged at 12 000 g for 20 min. The supernatant was then carefully decanted from the DNA pellet. To the pellet was added 100 μL of ethanol (−20 °C) followed by centrifuging at 12 000 g for 5 min. After decanting the ethanol, the pellet was vacuum dried and combined with 5 μL of sequence gel loading buffer, which was prepared by combining 800 μL of formamide, 100 μL of 0.1 N NaOH, 100 μL double distilled H_2O, and 1 mg each of xylene cyanol and bromophenol blue. The mixture was electrophoresed on 21 cm long × 0.25 mm thick 8% acrylamide (29:1, acrylamide to methylene bisacrylamide) 7 M urea denaturing gel employing 0.089 M pH 8.3 Tris borate buffer containing 25 mM of EDTA. The gel was run at 1700 V for 2 h.

Preparation of Blue DNA. To a solution of 70 mg of sonicated calf thymus DNA in 20 mL of pH 7.4, 0.05 M Tris buffer was added 20 mg PBI-A, dissolved in 2 mL of dimethyl sulfoxide, and 10 mg of 5% Pd on carbon. The mixture was degassed with argon, and then a stream of H_2 passed through the solution for 10 min. After reduction was complete (solution went from red to colorless), the excess H_2 was removed by purging with argon, and the reaction was incubated at 37 °C for 24 h. The reaction was opened to the air and extracted 3× with 50-mL portions of chloroform to remove hydrolysis products, and the aqueous layer was adjusted to 0.3 M sodium acetate (pH 5.1). The DNA pellet was obtained by diluting the sodium acetate solution with 3 volumes of ethanol, chilling the solution overnight at −20 °C, and finally centrifuging at 12 000 g for 20 min. The pellet was suspended in 100% ethanol and centrifuged at 12 000 g for 5 min and then dried, yield: 60% DNA recovered.

Reaction products were studied by HPLC on an ISCO 5 μm SAX reverse phase column, with 0.8 M KH_2PO_4 mobile phase, and the products were isolated by means of a Phenyl Bakerbond reverse phase column with aqueous methanol as the mobile phase. All the products derived from PBI were formed by hydrolysis. The structures of these products and their mechanism of formation will be the subject of another study.

Preparation of PBI 5′-dAMP Adduct. To a solution of 124.7 mg (0.289 mmol) of 5′-dAMP hydrated sodium salt in 20 mL of pH 7.4, 0.05 M Tris buffer was added 30 mg (0.115 mmol) of PBI (Z = OH), dissolved in 2 mL of dimethyl sulfoxide, and 10 mg of 5% Pd on carbon. Degassing and reduction was carried out as described above, and the reaction was incubated at 37 °C for 22 h. The reaction was opened to the air, filtered through Celite, and then placed on a 20-g Phenyl Bakerbond column prepared with water. The aminoquinone nucleotide adduct eluted from the column with water as the first blue band. The isolated product was rechromatographed on a 10-g column to remove 5′-dAMP, yield ~6 mg (8.5%). No other quinone–nucleotide adduct was formed in the reaction. Both ^1H NMR and ^{31}P NMR studies indicate that alkylation of phosphate oxygen had occurred: ^1H NMR dimethyl-d_6 sulfoxide δ 8.39 (1H, brs, C(8) adenine), 8.13 (1H,s, C(2) adenine), 7.65 and 7.00 (2H, 2brs, C(3′) hydroxy of 5′-dAMP, C(6) amino of PBI), 7.24 (2H, brs, C(6) adenine amine), 6.35 (1H, t, C(1′) anomeric proton, J = 6 Hz), 5.85 (1H, d, C(3) hydroxy, J = 6 Hz), 4.94 (1H, m, C(3) proton), 4.44 (1H, m, C(3′)), 4.17 and 4.07 (2H, 2m, C(1) diastereomeric methylene), 3.94 (1H, d, C(4′), J = 2.5 Hz), 3.83 (4H, m, C(6) ethyl bridge), 3.69 (2H, q, J = 5 Hz, C(5′)), 2.86 and 2.35 (2H, 2m, C(2) diastereomeric methylene), 2.73 and 2.26 (2H, 2m, C(2′) diastereomeric methylene), 1.93 (3H, s, C(7) methyl). Note that the amino group of adenine (two protons) is present and therefore amine alkylation could not have occurred. ^{31}P NMR (D_2O) provided a chemical shift of δ 3.46 (vs phosphoric acid) for the quinone nucleotide and a chemical shift of δ 6.98 (vs phosphoric acid) for 5′-dAMP. The 3.5 ppm upfield shift in the phosphorus resonance indicates that the quinone nucleotide is a phosphodiester.[19]

Antitumor Studies. Response of subrenal capsule HCT-116 colon tumor xenografts to PBI-C at 15 mg/Kg administered intraperitoneally every 4 days, starting on day 2 after tumor implantation, for a total of three treatments resulted in a T/C% value of 5, which indicates tumor inhibition (T/C% is defined as (ΔT/ΔC) × 100 where ΔT is the change in tumor weight in treated mice and ΔC is the change in tumor weight in control mice).

Responses of HCT-13 colon and RxF-303 renal implants to PBI-A and PBI-C, respectively were in the inactive range, T/C% > 10.

Response of ip-implanted LOX IMVI melanoma to 6.0 mg/kg dose of PBI-A administered intraperitoneally every 4 days, starting on day 1 after tumor implantation, for a total of three treatments was to increase life span 47% over controls.

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