Naturally Occurring Alkylresorcinols That Mediate DNA Damage and Inhibit Its Repair†

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ABSTRACT: A study of di- and trihydroxyalkylbenzenes and bis(dihydroxyalkylbenzenes) revealed that several compounds were capable of both mediating Cu^{2+}-dependent DNA cleavage and strongly inhibiting DNA polymerase β. The most potent DNA polymerase β inhibitors were bis(dihydroxyalkylbenzenes) **5** and **6**; compounds **3** and **4** were also reasonably potent. The length of the alkyl substituent was found to be a critical element for DNA polymerase β inhibition, since compounds **1** and **2** had shorter substituents than **3** and were completely inactive. Lineweaver−Burk plots revealed that **3**, **4**, and **6** exhibited mixed inhibition of DNA polymerase β with respect to both activated DNA and dTTP. Unsaturated bis(dihydroxyalkylbenzene) **5** was a pure noncompetitive inhibitor with respect to both substrates and associated avidly with the enzyme whether or not it was in complex with its substrate(s). Copper(II)-mediated DNA cleavage was the most pronounced for the trihydroxyalkylbenzene **3**, consistent with an earlier report [Singh, U. S., Scannell, R. T., An, H., Carter, B. J., and Hecht, S. M. (1995) *J. Am. Chem. Soc. 117*, 12691−12699]. Unsaturated bis(dihydroxyalkylbenzene) **5** was the next most active DNA cleaving agent, followed by the dihydroxyalkylbenzene **4**. The saturated bis(dihydroxyalkylbenzene) (**6**) did not cleave DNA well in a cell-free system under the conditions studied but nonetheless potentiated the effects of bleomycin to the greatest extent in cell culture studies. Interestingly, compound **5** produced a reduction in the numbers of viable cells when incubated in the presence of bleomycin and a further reduction in the numbers of viable cells in the presence of both bleomycin and Cu^{2+}. The same effect was noted to a lesser extent for compound **3** but not for **4** or **6**.

Currently utilized chemotherapeutic techniques include the use of cytotoxic drugs that specifically kill or inhibit the growth of rapidly dividing cell populations or that are delivered specifically to the site of a tumor (*1*). However, toxicity to the host generally limits treatment and can preclude the use of such drugs entirely. Further, cancer cells frequently develop mechanisms to resist the effects of applied cytotoxic agents. For DNA-damaging agents, for example, cancer cells can repair the damage inflicted to their DNA, thus lessening the effects of antitumor agents that function by this mechanism (*2*−*4*).

Targeting proteins involved in DNA repair in cancer cells constitutes a logical strategy for overcoming resistance to DNA-damaging agents employed for anticancer therapy. The investigation of cellular DNA repair mechanisms has thus been carried out to define the involvement of individual proteins in repair pathways. Proteins of special interest include those involved in repair processes such as base excision repair (BER)[1] (*5*), nucleotide excision repair (NER), and DNA mismatch repair (*6*, *7*). BER is responsible for the repair of damaged DNA after exposure to some DNA-damaging agents such as the bleomycins (*8*−*10*), monofunctional DNA alkylating agents (*11*, *12*), cisplatin (*13*, *14*), and neocarzinostatin (*10*). DNA polymerase β is an important enzyme in BER (*11*) and carries out excision of the 5′-terminal dRP group (*15*). The DNA substrate for this transformation is an apurinic/apyrimidinic (AP) site that has been cleaved on the 5′-side of the lesion by an AP endonuclease. Additionally, DNA polymerase β catalyzes a template-directed nucleotidyl transfer reaction to fill the gap (*16*).

The role of DNA polymerase β in DNA repair strongly suggests the validity of the enzyme as a target for combination chemotherapy; inhibition of this enzyme could potentiate the effects of DNA-damaging agents by blocking repair of the damaged DNA.

While numerous inhibitors of DNA polymerase β have been identified (*12*, *17*−*24*) and can be envisioned as prototypes for the development of agents capable of potentiating the action of clinically used antitumor agents such as bleomycin and cisplatin, another strategy might involve the identification of agents capable of mediating DNA damage and also blocking repair of the induced lesions. Recently, we reported (*17*) the isolation from *Panopsis rubescens* of bis(5-alkylresorcinols) [bis(dihydroxyalkylbenzenes), exemplified by **5** and **6**] (Figure 1) that inhibited calf thymus DNA polymerase β with IC_{50} values of 5.8−7.5 μM. Because we had previously shown that structurally related compounds

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[1] Abbreviations: BER, base excision repair; NER, nucleotide excision repair; dRP, 2′-deoxyribose phosphate; AP site, apurinic/apyrimidinic site; BLM, bleomycin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; dNTP, deoxyribonucleoside 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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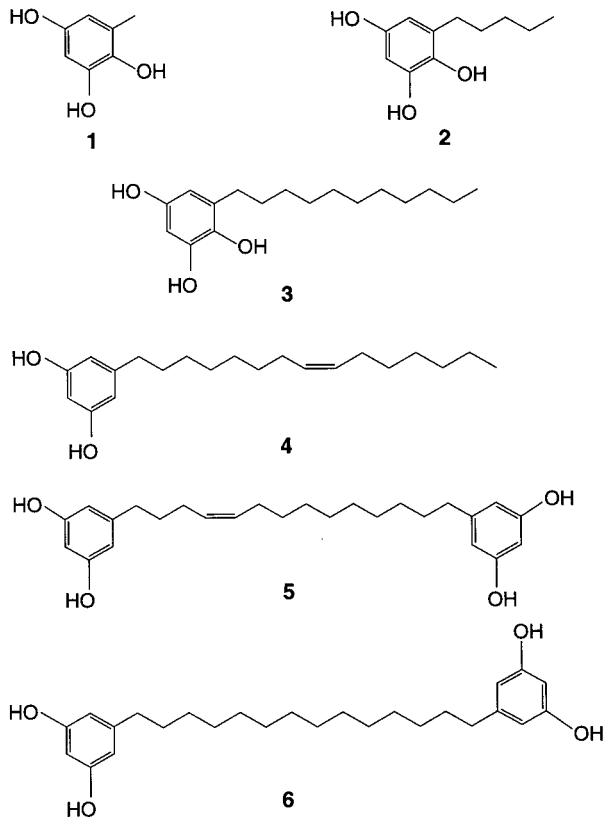

FIGURE 1: Structures of compounds **1**−**6**.

are capable of effecting DNA strand scission in the presence of Cu^{2+} (*25*−*28*), it seemed possible that some 5-alkylresorcinol derivatives might be able to mediate damage to chromosomal DNA and also block repair of the formed lesions. Presently, we describe the characterization of compounds **3**−**6** as DNA polymerase β inhibitors. In addition to their ability to inhibit this enzyme, we show that these compounds mediate Cu^{2+}-dependent DNA cleavage. These two activities are demonstrated to occur in a cell-free system and in cell culture. Compounds **3**−**6** potentiated the action of bleomycin in cell culture through inhibition of DNA polymerase β. Compounds **3** and **5** also caused reduction in the numbers of viable cultured cells upon admixture of Cu^{2+}, suggesting that they are capable of both inducing DNA damage and inhibiting its repair.

EXPERIMENTAL PROCEDURES

General Methods and Materials. Compounds **1**−**6** were dissolved in 1:1 DMSO−methanol for the DNA polymerase β inhibition assay and in 100% DMSO for kinetic studies. Compounds **3**−**6** were dissolved in 100% DMSO for the DNA cleavage assay and in 100% DMSO for the cell culture studies, with a final DMSO concentration of 0.25% in each culture. Recombinant rat liver DNA polymerase β was a gift from Xiangyang Wang and Hongge Wang, prepared as described previously (*29*, *30*). Antibiotic antimycotic solution, Hanks' balanced salt solution, Dulbecco's modified Eagle's medium containing 4500 mg of glucose/L, unlabeled dNTPs, and calf thymus DNA were purchased from Sigma Chemical Co.; calf thymus DNA was activated by the method described previously (*31*). [^{3}H]dTTP was purchased from ICN Pharmaceuticals. DEAE-cellulose paper (DE-81) was from Whatman. The CCL 46 certified cell line P388D_1 (mouse, lymphoid neoplasm) was purchased from American Type Culture Collection. Trypan blue dye and donor horse serum were from Gibco−BRL. Blenoxane, the clinically used mixture of bleomycins consisting predominantly of BLM A_2 and BLM B_2, was a gift from Bristol Myers Squibb Pharmaceuticals.

Agarose gels were quantified by use of Molecular Dynamics ImageQuant version 5.0 software. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

Active Principles. Compounds **1** and **2** were prepared as described previously (*28*). Compounds **3** and **4** were isolated from *Hakea trifurcata* (*25*, *27*) and **5** and **6** were isolated from *P. rubescens* (*17*) as reported previously. None of the compounds is entirely stable under ambient conditions; each was purified by HPLC for the experiments described.

DNA Polymerase β Inhibition Assay. To 50 μL of 62.5 mM 2-amino-2-methyl-1,3-propanediol buffer, pH 8.6, containing 10 mM $MgCl_2$, 1 mM DTT, 100 μg/mL BSA, 6.25 μM dNTPs including 0.04 Ci/mmol [^{3}H]dTTP, and 0.25 mg/mL activated calf thymus DNA was added 6 μL of a solution containing each test compound and 4 μL of recombinant rat liver DNA polymerase β preparation (6.9 units, 4.8×10^4 units/mg). After incubation at 37 °C for 60 min, the radiolabeled DNA product was collected on DEAE-cellulose paper (DE-81), dried, and rinsed successively with 0.4 M K_2HPO_4, pH 9.4, and 95% ethanol for radioactivity determination. For the kinetic studies, inhibitor constants (K_{is} and K_{ii}) were obtained by the same assay except incubation was for 20 min and the samples were immediately placed on ice before application to DE-81 paper. Additionally, dNTP concentrations were varied [25, 12.5, 6.25, 3.12, and 1.56 μM ([^{3}H]dTTP was added in proportion, such that its specific activity remained constant)] or the activated DNA concentration was varied (200, 100, 50, 25, and 12.5 μM nucleotide concentration).

DNA Cleavage Assay. Cleavage of pSP64 plasmid DNA was carried out in 25-μL reaction mixtures (total volume) containing 800 ng of DNA and the indicated amounts of $CuCl_2$ and compounds **3**−**6** in 10 mM Tris-HCl, pH 7.2. The reactions were incubated at 37 °C for 60 min, terminated by the addition of 5 μL of 0.125% bromophenol blue in 30% glycerol, and applied to a 1.0% agarose gel containing 0.7 μg/mL ethidium bromide. The gel was run in 89 mM Tris with 8.9 mM boric acid and 2.0 mM Na_2EDTA at 150 V for 2.5 h.

Assay for Effect of Bleomycin on Numbers of Viable Cells. P388D_1 cells were maintained as suspension cultures in 90% (v/v) Dulbecco's modified Eagle's medium containing 4500 mg of glucose/L, 10% (v/v) donor horse serum, and 100 IU of penicillin, 0.1 mg of streptomycin, and 0.25 μg of amphotericin B per milliliter at 37 °C in a 5% CO_2 in air atmosphere. To tissue culture flasks (having a total capacity of 12.5 mL) were added 5 mL of cell suspension containing $\sim 5 \times 10^5$ cells/mL; this was incubated for 1 h to stabilize the cells. Assays were carried out in 6-mL cultures containing the desired amounts of test compound and blenoxane (dissolved in ≤0.5 mL of medium). Cultures were incubated at 37 °C in a 5% CO_2 in air atmosphere for 6 h, followed by cell viability determination by trypan blue exclusion.

Assay in Mammalian Cells Using Copper. P388D_1 cells were maintained as described above for the BLM assay. To

Table 1: Kinetic Inhibition Constants Determined from the DNA Polymerase β Inhibition Assay[a,b]

compound	K_{is} (DNA)	K_{ii} (DNA)	K_{is} ([^{3}H]dTTP)	K_{ii} ([^{3}H]dTTP)
1	ND[c]	ND	ND	ND
2	ND	ND	ND	ND
3	7.7 ± 1.8	18 ± 2.0	64 ± 3.2	3.5 ± 1.2
4	17 ± 1.5	2.8 ± 2.0	37 ± 4.8	4.0 ± 1.4
5	1.3 ± 0.6	1.5 ± 1.0	5.4 ± 1.8	5.5 ± 4.3
6	4.3 ± 0.6	11 ± 1.8	42 ± 16	6.4 ± 3.9

[a] Reported as micromolar concentrations. [b] Standard error determined from a set of three experiments (mean ± SE). [c] ND, not determined.

12.5-mL tissue culture flasks was added 5 mL of cell suspension containing $\sim 5 \times 10^5$ cells/mL; this was incubated for 1 h to stabilize the cells. Assays were carried out in 6-mL cultures containing the indicated amounts of test compound and $CuCl_2$ (added from aqueous solutions of $\leq$ 100 μL volume). Cultures were incubated at 37 °C in a 5% CO_2 in air atmosphere for 18 h followed by cell viability determination by trypan blue exclusion.

Copper- and BLM-Dependent Mammalian Cell Culture Assay. P388D_1 cells were maintained as described above for the BLM assay. To 12.5-mL tissue culture flasks was added 2.5 mL of cell suspension at an initial concentration of $\sim 5 \times 10^5$ cells/mL; the cultures were incubated for 1 h to stabilize the cells. Assays were carried out in 3-mL cultures containing the indicated amounts of compound, blenoxane (dissolved in $\leq$0.5 mL of medium), and $CuCl_2$ (dissolved in $\leq$50 μL of water). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere for 6 h, followed by determination of cell viability by trypan blue exclusion.

RESULTS

IC_{50} Value Determination, Mode of Inhibition, and Kinetic Inhibition Constants. The IC_{50} values for compounds **1**−**6** obtained from the DNA polymerase β assay showed that the bis(5-alkylresorcinols) (**5** and **6**) were apparently the most potent inhibitors of DNA polymerase β (IC_{50} values of 7.5 and 5.8, respectively). Compounds **3** and **4**, having IC_{50} values of 13 and 14 μM, respectively, were apparently somewhat less potent as DNA polymerase β inhibitors. Compounds **1** and **2** did not inhibit DNA polymerase β at any tested concentration up to 250 μM.

Lineweaver−Burk plots revealed that compounds **3**, **4**, and **6** showed mixed inhibition with respect to activated DNA (Table 1). For **3** and **6** (Figure 2), the inhibition was intermediate between those for competitive and noncompetitive inhibition (*32*). In this situation, the K_{is} value (enzyme−inhibitor dissociation constant) was smaller than the K_{ii} value (enzyme−substrate−inhibitor dissociation constant), indicating that these inhibitors bound more strongly to DNA polymerase β alone than to the polymerase β−DNA complex (Table 1). Conversely, compound **4** had a smaller K_{ii} value, which indicated that the stronger interaction occurred when the enzyme was bound to its DNA substrate. In this case, the pattern of inhibition was intermediate between those for noncompetitive and uncompetitive inhibition. Compound **5** showed similar K_{is} and K_{ii} values (1.3 ± 0.6 and 1.5 ± 1.0 μM, respectively) demonstrating comparable association with the enzyme alone or with the enzyme−DNA binary complex, i.e., a pure noncompetitive pattern of inhibition (*32*).

The inhibition with respect to [^{3}H]dTTP for compounds **3**, **4**, and **6** was also mixed inhibition (Table 1, Figure 3). For these compounds, the K_{ii} values were significantly lower than the K_{is} values, indicating that the stronger association was for DNA polymerase β complexed with both DNA and dTTP. Compound **5** had nearly identical K_{is} and K_{ii} values with respect to [^{3}H]dTTP, again reflecting a pure noncompetitive pattern of inhibition.

DNA Cleavage Assay. An in vitro assay using pSP64 plasmid DNA was employed to test compounds **3**−**6** for their ability to cleave DNA in the absence and presence of Cu^{2+}. Relaxation of supercoiled pSP64 plasmid DNA was observed for compounds **3**−**5**, but only in the presence of Cu^{2+} (Figure 4). Compound **3** showed dose-dependent cleavage with 88% (form II) and 12% (form III) cleavage at 100 μM concentration and 89% (form II) and 2% (form III) cleavage at 50 μM concentration. Compound **5** was the next most potent agent, affording 56 and 45% cleavage (form II) at 100 and 50 μM concentrations, respectively. Compound **4** was slightly less potent [30% and 23% cleavage (form II) at 100 and 50 μM concentrations, respectively]; compound **6** cleaved DNA weakly, if at all. Indeed, a previous investigation in our laboratory showed that compounds such as 6-alkyl-1,2,4-trihydroxybenzene **3** were 50−100 times more efficient than the respective 5-alkyl-1,3-dihyroxybenzenes (5-alkylresorcinols) (**4**) as agents for Cu^{2+}-mediated DNA strand scission (*28*). However, there are no reports that compare the potencies of mono- versus bis(5-alkylresorcinols) or the potencies of the saturated versus unsaturated bis(5-alkylresorcinols) for DNA cleavage in the presence of Cu^{2+}. The present results show that the unsaturated bis(5-alkylresorcinol) **5** was more potent than mono-5-alkylresorcinol **4** and also more potent than the respective saturated species (**6**) in the DNA strand scission assay. That compound **6** cleaved DNA less efficiently than **5** is consistent with our earlier finding that unsaturation within the alkyl substituent tends to enhance efficiency of DNA cleavage (*26*). It should be noted, however, that the DNA cleavage observed for compounds **4**−**6** must require the conversion of each to the respective trihydroxylated benzenes (*28*) and there can be no assurance that this conversion occurred to the same extent for each compound.

Potentiation of the Ability of Bleomycin To Reduce the Numbers of Viable Cells. Cell culture studies with a transformed cell line (P388D_1) were carried out to further characterize compounds **3**−**6** (Figure 5). These experiments investigated the ability of compounds **3**−**6** to potentiate the effects of bleomycin, when both types of agents were used concurrently at concentrations of each that were nontoxic. Bleomycin is an antitumor antibiotic used clinically for the treatment of certain tumors (*33*).

In our assay, the agent that produced the greatest reduction in the number of viable cells was compound **6** (30% decrease) when the cells were treated concurrently with 10 μM **6** and 75 nM bleomycin. Compounds **4** and **5** exhibited slightly less potency (22% and 19% decreases in numbers of viable cells, respectively). Compound **3** was the least potent, producing only a 16% decrease in the number of viable cells. Thus compounds **3**−**6** all potentiated the effects of BLM in reducing the number of viable cells when employed at a concentration (10 μM) that had no observable effect on the cells in the absence of BLM. It may be noted, however, that sublethal damage could not have been detected in this assay system. That the mechanism of potentiation by

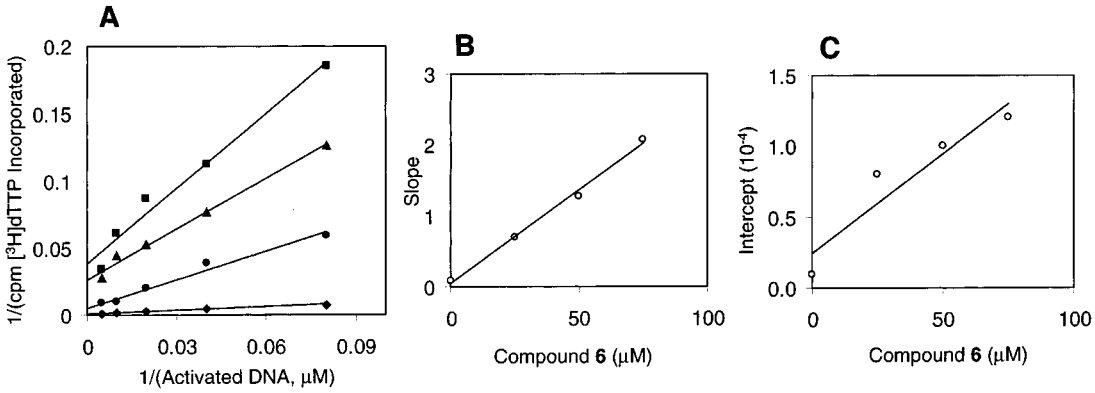

FIGURE 2: Kinetic analysis of compound **6** with respect to activated DNA. (A) Primary data are displayed in a Lineweaver−Burk plot: (◆) 0, (●) 25, (▲) 50, and (■) 75 μM **6**. Replots of the slopes and intercepts from the double-reciprocal plot were constructed for determination of K_{is} (B) and K_{ii} (C) values, respectively. The plots represent one trial from a set of three experiments.

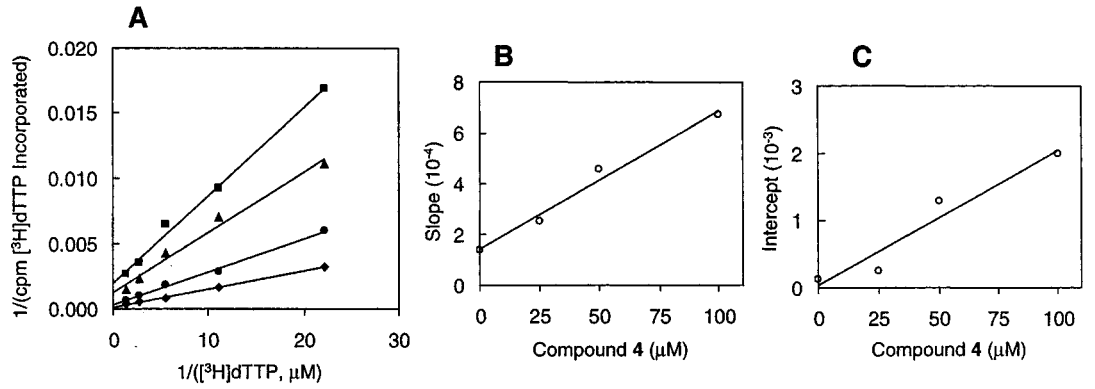

FIGURE 3: Kinetic analysis of compound **4**, with respect to [^{3}H]dTTP. (A) Primary data are displayed in a Lineweaver−Burk plot: (◆) 0, (●) 25, (▲) 50, and (■) 100 μM **4**. Replots of the slopes and intercepts from the double-reciprocal plot were constructed for determination of K_{is} (B) and K_{ii} (C) values, respectively. The plots represent one trial from a set of three experiments.

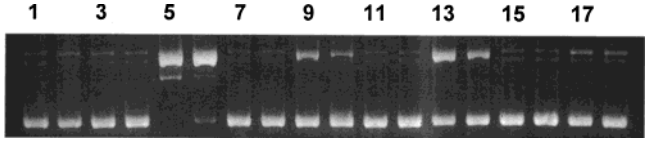

FIGURE 4: Strand scission of supercoiled pSP64 plasmid DNA by compounds **3**−**6** in the absence and presence of Cu^{2+}. DNA samples were analyzed on a 1% agarose gel with 0.7 μg/mL ethidium bromide (150 V, 2.5 h). Lane 1, DNA alone; lane 2, 100 μM Cu^{2+}; lane 3, 100 μM **3**; lane 4, 50 μM **3**; lane 5, 100 μM **3** + 100 μM Cu^{2+}; lane 6, 50 μM **3** + 100 μM Cu^{2+}; lane 7, 100 μM **4**; lane 8, 50 μM **4**; lane 9, 100 μM **4** + 100 μM Cu^{2+}; lane 10, 50 μM **4** + 100 μM Cu^{2+}; lane 11, 100 μM **5**; lane 12, 50 μM **5**; lane 13, 100 μM **5** + 100 μM Cu^{2+}; lane 14, 50 μM **5** + 100 μM Cu^{2+}; lane 15, 100 μM **6**; lane 16, 50 μM **6**; lane 17, 100 μM **6** + 100 μM Cu^{2+}; lane 18, 50 μM **6** + 100 μM Cu^{2+}.

these species involved inhibition of DNA polymerase β was consistent with the finding that compound **6**, the most potent DNA polymerase β inhibitor, also potentiated the action of BLM the most effectively.

Copper-Dependent Reduction in the Numbers of Viable Cells. The in vitro DNA cleavage assays with Cu^{2+} prompted us to investigate the possibility that compounds **3**−**6** also could reduce the numbers of viable P388D$_1$ cells by direct DNA cleavage. Since the DNA cleavage activity of all four compounds was found to be Cu^{2+}-dependent (Figure 4), the ability of Cu^{2+} to potentiate the effects of **3**−**6** in mammalian cell culture was studied. Accordingly, the cells were incubated separately with Cu^{2+} and test compounds at concentrations of each that produced little change in the numbers of viable cells and then with the combination of the two (Figure 6). Compound **3** produced the greatest decrease in number of viable cells (35%) in the presence of Cu^{2+}, while **4** and **5** were slightly less active (29% and 25% decrease in cell

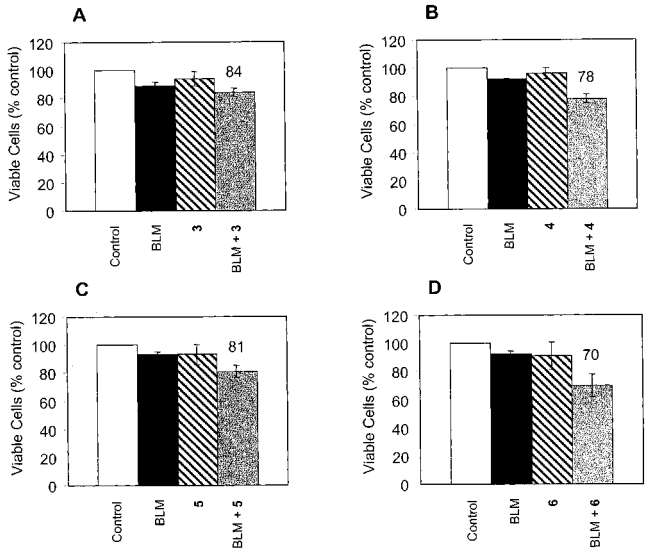

FIGURE 5: Effects of compounds **3**−**6** on BLM-induced reduction in the number of viable P388D$_1$ cells. Cells were treated as described for 6 h. Viability was assessed by trypan blue exclusion. White bars, control (no treatment); black bars, 0.075 μM blenoxane; hatched bars, 10 μM test compound; dark gray bars, 10 μM test compound + 0.075 μM BLM. Results are presented as percent of control (untreated) cells and standard error (mean ± SE), determined from three experiments. Panels A−D utilized compounds **3**−**6**, respectively.

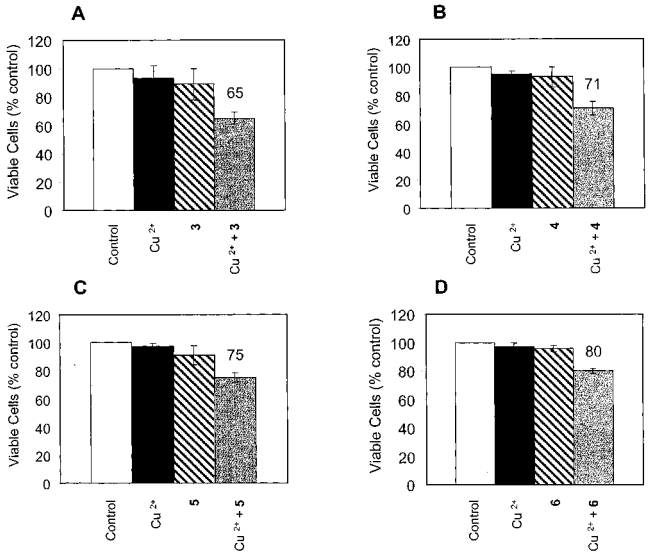

FIGURE 6: Effects of compounds **3**−**6** plus Cu^{2+} on $P388D_1$ cells. Cells were treated as described for 18 h. Viability was assessed by trypan blue exclusion. White bars, control (no treatment); black bars, 40 μM Cu^{2+}; hatched bars, 10 μM test compound; dark gray bars, 10 μM test compound + 40 μM Cu^{2+}. Results are presented as percent of control (untreated) cells and standard error (mean ± SE), determined from three experiments. Panels A−D utilized compounds **3**−**6**, respectively.

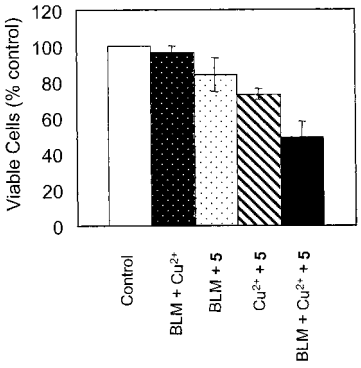

FIGURE 7: Effect of compound **5** plus Cu^{2+} on BLM-induced reduction in the numbers of viable $P388D_1$ cells. Cells were treated as described for 6 h. Viability was assessed by trypan blue exclusion. White bar, control (no treatment); black bar with white dots, 0.075 μM BLM + 40 μM Cu^{2+}; white bar with black dots, 0.075 μM BLM + 10 μM **5**; hatched bar, 40 μM Cu^{2+} + 10 μM **5**; black bar, 0.075 μM BLM + 40 μM Cu^{2+} + 10 μM **5**. Results are presented as percent of control (untreated) cells (mean ± SE), determined from three experiments.

numbers, respectively). Compound **6** afforded the smallest decrease in numbers of viable cells (20%). This pattern closely resembles the order of potencies observed for DNA strand scission in the in vitro DNA cleavage assay; compound **3** was the most potent and compound **6** was the least potent DNA cleaving agent in the presence of Cu^{2+} (Figure 4).

Potentiation of Reduction in Numbers of Viable Cells by a Dual Mechanism. Since compounds **3**−**6** can mediate Cu^{2+}-dependent DNA strand scission and inhibit its repair, it seemed possible that the effects on cell growth inhibition might well reflect this dual mechanism. Therefore, experiments were carried out to determine whether alkylresorcinols that inhibited DNA polymerase β exhibited an enhanced reduction in the numbers of viable cells under conditions that also permitted these compounds to effect DNA damage. The contributions of these effects to the reduction in numbers of viable cells was also compared to that produced by potentiation of the reduction in numbers of viable cells by BLM through inhibition of the repair of BLM-induced DNA damage. As shown for compound **5** (Figure 7 and Supporting Information Figure 1), in the presence of both 75 nM BLM and 40 μM Cu^{2+} the number of viable cells was 96% of that in the untreated control; separate incubations with BLM + **5** and Cu^{2+} + **5** reduced the number of viable cells to 84% and 73% of the control, respectively. However, when BLM + Cu^{2+} + 10 μM **5** were added together, the number of viable cells was reduced to 49%. Thus, the data are consistent with the interpretation that the observed diminution in the numbers of viable cells may be due to the additive effects of DNA cleavage by BLM and by **5**, as well as to the inhibition of repair of the DNA damage induced by these agents. Likewise, compound **3** was a strong DNA cleaving agent and also inhibited DNA polymerase β; the reduction in numbers of viable cells was greater in the presence of BLM + Cu^{2+} + **3** than with either BLM + **3** or Cu^{2+} + **3** (Supporting Information Figure 2). In comparison, no consistent effect of Cu^{2+} in potentiating the effects of BLM + **4** or BLM + **6** could be observed.

DISCUSSION

DNA polymerase β and other important DNA repair enzymes have been studied intensively to define their possible roles in cellular resistance to cancer chemotherapeutic agents. Selective inhibition of DNA polymerase β has been suggested as a strategy for increasing the effectiveness of chemotherapeutic agents by preventing repair of the damaged DNA (*17*−*19*, *24*, *34*, *35*). Consistent with this thesis, DNA polymerase β has been shown to be overexpressed in cells exposed to DNA-damaging agents such as cisplatin, melphalan, and mechlorethamine (*4*).

As noted above, a related strategy might involve the development of therapeutic agents capable of damaging DNA and simultaneously blocking the repair of that DNA damage through inhibition of DNA polymerase β. Our studies indicate that compounds **3**−**6** are agents that possess such dual activities: Cu^{2+}-mediated DNA cleavage activity and inhibition of DNA polymerase β to block repair of the damaged DNA. Further, the lack of cell growth inhibition by compounds **3**−**6** in the absence of BLM implies that the agents do not interfere with other enzymic activities under the conditions tested, at least not to the extent that would affect cell growth.

The IC_{50} values for **3**−**6** indicate that these compounds are good inhibitors of DNA polymerase β. Since compounds **1** and **2**, analogues of **3** but with shorter alkyl substituents (*28*), did not inhibit DNA polymerase β, it is evident that the side chain and its length are essential elements for DNA polymerase β inhibition. It may be noted that a series of anacardic acids and related species have been found to inhibit DNA polymerase β; these compounds also have hydroxylated benzene rings attached to alkyl substituents of lengths comparable to those present in compounds **4**−**6** (*28*). These compounds also potentiated the effects of bleomycin and cisplatin in short- and long-term cell culture experiments and blocked unscheduled DNA synthesis induced by bleomycin and cisplatin (*24*, *36*). Numerous additional naturally occur-

ring inhibitors of polymerase β were found to have comparable effects, arguing strongly that the effects observed here actually are due to the ability of **3**−**6** to potentiate the effects of bleomycin.

Detailed steady-state kinetic analysis (Table 1, Figure 2) revealed that compounds **3**, **4**, and **6** show mixed inhibition with respect to the DNA primer−template (activated DNA). Compounds **3** and **6**, the two saturated analogues, bound more tightly to DNA polymerase β when the enzyme was not complexed with DNA (lower K_{is} values as compared to the K_{ii} values). Compound **4**, an unsaturated mixed inhibitor, appeared to associate more strongly with the enzyme when it was complexed with activated DNA (lower K_{ii} value). Compound **5**, in contrast, was purely noncompetitive with respect to activated DNA.

Kinetic inhibition analysis with respect to dTTP showed that compounds **3**, **4**, and **6** were also mixed inhibitors (Figure 3); association with the enzyme was most efficient when it was complexed to (DNA and) dTTP (*37*). DNA polymerase β catalyzes template-directed nucleotidyl transfer reactions. Crystal structures of DNA polymerase β (*38*, *39*) have aided in the conclusion that prior to DNA template−primer association, DNA polymerase β is in an "open conformation" with the three subdomains of the 31 kDa polymerase domain positioned so as to enclose a U-shaped cleft. DNA binding causes the protein to adopt a "closed doughnutlike conformation" in which the 8 kDa domain with 5′-dRP excision activity (*15*, *16*) associates with the C-terminal thumb domain (*40*). This conformational change in part encourages formation of the active site for nucleotide triphosphate incorporation. Our data suggest that the presence of a nucleotide triphosphate in the active site was not required for drug association for compounds **3**, **4**, and **6**, as seen from the K_{is} values with respect to dTTP, but that the strength of association with these inhibitors is enhanced by the presence of dTTP, as seen from the smaller K_{ii} values overall for compounds **3**, **4**, and **6** with respect to dTTP. The exception was compound **5**, which was purely noncompetitive with respect to dTTP.

The ability to mediate DNA strand scission was another activity investigated for compounds **3**−**6** (Figure 4). Compound **3**, the 6-alkyl-1,2,4-trihydroxybenzene, was the most potent DNA cleaving agent (Figure 4). This was expected, since it is believed to be the trihydroxybenzene moiety that catalyzes Cu^{2+}-dependent DNA strand scission, as shown previously (*28*). According to this study the resorcinols, such as **4**, first require oxygenation of the benzene nucleus to yield a reactive trihydroxylated benzene (*28*). Indeed, 5-alkylresorcinol **4** induced significantly less DNA cleavage than **3**, but again only in the presence of Cu^{2+}. Interestingly, unsaturated bis(5-alkylresorcinol) **5** mediated significantly more cleavage than the unsaturated mono-5-alkylresorcinol (**4**). Further, **5** was also more efficient in mediating DNA cleavage than its saturated counterpart **6**. These results suggest that, for the resorcinols, the presence of two benzene moieties enhances the efficiency of DNA cleavage; either of the two benzene rings can putatively undergo oxygenation to afford a trihydroxylated alkylbenzene, which subsequently reduces Cu^{2+} to produce more (on a molar basis) of the reactive oxygen species that are believed to actually effect DNA strand scission (*28*). Additionally, unsaturation in the alkyl substituent of the bis(5-alkylresorcinols) may also facilitate DNA cleavage. Since several DNA minor-groove binding agents have been shown to associate with DNA via hydrophobic interactions (*41*−*46*), it is possible that the double bond imparts an orientation to the alkyl substituent conducive to hydrophobic association in the minor groove of DNA.

Cell culture studies provide a means for evaluating the effects of specific biochemical mediators in a cellular context. Using $P388D_1$ mouse lymphoid neoplasm cells, compounds **3**−**6** were tested to determine the extent to which they could potentiate a reduction in the number of viable cells by BLM (Figure 5). The bleomycins are glycopeptide-derived antitumor antibiotics originally isolated from cultures of *Streptomyces verticillus* (*47*). The bleomycins are believed to mediate their antitumor effects by mediating DNA strand scission (*48*). DNA polymerase β, a 39 kDa gap-filling enzyme involved in base excision repair (*11*, *15*, *16*), has been reported to be responsible for the repair of single-strand breaks caused by bleomycin (*8*−*10*). Since compounds **3**−**6** were found to inhibit DNA polymerase β in a cell-free system, it seemed logical to anticipate that they might potentiate effects of BLM in $P388D_1$ cells by inhibiting this repair enzyme following BLM-mediated DNA damage. Several DNA polymerase β inhibitors have been identified to date (*12*, *17*−*24*, *34*, *35*); anacardic acid and three structurally related derivatives (*24*), as well as mispyric acid (*18*) and a number of other naturally occurring agents, have been shown to potentiate the effects of DNA-damaging agents, such as BLM, in cell culture. Compounds **3**−**6** were utilized to potentiate the action of BLM as a cytotoxic agent. All four compounds were utilized at 10 μM concentration, at which they lacked any ability to reduce the number of viable $P388D_1$ cells in culture. When coadministered with a nontoxic dose of BLM (75 nM), each of the four compounds (**3**−**6**) reduced the number of viable cells after a 6-h incubation. It may be noted that the greatest and least effects were promoted by compounds **6** and **3**, respectively. These were also the strongest and weakest DNA polymerase β inhibitors, supporting the interpretation that diminution of the numbers of viable cells resulted from inhibition of the repair of BLM-mediated DNA damage in the treated cells.

Cell culture assays were also carried out in the presence of added Cu^{2+} to determine whether compounds **3**−**6** could also diminish the numbers of viable cells by mediating Cu^{2+}-dependent DNA cleavage in the treated cells (Figure 6). Under these conditions, compound **3** was quite effective in reducing the numbers of viable cells, in keeping with its exceptional potency as a DNA cleavage agent (Figure 4). Compounds **4** and **5** were less potent, while compound **6** was the least potent, as expected.

The treatment of $P388D_1$ cells concurrently with bleomycin, Cu^{2+}, and compounds **3**−**6** were carried out to investigate whether they exhibited effects in cell culture consistent with their abilities to both damage DNA and block polymerase β-mediated DNA repair. Those compounds that cleaved DNA most efficiently in the in vitro assay (**3** and **5**, Figure 4) significantly reduced the numbers of viable cells when the cells were treated simultaneously with BLM, Cu^{2+}, and otherwise noninhibitory concentrations of the compounds (Figure 7 and Supporting Information Figure 2). In comparison, Cu^{2+} had no consistent effect on the potentiation of BLM-mediated diminution of cell viability by compounds

4 or **6**. Thus the cellular effects of those compounds appear to be due predominantly to their activities as DNA polymerase β inhibitors. The notable dissimilarity in activity between **3** and **5** as compared to **4** and **6** argues that trihydroxyalkybenzene **3** and unsaturated bis(dihydroxyalkybenzene) **5** act in cell culture to both induce copper-dependent DNA damage and inhibit the repair of such lesions. The in vitro DNA cleavage data and the cell culture experiments, along with the low IC_{50} value, argue that compound **5** was the most effective agent for mediating copper-dependent DNA damage as well as blocking the repair of this DNA damage through inhibition of DNA polymerase β.

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SUPPORTING INFORMATION AVAILABLE

Two figures showing additional cell culture bioassay data for compounds **3** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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