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Bioorganic & Medicinal Chemistry 12 (2004) 4253–4258
Bioorganic & Medicinal Chemistry

Plant sterols as selective DNA polymerase β lyase inhibitors and potentiators of bleomycin cytotoxicity

Shi-Sheng Li, Zhijie Gao, Xizhi Feng, Shannon H. Jones and Sidney M. Hecht*

Departments of Chemistry and Biology, University of Virginia, Charlottesville, VA 22901, USA

Received 6 March 2004; accepted 2 May 2004
Available online 15 June 2004

Abstract—In a survey of crude plant extracts for DNA polymerase β lyase inhibitors, the hexanes extracts of *Cladogynus orientalis*, *Hymenache donacifolia*, and *Heteropsis integerrima*, and the methyl ethyl ketone extract of *Acacia pilispina* were found to exhibit good inhibition of the dRP lyase activity of DNA polymerase β. Bioassay-guided fractionation of these extracts led to the isolation of three DNA polymerase β lyase inhibitory phytosterols, namely stigmasterol (**1**) and β-sitosterol (**2**), isolated from the hexanes extracts, and β-sitosterol-β-D-glucoside (**3**), isolated from the methyl ethyl ketone extract. Compounds **1**–**3** inhibited the DNA polymerase β lyase activity with IC_{50} values of 43.6, 43.3, and 72.4 μM, respectively. Compounds **1** and **2** were found capable of potentiating the action of bleomycin in cultured human tumor cells, consistent with the possibility that lyase inhibitors may find utility in vivo.

1. Introduction

Many chemotherapeutic regimens currently in use for the treatment of cancers depend at least in part on the use of DNA damaging agents.[1] The clinical efficacy of these agents is often severely impaired by cellular resistance mechanisms. For example, DNA damage repair mediated through the base excision repair (BER) pathway in cancer cells plays an important role.[2] Cells contain three types of excision repair mechanism that act on specific DNA lesions, namely BER, nucleotide excision repair (NER), and mismatch repair (MMR).[3] The DNA BER pathway is the predominant repair pathway that protects cells from the deleterious effects of DNA nucleobase damage resulting from base oxidization or alkylation.[4] BER is a multi-step process that is initiated by the removal of modified base by a DNA glycosylase, followed by DNA backbone incision 5′ to the lesion by an apurinic/apyrimidinic (AP) endonuclease. Subsequently, the residual 2-deoxyribose phosphate (dRP) moiety at the 5′-end of the cleaved strand can be either removed by a dRP lyase activity or displaced by DNA polymerase action, leading to 'single-nucleotide' or 'long patch' repair, respectively.[3–5] DNA polymerase β is the major DNA polymerase involved in 'single-nucleotide' BER of lesions in the nuclear DNA of tumor cells produced by anticancer agents such as bleomycin, cisplatin, neocarzinostatin, and mono-functional DNA alkylating agents.[6–20]

DNA polymerase β is the smallest eukaryotic polymerase and is composed of a single 39-kDa-polypeptide chain containing 335 amino acid residues, which can be proteolyzed into two specialized N- and C-terminal domains.[3] Functionally, the 31 kDa C-terminal domain contains the polymerase activity, including double-stranded DNA (dsDNA)-binding by a helix–hairpin–helix motif, nucleotidyl transferase activity, and deoxynucleotide triphosphate (dNTP) selection.[21] The 8 kDa N-terminal domain also mediates a number of functions, including dRP lyase activity, single-stranded DNA binding and monovalent metal binding, as well as gapped dsDNA binding.[4,22–25] Compelling evidence indicates that both the polymerase and 5′-dRP lyase activities of DNA polymerase β are essential for the mammalian 'single-nucleotide (short-patch)' BER in vivo,[8] and that the 5′-dRP lyase activity is the rate-limiting step during short-patch BER.[4,6,7,21,22]

Recent studies of the catalytic mechanism of 5′-dRP lyase of DNA polymerase β by site-directed mutagenesis and MS/MS mapping of the trapped covalently

Keywords: Sterols; Nucleic acids; Enzyme inhibitors; Chemotherapy.
* Corresponding author at present address: Department of Chemistry, University of Virginia, Charlottesville, VA 22901, USA. Tel.: +1-434-924-3906; fax: +1-434-924-7856; e-mail: sidhecht@virginia.edu

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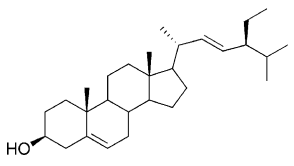
doi:10.1016/j.bmc.2004.05.016

cross-linked protein–DNA complex intermediate formed between the primary amine of Lys72 in the 8 kDa domain of DNA polymerase β and the aldehydic C-1′ carbonyl group of AP DNA, have provided direct evidence in support of a proposed β-elimination catalytic mechanism, and unequivocally assigned Lys72 as the enzymic participant in Schiff base formation.[26–29] This type of uncatalyzed β-elimination reaction ($t_{1/2} \geqslant 2$ h in vitro) is a rather slow process,[22] suggesting that the dRP lyase activity of DNA polymerase β in tumor cells may be pivotal to the short-patch BER involved in the resistance of cancer cells to DNA damaging anticancer agents. Recently, it was found that only the 5′-dRP lyase activity of DNA polymerase β was required to reverse methylating agent (methyl methanesulfonate, MMS) induced hypersensitivity in DNA polymerase β null cells.[6] Thus, there is an obvious need for DNA polymerase β inhibitors that specifically target the 5′-dRP lyase activity. In contrast, the DNA polymerase β inhibitors described to date were selected as inhibitors of the polymerase activity of the enzyme.[30–32]

During a survey of plant metabolites for inhibitors of the 5′-dRP lyase activity of DNA polymerase β, the hexanes extracts of *Cladogynus orientalis* (Euphorbiaceae), *Hymenache donacifolia* (Poaceae), and *Heteropsis integerrima* (Araceae), and the methyl ethyl ketone extract of *Acacia pilispina* (Leguminosae), were found to exhibit good inhibition to the dRP lyase activity of DNA polymerase β. Subsequent bioassay-guided fractionation of these extracts, using an assay to detect DNA polymerase β 5′-dRP-excision (lyase) inhibition, resulted in the isolation of three DNA polymerase β lyase inhibitory phytosterols, namely stigmasterol (**1**) and β-sitosterol (**2**) from *C. orientalis*, *H. donacifolia* and *H. integerrima*, and β-sitosterol-β-D-glucoside (**3**) from *A. pilispina*. Compounds **1**–**3** exhibited inhibitory activity against DNA polymerase β lyase with IC_{50} values of 43.6, 43.3, and 72.4 μM, respectively. Reported herein are the isolation of compounds **1**–**3** and their in vitro characterization as DNA polymerase β lyase inhibitors.



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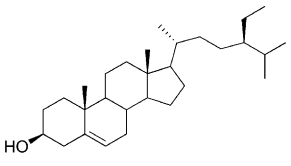

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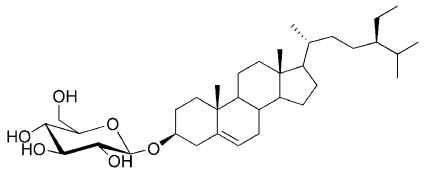

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2. Results and discussion

The crude plant materials were soaked successively with hexanes, methyl ethyl ketone, methanol, and water. The hexane extracts of *C. orientalis*, *H. donacifolia* and *H. integerrima*, and the methyl ethyl ketone extract of *A. pilispina*, were found to inhibit the dRP lyase activity of DNA polymerase β. Diaion HP-20 (a macroreticular-divinylbenzene co-polymer) and diol (a type of porous silica particles with chemically bonded dihydroxypropyl groups on the surface) resins were employed in the bioassay-guided multi-step fractionation of the hexanes crude extracts, which provided the inhibitors **1** and **2**. The methyl ethyl ketone extract of *A. pilispina* was fractionated initially on a polyamide 6S column, which was washed successively with H_2O, 1:1 H_2O–MeOH, 1:4 CH_2Cl_2–MeOH, 1:1 CH_2Cl_2–MeOH, and 9:1 MeOH–NH_4OH. The 1:4 CH_2Cl_2–MeOH and the 9:1 MeOH–NH_4OH fractions strongly inhibited the lyase activity of DNA polymerase β. The final fraction contained polyphenols, which tend to bind DNA and protein non-specifically,[32–34] and are not specific inhibitors of the enzyme. Thus, the 1:4 CH_2Cl_2–MeOH fraction was fractionated further on an HP-20 column, which was washed successively with 40% aq MeOH, 60% aq MeOH, 80% aq MeOH, 90% aq MeOH, and MeOH. The MeOH fraction showed the strongest inhibition of the DNA polymerase β lyase activity, and deposited crystals of pure **3** from 1:1 CH_2Cl_2–MeOH.

Through comparison of their ^1H and ^{13}C NMR, and MS spectral data with literature reports, the chemical structures of the isolated inhibitors were determined to be stigmasterol (**1**),[35–37] β-sitosterol (**2**),[35–37] and β-sitosterol-β-D-glucoside (**3**).[38,39] The optical rotations were $[\alpha]_D^{25}$ −52.3 (*c* 0.1, $CHCl_3$) for **1**; $[\alpha]_D^{25}$ −35.8 (*c* 0.1, $CHCl_3$) for **2**; $[\alpha]_D^{25}$ −48.2 (*c* 0.1, $CHCl_3$) for **3**.

Compounds **1**–**3** are structural analogues and belong to the same class of phytosterols. They displayed inhibition of the dRP lyase activity of DNA polymerase β in the in vitro assay employed (Fig. 1A) in a dose-dependent manner (exemplified in Fig. 1B). Their IC_{50} values were 43.6, 43.3, and 72.4 μM, respectively (Table 1). The virtually identical inhibitory potencies of compounds **1** and **2** argue that the presence of the olefin at the C-17 side chain of compound **1** is not a determinant of the inhibitory potential. The rather different IC_{50} values for compound **2** versus **3** indicates that the 3-hydroxyl group and its accessibility may be an important determinant of the potency of DNA polymerase β lyase inhibition by compound **2**.

It was of interest to determine whether these dRP lyase inhibitors also affected the polymerase activity of DNA polymerase β. In the DNA polymerase β inhibition assay utilized, none of these compounds exhibited inhibitory activity at concentrations up to 100 μg/mL. This indicates that compounds **1**–**3** represent prototype DNA polymerase β inhibitors that selectively block the dRP lyase activity of DNA polymerase β, as compared with the polymerase activity. They may specifically target the N-terminal domain of DNA polymerase β, thus

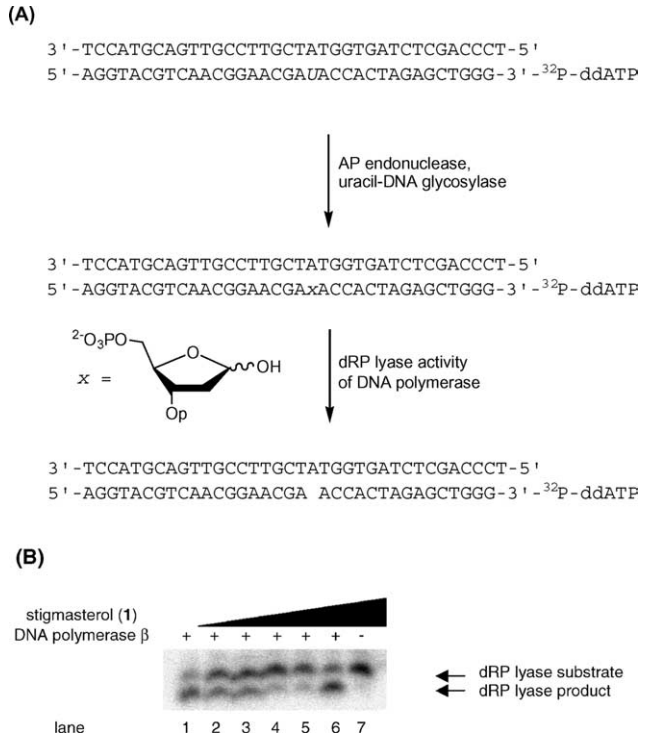

Figure 1. dRP lyase assay of DNA polymerase β. (A) Illustration of the substrate employed for DNA polymerase β lyase. The dRP lyase substrate and dRP lyase product was analyzed by gel electrophoresis. Since the 3′-end of the AP site (uridine)-containing strand was labeled with [α-^{32}P]ddATP, the dRP lyase substrate, and product could be visualized by autoradiography. (B) A typical autoradiogram of the reaction mixtures resolved by polyacrylamide gel electrophoresis. Lanes 1–5, inhibitor **1** was added to the DNA polymerase β dRP lyase assay at five different concentrations ranging from 5–78 μM (triangle); lane 6, DNA polymerase β and dRP lyase substrate without addition of the inhibitor; lane 7, dRP lyase substrate alone. The positions of dRP lyase substrate and dRP lyase product in the gel are indicated.

Table 1. DNA polymerase β lyase inhibitory activity for phytosterols **1**–**3**

Compound	IC_{50} (μM)
1	43.6
2	43.3
3	72.4

blocking the lyase activity of the enzyme. The dRP lyase activity of DNA polymerase β is the rate-limiting step in 'single-nucleotide' BER of lesions generated by anticancer agents in the DNA of tumor cells.[4,6,7,21,22] Such compounds are, therefore, of interest in the search for agents that may be clinically useful for adjuvant cancer therapy to facilitate the action of DNA damaging antitumor agents.

Compounds **1** and **2** were also tested for their ability to potentiate the cytotoxicity of bleomycin (BLM) in cultured A549 cells (derived from a human lung carcinoma). Accordingly, A549 cells were incubated for 48 h in the presence of bleomycin alone, compound **1** or **2** alone, and BLM + **1** or **2**. As shown in Table 2, bleomycin and the inhibitors exhibited minimal toxicity (cell growth inhibition $\leqslant 5\%$) toward the cultured cells when employed at 0.75 and 6.25–12.5 μM concentrations, respectively. However, in the presence of bleomycin + compound **1** or **2**, cell growth inhibition was increased markedly. Likewise, compound **1** or **2** alone exhibited ~11–21% cell growth inhibition at 25–50 μM concentrations, while in combination with 0.75 μM bleomycin, the cell growth inhibition was increased to ~26–35%. Thus, phytosterols **1** and **2** were capable of potentiating the action of bleomycin in a cultured human tumor cell line. This finding is consistent with the possibility that inhibitors of this type could be of utility as adjuvants in chemotherapeutic regimens.

These phytosterols have been reported previously to lower endogenous cholesterol levels when employed at high concentrations, and to possess anti-inflammatory activity and immune-modulating properties.[40] An early finding revealed that β-sitosterol (**2**) was able to inhibit the growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle,[41] which highlights the potential importance of revisiting this group of long-studied sterols as sources of novel ligands capable of targeting specific cellular receptors. The present study demonstrates that these phytosterols are selective DNA polymerase β dRP lyase inhibitors and can potentiate the action of bleomycin in cultured human tumor cells. This finding may facilitate the development of novel agents capable of selectively blocking DNA repair in complex biological systems.

3. Experimental

3.1. General methods

Polyamide 6S (pour density 0.25 g/mL, a product of Riedel-del Haen, Germany) was obtained from Crescent Chemical Co. (Hauppauge, NY). LiChroprep Diol resin (40–63 μm) (a product of E. Merck, Germany) was purchased from EM Separations Technology

Table 2. Potentiation of bleomycin cytotoxicity in A549 cells by phytosterols **1** and **2**

Compound	Cell growth inhibition (% of control)			
	6.25 μM	12.5 μM	25.0 μM	50.0 μM
Stigmasterol (**1**)	0	0	11.0	19.7
1 + BLM (0.75 μM)[a]	12.2	19.7	29.2	35.0
β-Sitosterol (**2**)	3.6	5.4	10.8	20.7
2 + BLM (0.75 μM)[a]	24.3	22.5	26.1	32.4

[a] Cell growth inhibition of BLM at 0.75 μM was 5.1%.

(Gibbstown, NJ). Diaion HP-20 resin was purchased from Mitsubishi Chemical Corp. (Tokyo, Japan). Baker-bond C_{18} reversed-phase resin (32–60 μm) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Hexanes, dichloromethane, and methanol of analytical grade for open column chromatography were purchased from Fisher Scientific (Fair Lawn, NJ). ^{1}H and ^{13}C NMR spectra were obtained on a Varian NMR spectrometer. Mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer. Unlabeled dNTPs and calf thymus DNA were purchased from Sigma Chemicals; [^{3}H]dTTP (0.04 Ci/mmol) was from ICN Pharmaceuticals. [α-^{32}P]ddATP (3000 Ci/mmol) was purchased from ICN Biomedicals, Inc. AP endonuclease was from TREVIGEN, Inc. (Gaithersburg, MD). Uracil-DNA glycosylase was purchased from New England Biolabs. Synthetic oligodeoxyribonucleotides were obtained from Integrated DNA technologies, Inc. (Coralville, IA) DEAE-cellulose paper (DE-81) was purchased from Whatman (Abingdon, MD). Distilled, de-ionized water from a Milli-Q system was used for all aqueous manipulations. The human lung carcinoma cell line A549 was obtained from American Type Culture Collection (ATCC).

3.2. Preparation of plant crude extracts

Stem wood of *C. orientalis* was collected in Thailand in February 1980. Roots, stem, leaves and flowers of *H. donacifolia* were collected in Peru in March 1980. Roots, stem, leaves and flowers of *H. integerrima* were collected in Ecuador in 1980. Stem, leaves and flowers of *A. pilispina* were collected in Ethiopia. The dried plant materials were soaked at room temperature successively with hexanes, methyl ethyl ketone, methanol, and water, and the solutions were concentrated under diminished pressure to afford the extracts.

3.3. Bioassay-guided fractionation and isolation of DNA polymerase β lyase inhibitors

By the use of a bioassay to specifically monitor the 5′-dRP excision (lyase) function of DNA polymerase β, the hexanes extracts of *C. orientalis*, *H. donacifolia*, and *H. integerrima*, and the methyl ethyl ketone extract of *A. pilispina* were found to contain potent inhibitory activity to the DNA polymerase β dRP lyase function. Subsequent fractionation and isolation were guided by the DNA polymerase β lyase assay; typical sets of experiments are described below.

3.3.1. *Cladogynus orientalis*. The hexanes extract (20 mg) was first fractionated on a Diaion HP-20 column (45 × 1.5 cm), which was washed successively with 70% aq MeOH, 80% aq MeOH, 90% aq MeOH, and MeOH. The MeOH fraction (7.5 mg), which exhibited the strongest DNA polymerase β lyase inhibition, was applied to a diol open column (40 × 1.8 cm) for further fractionation, using 5:95 CH_2Cl_2–hexanes, 10:90 CH_2Cl_2–hexanes, 20:80 CH_2Cl_2–hexanes, 30:70 CH_2Cl_2–hexanes, 50:50 CH_2Cl_2–hexanes, CH_2Cl_2 and MeOH as the eluants. The 10:90 CH_2Cl_2–hexanes (2.1 mg) and 20:80 CH_2Cl_2–hexanes (1.7 mg) fractions displayed the strongest inhibition of DNA polymerase β lyase. The 20:80 CH_2Cl_2–hexanes fraction (1.7 mg) was applied to a diol open column (25 × 0.8 cm) using 15:85 CH_2Cl_2–hexanes as the isocratic eluant, and afforded pure **1** (0.6 mg; 0.0038% of the weight of the dried plant material) and **2** (1.0 mg; 0.0063% of the weight of the dried plant material). The 10:90 CH_2Cl_2–hexanes fraction (2.1 mg) was crystallized from 30:70 CH_2Cl_2–MeOH to afford **3** (1.5 mg; 0.0094% of the weight of the dried plant material). Their physico-chemical and spectral data were the same as those reported in the literature.[35–37]

3.3.2. *Hymenache donacifolia*. The hexanes extract (110 mg) was applied to a diol open column (45 × 1.5 cm), which was washed successively with hexanes, 1:1 hexanes–CH_2Cl_2, CH_2Cl_2, 99:1 CH_2Cl_2–MeOH and MeOH. The 1:1 hexanes–CH_2Cl_2 fraction (46.8 mg) showed the strongest inhibitory effect on the lyase activity of DNA polymerase β and was subjected to further fractionation through a diol column (48 × 1.2 cm), which was washed successively with 80:20 hexanes–CH_2Cl_2, 60:40 hexanes–CH_2Cl_2, 40:60 hexanes–CH_2Cl_2, 20:80 hexanes–CH_2Cl_2, CH_2Cl_2, and MeOH. The 60:40 hexanes–CH_2Cl_2 fraction (23.2 mg) contained the inhibitory activity toward DNA polymerase β lyase and was identified as a mixture of **1** and **2** by comparing its ^{1}H and ^{13}C NMR data with those reported in the literature.[35–37]

3.3.3. *Heteropsis integerrima*. A diol open column (45 × 1.5 cm) was employed initially to fractionate the hexanes extract (111 mg). The column was washed with hexanes, 1:1 hexanes–CH_2Cl_2, CH_2Cl_2, 99:1 CH_2Cl_2–MeOH, and MeOH. The 1:1 hexanes–CH_2Cl_2 fraction (27 mg) showed the strongest DNA polymerase β lyase inhibition and was fractionated further using a diol column (48 × 1.2 cm), which was washed successively with 80:20 hexanes–CH_2Cl_2, 60:40 hexanes–CH_2Cl_2, 40:60 hexanes–CH_2Cl_2, 20:80 hexanes–CH_2Cl_2, CH_2Cl_2, and MeOH. The results of DNA polymerase β lyase assay indicated that the inhibitors were enriched in the 60:40 hexanes–CH_2Cl_2 fraction (3.4 mg), which was identified as a mixture of **1** and **2** by comparing its ^{1}H and ^{13}C NMR data with those reported in the literature.[35–37]

3.3.4. *Acacia pilispina*. The methyl ethyl ketone extract (110 mg) was fractionated initially using a polyamide 6S column (50 × 2.5 cm), which was washed successively with H_2O, 1:1 H_2O–MeOH, 1:4 CH_2Cl_2–MeOH, 1:1 CH_2Cl_2–MeOH, and 9:1 MeOH–NH_4OH. The 1:4 CH_2Cl_2–MeOH fraction (54 mg) strongly inhibited the lyase activity of DNA polymerase β and was fractionated further on a Diaion HP-20 column (45 × 1.5 cm), which was washed successively with 40% aq MeOH, 60% aq MeOH, 80% aq MeOH, 90% aq MeOH, and

MeOH. The MeOH fraction (10.8 mg) showed the strongest inhibition of DNA polymerase β lyase; crystallization from 1:1 CH_2Cl_2–MeOH afforded **3** (8.0 mg; 0.082% of the weight of the dried plant material) as colorless microcrystals. The physico-chemical and spectral data of this compound were identical with those reported previously.[38,39]

3.4. DNA polymerase β lyase inhibition assay

3.4.1. 3′-[^{32}P]-End labeling. A 36-base pair oligodeoxyribonucleotide (Fig. 1A) containing a uridine at position 20 on one strand was ^{32}P radiolabeled at its 3′-end with terminal deoxynucleotidyltransferase using [α-^{32}P]-ddATP as a substrate. The product was then purified using a 20% denaturing polyacrylamide gel. The band of interest was visualized by autoradiography and excised from the gel. After removal by the 'crush and soak' method, these oligodeoxyribonucleotides were then annealed to their complementary strands by heating the solution at 70 °C for 3 min, followed by slow cooling to 25 °C.

3.4.2. Apurinic site preparation. A nicked AP site was created in a reaction mixture (200 μL total volume) that contained 354 nM 3′-[^{32}P]-end labeled double-stranded oligodeoxynucleotide having an uridine at position 20, 10 mM K Hepes, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10 mg/mL of bovine serum albumin, 3 units of AP endonuclease, and 2.4 units of uracil–DNA glycosylase. After incubation at 37 °C for 20 min, the ^{32}P-end labeled double-stranded oligodeoxynucleotide containing an AP site at position 20 was ready for the dRP-excision assay.

3.4.3. dRP-Excision assay. dRP-Excision activity was determined using a reaction mixture (5 μL total volume) that contained 354 nM ^{32}P-labeled DNA substrate containing a nicked AP site at position 20, 0.17 unit of rat DNA polymerase β, and the test samples (crude extracts, fractions, or compounds **1**–**3**, dissolved in DMSO, diluted a final DMSO concentration of 1.5% in the reaction mixture). After incubation at room temperature for 30 min, the reaction was terminated. The reaction mixture was treated with 0.5 M $NaBH_4$ to a final concentration of 50 mM, and then incubated at room temperature for 10 min. After additional incubation at 75 °C for 20 min to destroy excess $NaBH_4$, the reaction products were separated on a 20% denaturing polyacrylamide gel and visualized by autoradiography. To quantify the product, gels were scanned on a Molecular Dynamics phosphorimager, and the data were analyzed using ImageQuant software.

3.4.4. DNA polymerase inhibition assay. Compounds **1**–**3** were dissolved in DMSO. Sample (6 μL) and 4 μL of rat DNA polymerase β (6.9 units, 48,000 units/mg) were added to the 50-μL reaction solution (total volume), which consisted of 6.25 μM dNTPs, 0.04 Ci/mmol [^{3}H]dTTP, 62.5 mM 2-amino-2-methyl-1,3-propanediol buffer, pH 8.6, 10 mM $MgCl_2$, 1.0 mM DTT, 0.1 mg/mL bovine serum albumin, and 0.25 mg/mL activated calf thymus DNA. After incubation at 37 °C for 1 h, the radioactive DNA product was collected on DEAE-cellulose filters (DE-81) and dried. The filters were washed three times with 0.4 M K_2HPO_4, pH 9.4, and then with H_2O, and briefly with 95% ethanol, and then used for determination of radioactivity.

3.5. Potentiation of the action of bleomycin in cultured cells

Cytotoxicity was determined by the MTT method. A549 cells were grown at 37 °C under a 5% CO_2 atmosphere in Kaighn's modification of Ham's F12 medium (F12K) with 2 mM L-glutamine supplemented with 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum. Culture samples (200 μL) containing approximately 10^4 A549 cells were placed in a 96-well culture plate and treated with the appropriate concentration of DMSO (the control) or compound **1**, **2**, and blenoxane (the clinically used mixture of BLMs, consisting primarily of BLM A_2 and BLM B_2). The cultures were incubated at 37 °C for 48 h under a 5% CO_2 atmosphere. After removal of the culture medium and addition of 15 μL of MTT (5 mg/mL) to each well, the samples were incubated at 37 °C for an additional 4 h under a 5% CO_2 atmosphere. DMSO (200 μL) was added to each well. The OD_{570} value was obtained from a microplate reader. The results were expressed as 'percent growth inhibition' according to the formula $[(N_c - N_e)/N_c] \times 100\%$, where N_c was the OD_{570} value counted in the control culture and N_e was the OD_{570} value in the treated culture.

Acknowledgements

This work was supported by NIH Research Grant CA50771 awarded by the National Cancer Institute.

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