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Inhibitors of DNA polymerase β: Activity and mechanism

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Abstract—Bioassay-guided fractionation of extracts prepared from *Couepia polyandra* and *Edgeworthia gardneri* resulted in the isolation of the DNA polymerase β (pol β) inhibitors oleanolic acid (**1**), edgeworin (**2**), betulinic acid (**3**), and stigmasterol (**4**). Study of these pol β inhibitors revealed that three of them inhibited both the lyase and polymerase activities of DNA polymerase β, while stigmasterol inhibited only the lyase activity. Further investigation indicated that the four inhibitors had substantially different effects on the DNA–pol β binary complex that is believed to be an obligatory intermediate in the lyase reaction. It was found that the inhibitors potentiated the inhibitory action of the anticancer drug bleomycin in cultured A549 cells, without any influence on the expression of pol β in the cells. The results of the unscheduled DNA synthesis assay support the thesis that the potentiation of bleomycin cytotoxicity by DNA pol β inhibitors was a result of an inhibition of DNA repair synthesis.

1. Introduction

DNA repair in mammalian cells is a multi-pathway process that protects the integrity of DNA in the presence of DNA damaging agents. Many currently used anticancer therapeutic agents rely on the ability to create DNA lesions, leading to cancer cell death. The ability of cancer cells to repair such DNA damage is a major cause of resistance to these clinically used antitumor agents.[1–3] One strategy to address this issue involves targeting proteins involved in DNA repair in cancer cells, thereby overcoming resistance to DNA damaging agents employed for anticancer therapy. Implementation of a strategy for adjuvant chemotherapy would demand that the agents so employed lacked significant intrinsic cytotoxic activity, but rather potentiated the cytotoxic action of the DNA damaging agents.

Three types of excision repair have been found to target specific DNA lesions, namely base excision repair (BER),[4] nucleotide excision repair (NER)[5,6] and DNA mismatch repair.[7] BER is a major pathway employed for the repair of damaged bases in DNA[8,9] produced by antitumor agents such as bleomycin,[10,11] monofunctional DNA alkylating agents,[12,13] cisplatin,[14,15] and neocarzinostatin.[16] Pol β plays important roles in the BER pathway;[17] it excises the 5′-terminal dRP[18,19] from a preincised apurinic or apyrimidinic (AP) site after the modified base has been removed by DNA glycosylase and AP endonuclease (Fig. 1). Further, polymerase β catalyzes a template-directed nucleotidyl transfer reaction to fill in the resulting gap.[17,20] However, at least one abasic lesion, a deoxyribonolactone, cannot be repaired efficiently, and can actually lead to crosslinking of the repair enzyme to the DNA lesion.[21,22]

Therefore, pol β constitutes a promising target for adjuvant cancer therapy. It is logical to think that the efficacy of clinically used DNA damaging agents could be enhanced by blocking the repair of damaged DNA through inhibition of this enzyme. A number of inhibitors have been identified including inhibitors of both the polymerase[23–32] and lyase[33–39] activities of the enzyme. Consistent with the thesis that the inhibition of pol β should enhance the cytotoxicity of DNA damaging agents used for cancer chemotherapy, a number of the identified inhibitors were shown to enhance the cytotoxicity of the DNA damaging agent bleomycin (BLM) toward cultured mammalian cells,[24,27,30–33,35,36] although the extent of potentiation of BLM toxicity did not seem to correlate in a simple way with the potency of pol β inhibition by individual agents. However, there are only limited data concerning the interactions between pol β, pol β inhibitors and DNA substrate,[28,32,40] or regarding the effect of pol β inhibitors on DNA synthesis or pol β expression in cultured cells.

Keywords: Polymerase inhibitor; Inhibition mechanism; Enzyme–DNA covalent complex; Antitumor agent potentiation; Natural products.
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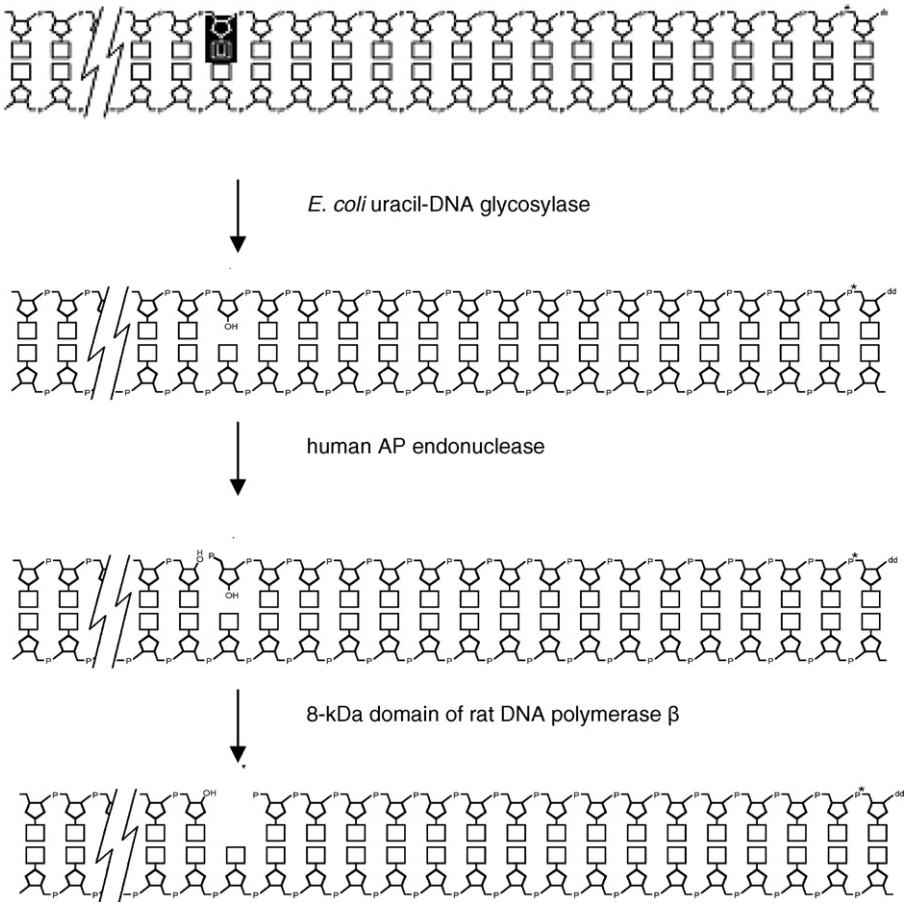


Figure 1. Formation of AP site and excision of the dRP group.

To facilitate an understanding of the consequences of inhibition of the polymerase and lyase activities of pol β, we focused on four natural products (**1**–**4**) (Fig. 2) that were isolated recently as inhibitors of the lyase activity of DNA polymerase β.[34,36] Two of these (oleanolic acid (**1**) and betulinic acid (**3**)), had also previously been found to inhibit the polymerase activity of pol β.[30,31] In the present study, the pol β inhibitory activities of these compounds were determined using an assay that permitted each activity to be monitored under the same reaction conditions. Also investigated under uniform conditions was the ability of each of the four compounds to potentiate the activity of bleomycin. To permit an analysis of the molecular basis of the observed effects, the expression of pol β in cultured A549 cells that had been exposed to the pol β inhibitors was studied as was the impact of these inhibitors on unscheduled DNA synthesis in A549 cells. On the basis of these data, the molecular mechanisms and the biological characteristics of these inhibitors are discussed.

2. Results

2.1. Synthesis of edgeworin (2)

Edgeworin was synthesized as outlined in Scheme 1. Briefly 7-hydroxycoumarin (**7**) was treated with ethyl bromoacetate to afford ethyl ester **8** in quantitative yield. Saponification gave **9**, which was condensed with 2-hydroxy-4-(2-methoxyethoxymethoxy)benzaldehyde (**6**) to afford MEM-protected edgeworin (**10**). Removal of the protecting group afforded edgeworin (**2**) in 83% yield.

2.2. Construction of 3′-end labeled DNA substrate

To investigate the inhibition of pol β lyase and polymerase activities, a 34-nt oligodeoxyribonucleotide having a deoxyuridine at position 16 was employed (Fig. 3). The DNA substrate was labeled at 3′-end using [α-^{32}P]ddATP in the presence of terminal deoxynucleotidyltransferase. After purification, the substrate was exposed to uracil–DNA glycosylase and AP endonuclease to remove the uracil moiety and create an AP site. This substrate was used to examine the pol β lyase and polymerase activities, as well as the formation of the DNA–pol β binary complex.

2.3. Inhibition of pol β lyase and polymerase activities

Previous work showed that compounds **1**–**4** exhibited moderate inhibition of pol β lyase activity;[34,36] two of them (**1** and **3**) were also found to inhibit the polymerase activity of the enzyme.[30,31] In the earlier work, these inhibitory activities were studied using two separate assays carried out under quite different conditions. In order to study the two properties under comparable

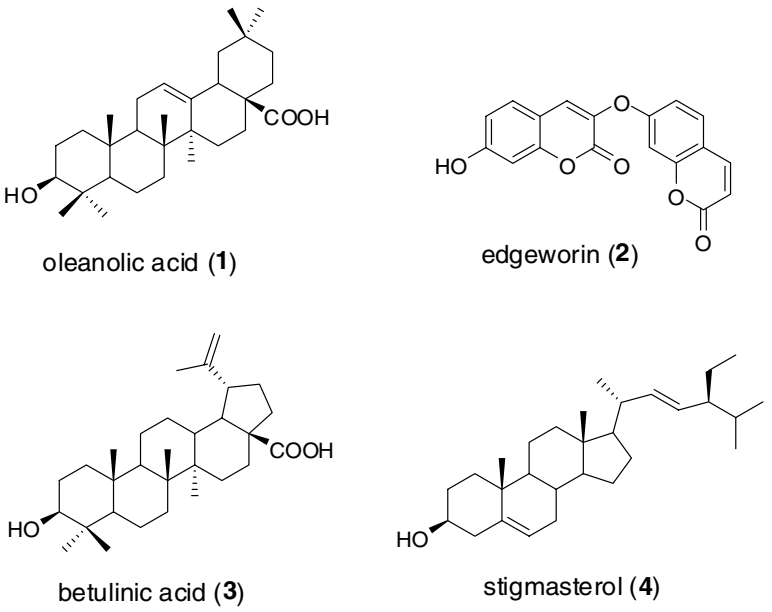
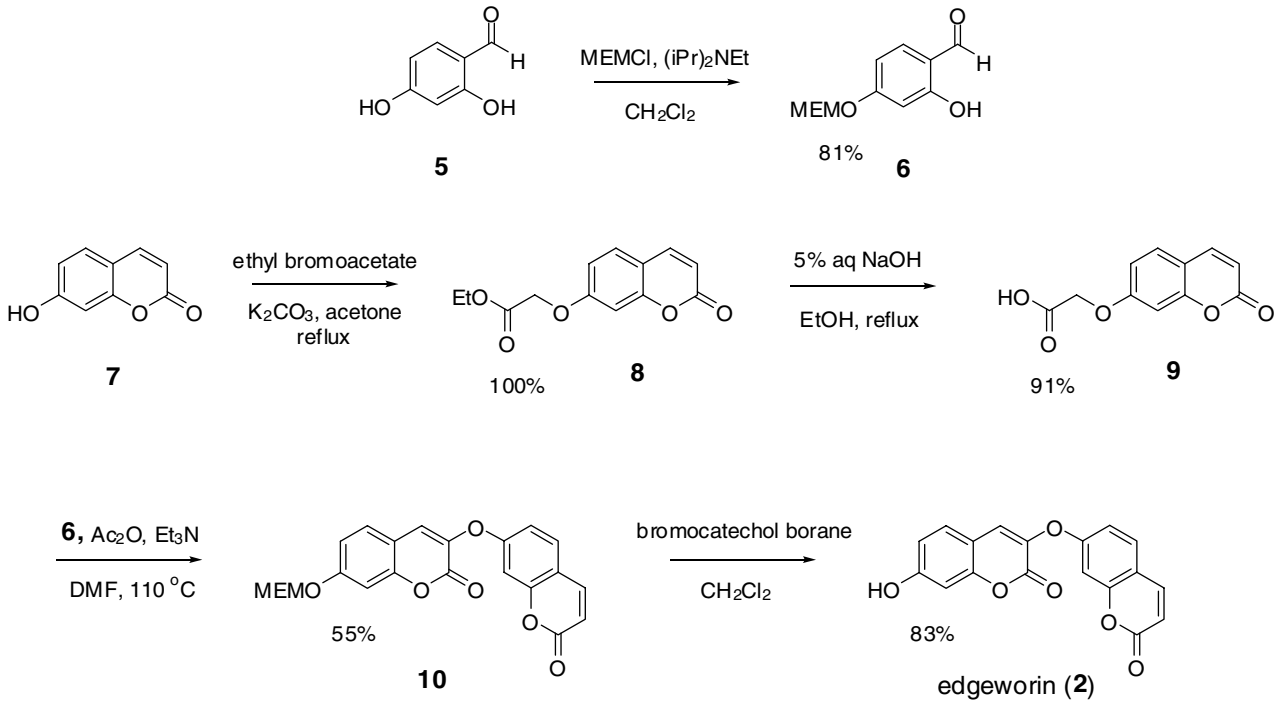


Figure 2. Structures of polymerase β inhibitors **1**–**4**.



Scheme 1. Synthesis of edgeworin (**2**).

conditions, an assay was constituted under which the two activities could be studied in parallel (Fig. 3).[40] As shown in Figures 3C and D, oleanolic acid (**1**) exhibited the strongest inhibition of both lyase and polymerase activities of pol β under these assay conditions. Compound **2** also inhibited the lyase and polymerase activities to roughly comparable extents, while compound **3** was a more potent inhibitor of the lyase activity than the polymerase activity. Compound **4** was a weak pol β lyase inhibitor but exhibited no detectable polymerase inhibitory activity. The IC_{50} values of these compounds for lyase and polymerase activities are summarized in Table 1.

2.4. Effect on the interaction between DNA substrate and pol β

The amino-terminal 8-kDa domain of DNA pol β catalyzes the excision of dRP groups from 5′-incised AP sites via a β-elimination reaction following the formation of a Schiff base between an aldehyde group of the AP site and an amino group of the enzyme

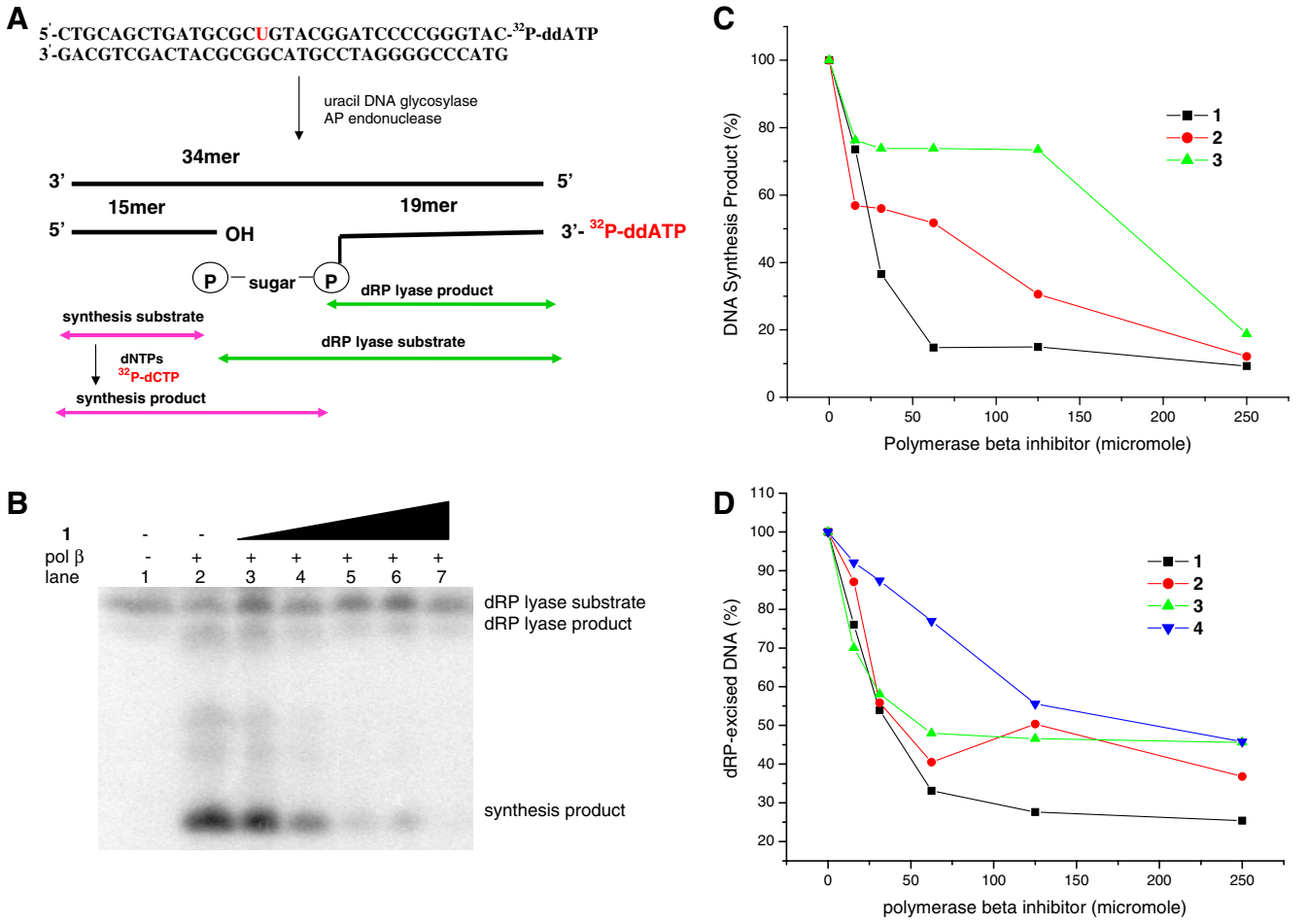


Figure 3. Inhibition of pol β lyase and polymerase activities by pol β inhibitors. The reaction conditions and analyses are described in the Experimental. (A) Schematic representation of the substrate for the pol β lyase–polymerase assay: the ^{32}P-labeled dRP lyase substrate, products after AP endonuclease cleavage (19-mer having a 3′-^{32}P label and 5′-sugar phosphate (AP site) + 15-mer). The 15-mer is the DNA synthesis substrate, while the 19-mer is the lyase substrate. Also shown schematically are the products of the lyase and polymerase reactions. (B) dRP lyase and DNA polymerase activities of pol β. Lane 1 is a control in the absence of pol β, lane 2 contained pol β without inhibitor, lanes 3–7 contained pol β with increasing concentration of **1** (15, 31, 62, 125, and 250 μM, respectively). (C) The inhibition of pol β polymerase activity by compounds **1**, **2**, and **3**. (D) The inhibition of pol β dRP excision activity by compounds **1**, **2**, **3**, and **4**.

Table 1. Inhibition of the catalytic activities of DNA polymerase β

Compound	Lyase inhibition IC_{50} (μM)	Polymerase inhibition IC_{50} (μM)
Oleanolic acid (**1**)	26.25 ± 5.3	24.98 ± 3.3
Edgeworin (**2**)	38.88 ± 5.1	31.43 ± 2.9
Betulinic acid (**3**)	33.7 ± 4.9	46.25 ± 3.1
Stigmasterol (**4**)	60.28 ± 5.3	>250

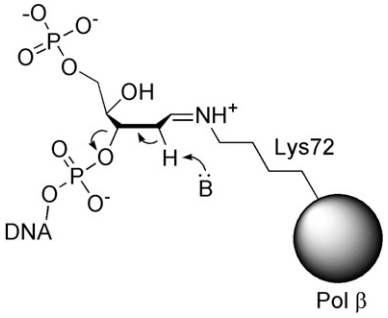


Figure 4. Schiff base complex formed between incised apurinic acid DNA lesion and lysine 72 of the N-terminal domain of polymerase β.

(Fig. 4).[17,18,41,42] The β-elimination is relatively facile, such that the Schiff base intermediates can be difficult to observe and monitor. However, treatment with $NaBH_4$ can reduce and thereby stabilize the formed imine, thus permitting its assay by polyacrylamide gel electrophoresis.

To investigate the effect of inhibitors on the covalent binding between pol β and DNA substrate, the formation of the DNA–pol β binary complex was studied using a DNA binding mobility shift assay. Pol β was assayed for its ability to bind with DNA substrates in the presence or absence of inhibitors. A 3′-^{32}P labeled double-stranded oligonucleotide containing a 5′-incised AP site was incubated with pol β and inhibitors. After reducing the Schiff base intermediates by treatment with $NaBH_4$, the samples were analyzed by native polyacrylamide gel electrophoresis (Fig. 5). The result showed that the amount of DNA–pol β binary complex was generally diminished as the concentrations of compounds **1**–**4** were increased (Fig. 5C). However, there were clear differences between the four compounds. While there

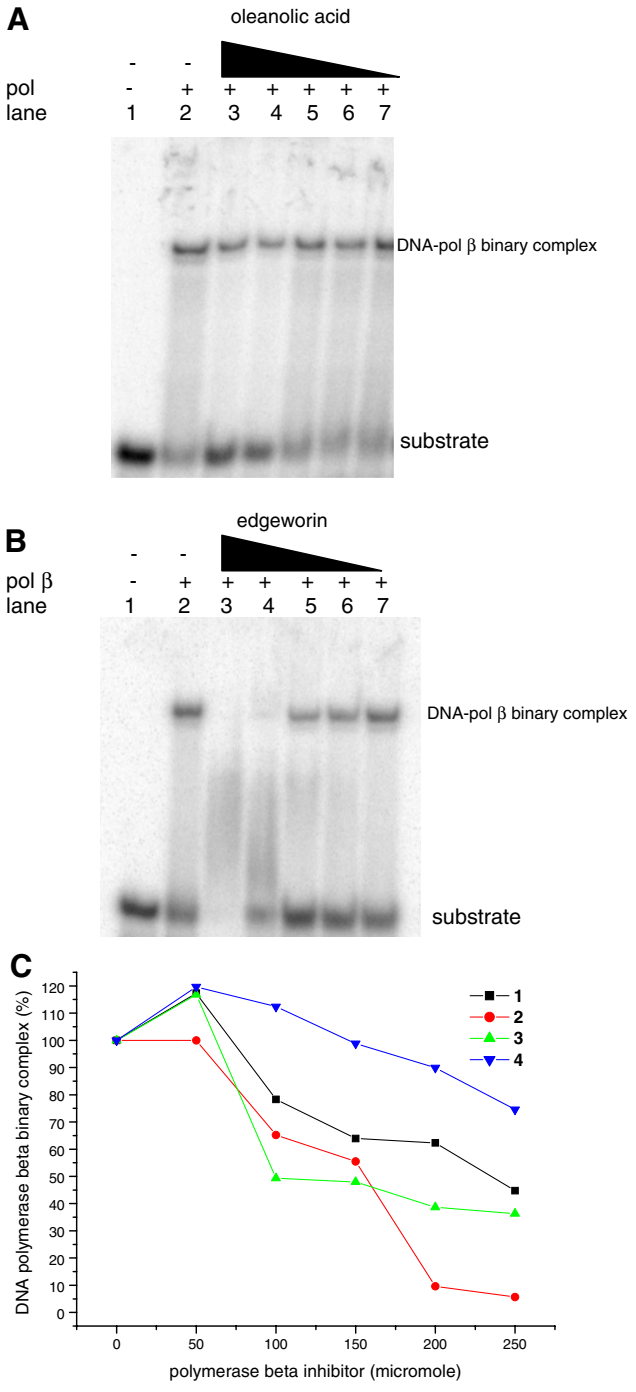


Figure 5. Inhibition of DNA–pol β binary complex by pol β inhibitors. (A) The formation of DNA–pol β binary complex in the presence of oleanolic acid (**1**). Lane 1, no pol β; lane 2, with pol β; lanes 3–7 pol β with varying concentrations of **1** (1ane 3, 250 μM; lane 4, 200 μM; lane 5, 150 μM; lane 6, 100 μM; lane 7, 50 μM). (B) Effect of edgeworin (**2**) on binary complex formation. Lane 1, no pol β; lane 2, with pol β; lanes 3–7 pol β with varying concentration of **2** (1ane 3, 250 μM; lane 4, 200 μM; lane 5, 150 μM; lane 6, 100 μM; lane 7, 50 μM). (C) Diminution of the concentration of the DNA–pol β binary complex by **1**, **2**, **3**, and **4**.

was virtually no enzyme–DNA binary complex present at the two highest concentrations of **2** (Figs. 5B and C), compound **1** had much less effect on the enzyme–DNA covalent binary complex (cf Figs. 5A and B). Compound **4** had only a slight effect on the enzyme–DNA covalent binary complex.

2.5. Potentiation of the cytotoxicity of bleomycin

Three of the four compounds studied here were only weakly cytotoxic to A549 cells (Table 2) while betulinic acid (**3**) was moderately cytotoxic. Given that betulinic acid has been shown to function at several critical cellular loci,[43–45] the observation of greater cytotoxicity for this compound is unsurprising. However, the modest cytotoxicities observed for compounds **1**, **2**, and **4** support the thesis that inhibitors of pol β function need not be strongly cytotoxic, and can potentially be used for adjuvant chemotherapy. To examine the ability of the compounds to potentiate the cytotoxicity of BLM, each was tested at a concentration which exhibited minimal cytotoxic activity. A549 cells in log phase growth were cultured with 1 mM BLM and 25 μM **1**, 6 μM **2**, 3 μM **3**, or 6 μM **4**; cell numbers were determined after 48 h. All four compounds were found to potentiate the cytotoxic activity of blenoxane (Table 3). The extent of potentiation of cytotoxicity ranged from a maximum of 44% for oleanolic acid (**1**) to 15% for stigmasterol (**4**).

2.6. Effect of compounds 1–4 on the expression of pol β in A549 cells

To confirm that the potentiation of BLM cytotoxicity by these compounds resulted from the inhibition of pol β itself rather than an effect at the level of pol β expression, the expression of pol β was determined. Western blot experiments performed in whole cell extracts collected after 48-h incubation with 25 μM **1**, 6 μM **2**, 3 μM **3**, and 6 μM **4**, respectively, showed the same expression of pol β in treatment groups, compared to control. Anti-β actin antibody was used to assure the equivalence of protein loading (Fig. 6).

2.7. Unscheduled DNA synthesis

Previous studies documented that in the presence of 2 mM nicotinamide and 10 mM hydroxyurea, optimal

Table 2. Cytotoxicity of polymerase β inhibitors toward cultured A549 cells

Compound	Cytotoxicity IC_{50} (μM)
Oleanolic acid (**1**)	>100
Edgeworin (**2**)	80.2 ± 2.1
Betulinic acid (**3**)	6.65 ± 2.3
Stigmasterol (**4**)	98.2 ± 2.5

Table 3. Potentiation of the cytotoxicity of bleomycin by polymerase β inhibitors

Compound	Fold enhancement of growth inhibition by 1 mM BLM
Oleanolic acid (**1**) (25 μM)	1.44 ± 0.08
Edgeworin (**2**) (6 μM)	1.27 ± 0.06
Betulinic acid (**3**) (3 μM)	1.32 ± 0.24
Stigmasterol (**4**) (6 μM)	1.15 ± 0.04

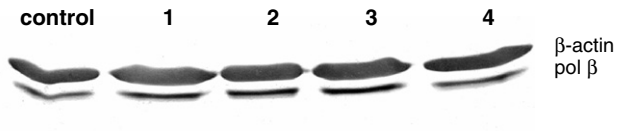


Figure 6. Western blot assay for quantification of pol β in A549 cells treated with polymerase β inhibitors **1**–**4**. The reaction conditions and analyses are described in the Experimental section.

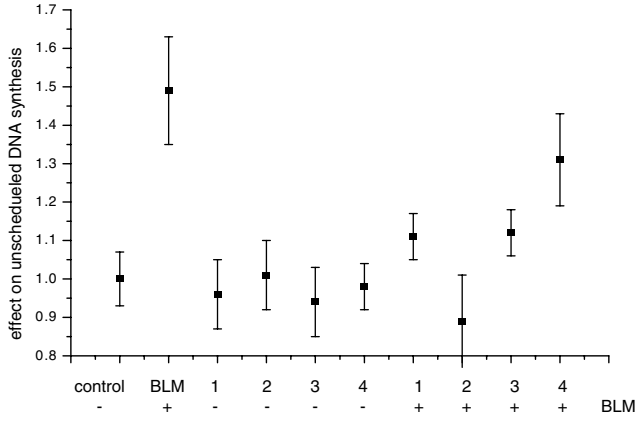


Figure 7. Suppression of BLM-induced unscheduled DNA synthesis in cultured A549 cells.

unscheduled DNA synthesis is measured with maximal suppression of replicative synthesis.[46,47] It has been shown that following treatment of cells with a DNA damaging agent, the amount of unscheduled DNA synthesis represents DNA repair synthesis.[48,49] This technique has been used previously to measure the increase in cellular repair synthesis induced following BLM treatment.[50–53] In the present study, this technique was applied to determine the ability of DNA pol β inhibitors to inhibit BLM-induced DNA repair synthesis. Treatment with 25 μM **1** alone had little effect on unscheduled DNA synthesis occurring in the cells. BLM, on the other hand, induced DNA synthesis effectively following brief treatment at 10 mM concentration. When the cells were exposed to both 10 mM BLM and 25 μM compound **1**, there was strong inhibition of BLM-induced DNA repair synthesis (Fig. 7). Compounds **2** (6 μM) and **3** (3 μM) also suppressed BLM-induced unscheduled DNA synthesis, but a 6 μM concentration of **4** was less effective in suppressing BLM-induced unscheduled DNA synthesis.

3. Discussion

DNA–polymerase β is a single polypeptide chain of 39 kDa that folds into two domains, each of which contains an active site. The dRP excision activity resides in the N-terminal (8 kDa) domain, while the active site for DNA synthesis resides within the larger (31 kDa) C-terminal domain. While the lyase activity is believed to be rate-limiting, suggesting that it may be the more logical target for inhibition, many inhibitors of pol β can act on both active sites,[30,31,40] confounding efforts to define the basis for the cellular effects of pol β inhibitors. Moreover, the assays employed historically for measuring the two activities have employed rather different experimental conditions,[18] further complicating the analysis. For some polymerase β inhibitors there are also 'off-target effects' such as DNA cleavage;[54–56] while potentially of great utility, these do not facilitate an analysis of the cellular consequences of individual biochemical effects.

In the present study, we have employed four compounds (**1**–**4**) isolated based on their lyase inhibitory activity. Three of these compounds (**1**–**3**) also inhibited the DNA polymerase activity of the enzyme (Fig. 3 and Table 1). Critically, the measurement of inhibition was made for both activities under identical experimental conditions.

The use of pol β inhibitors as adjuvants for chemotherapy with clinically used DNA damaging agents demands that they be relatively non-toxic when administered as single agents. In fact, compounds **1**, **2**, and **4** were poorly cytotoxic toward cultured A549 cells (Table 2), consistent with this requirement. Betulinic acid (**3**), which functions at a number of cellular loci,[43–45] unsurprisingly exhibited somewhat greater cytotoxicity. When these compounds were used at relatively non-toxic concentrations to potentiate the cytotoxicity of 1 mM BLM, all of them did increase the observed cyctotoxicity. Oleanolic acid (**1**), which exhibited the strongest inhibition of both enzyme activities (Table 1) and the least cytotoxicity toward A549 cells as a single agent, afforded the best potentiation of BLM (Table 3). Edgeworin (**2**) and betulinic acid (**3**), which inhibited both pol β activities to comparable extents, but less potently than **1**, also gave slightly less potentiation of BLM than **1**. Not surprisingly, stigmasterol (**4**), which inhibited the lyase activity of pol β poorly, and failed to inhibit the polymerase activity detectably, gave little potentiation of the cytotoxicity of BLM toward A549 cells. Stigmasterol was also the least potent of the four compounds in inhibiting BLM-induced unscheduled DNA synthesis.

The present data strongly support the thesis that inhibitors of polymerase β may be used to potentiate the cytotoxicity of DNA damaging agents in spite of their lack of strong intrinsic cytotoxicity, and that the effect is mediated at the level of impaired DNA repair. While the present study does not permit a definitive analysis of the relative effects on potentiation caused by inhibition of the polymerization and lyase activities, it may be noted that some potentiation was obtained with stigmasterol, a weak lyase inhibitor lacking any measurable polymerase inhibitory activity.

Finally, it may be noted that the compounds studied had different effects on the stability of the DNA–pol β covalent binary complex (Fig. 5), suggesting that they may inhibit the lyase activity using somewhat different molecular strategies. In this context, it may be noted that the DNA–protein covalent binary complex formed as an obligatory intermediate in the lyase reaction bears for-

mal analogy to the DNA–protein complex formed between topoisomerase I and its DNA substrate. This complex is poisoned by the binding of camptothecin, an interaction that is believed to form the basis for the clinically useful antitumor activity of the camptothecins.[57] Thus, an alternative strategy for exploiting polymerase β targeting for more effective antitumor therapy might involve the identification of compounds that bind tightly to the DNA–pol β covalent binary complex. In fact, bioactive isomalabaricone triterpenoids have been found to exhibit this property, and exhibited fairly strong cytotoxicity toward A2780 ovarian cancer cells as single agents.[58]

4. Experimental

4.1. General methods and materials

Compounds **1** and **4** were purchased from Sigma Chemicals; compound **3** was from Aldrich. Compound **2** was synthesized as described below (Scheme 1). Experiments requiring anhydrous conditions were performed using flame-dried glassware and under a nitrogen atmosphere. Chemical reagents were purchased from Aldrich Chemical Co. Anhydrous CH_2Cl_2 was distilled from CaH_2 prior to use. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity 500 MHz NMR spectrometer and a Varian Gemini 300 NMR spectrometer. All δ values are given in ppm and are relative to tetramethylsilane; J values are recorded in Hz. High resolution mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported, in part, by a Grant (DRR-00180) from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health. Thin layer chromatography was performed using Merck silica gel F_{254} pre-coated plates with spots visualized using UV light (254 nm) or by dipping the plates in a vanillin staining reagent. Silicycle (Quebec City, Quebec) ultra pure silica gel, mesh size 35–75 μm, was used for column chromatography.

These compounds were initially dissolved in DMSO for the DNA pol β lyase–polymerase assay, DNA binding mobility shift assay, and in DMSO for the cell culture studies with a final DMSO concentration <0.05% in each culture. Recombinant rat liver pol β was a gift from Dr. Xiangyang Wang and Hongge Wang, prepared as described previously.[59,60] The oligodeoxyribonucleotide (34-mer) was purchased from Integrated DNA Technologies. [α-^{32}P]ddATP (5000 Ci/mmol), [α-^{32}P]dCTP (6000 Ci/mmol) and [^{3}H]thymidine (80 Ci/mmol) were purchased from Amersham Pharmacia Biotech. AP endonuclease and anti-human DNA polymerase β antibody were purchased from Trevigen. Uracil–DNA glycosylase was from New England Biolabs; terminal deoxynucleotidyltransferase was obtained from Invitrogen. The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection. Kaighn's modification of Ham's F2 medium, Hank's balanced salt solution, donor horse serum, aprotinin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, anti-β-actin antibody, hydroxyurea, nicotinamide, and trichloroacetic acid were from Sigma Chemicals. Ninety-six-well plates and 150 cm^2 flasks were from Fisher. 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. Bio-Rad protein assay dye reagent, PVDF membrane, and Immun-Blot Assay kit were from Bio-Rad. Whatman GF/C filters were from Fisher.

Denaturing, native gels and immunoblots were quantified by use of Molecular Dynamics ImageQuant version 5.0. Scintillation counting was performed on a Beckman LS-100C instrument using Beckman Ready Safe scintillation fluid. The microplate reader was from Bio-Rad. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

4.2. Chemical synthesis of edgeworin (2)

4.2.1. 2-Hydroxy-4-(2-methoxyethoxymethoxy)benzaldehyde (6). To a stirred suspension containing 3.0 g (21.7 mmol) of 2,4-dihydroxybenzaldehyde (**5**) in 20 mL of dry CH_2Cl_2 was added 3.78 mL (2.80 g, 21.7 mmol) of *N*,*N*-diisopropylethylamine, followed by the dropwise addition of 2.48 mL (2.71 g, 21.7 mmol) of MEMCl over a period of 5 min. After stirring at room temperature under nitrogen overnight, the reaction mixture was partitioned in 400 mL of 1:1 methylene chloride–water; the aqueous phase was then extracted using three 100-mL portions of methylene chloride. The combined organic extract was washed with 200 mL of brine solution and then dried over $MgSO_4$, filtered and concentrated under diminished pressure. The residue was then purified by flash chromatography on a silica gel column (30 × 4 cm); elution with 1:1 ethyl acetate–hexanes gave the desired product **6** as a colorless oil: yield 3.96 g (81%); silica gel TLC R_f 0.8 (1:1 ethyl acetate–hexanes); ^1H NMR ($CDCl_3$) δ 3.34 (s, 3H), 3.55 (m, 2H), 3.81 (m, 2H), 5.30 (s, 2H), 6.59 (s, 1H), 6.63 (dt, 1H, J = 8.7 and 1.8 Hz), 7.44 (d, 1H, J = 8.7 Hz), 9.70 (s, 1H), and 11.34 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 59.3, 68.5, 71.7, 93.3, 103.6, 109.2, 116.2, 135.6, 164.3, 164.5 and 194.9.

4.2.2. Ethyl 2-(2-oxo-2*H*-chromen-7-yloxy)acetate (8). To a solution containing 5.00 g (30.9 mmol) of 7-hydroxycoumarin (**7**) and 5.15 g (30.9 mmol) of ethyl bromoacetate in 150 mL of acetone was added 4.41 g (31.5 mmol) of anhydrous K_2CO_3. The reaction mixture was heated at reflux for 4 h, after which time the cooled reaction mixture was filtered to remove the precipitate. The filtrate was then concentrated under diminished pressure to afford a crude colorless solid. The crude material was crystallized from abs EtOH to afford **8** as colorless needles: yield 7.64 g (100%); silica gel TLC R_f 0.48 (1:1 ethyl acetate–hexanes); ^1H NMR ($CDCl_3$) δ 1.15 (t, 3H, J = 6.9 Hz), 4.11 (q, 2H, J = 6.9 Hz), 4.76 (s, 2H), 6.12 (d, 1H, J = 9.6 Hz), 6.76 (d, 1H, J = 1.8 Hz), 6.83 (dd, 1H, J = 8.7 and 2.7 Hz), 7.47 (d, 1H, J = 8.7 Hz) and 7.77 (d, 1H, J = 9.6 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 61.4, 65.3, 102.2, 111.1, 113.0, 113.4, 130.0, 145.0, 154.7, 160.5, 161.0 and 167.6.

4.2.3. 2-(2-Oxo-2*H*-chromen-7-yloxy)acetic acid (9). To a solution containing 7.64 g (30.8 mmol) of **8** in 150 mL of ethanol was added 22 mL of 5% aq NaOH. The reaction mixture was stirred at reflux for 2 h and the cooled reaction mixture was concentrated under diminished pressure. The residue was diluted with 150 mL of H_2O and acidified with 6 N HCl. Upon becoming acidic a thick white ppt formed; this was collected by filtration, dried and crystallized from abs EtOH to afford **9** as colorless needles: yield 6.20 g (91%); ^{1}H NMR (DMSO-d_6) δ 4.86 (s, 2H), 6.32 (d, 1H, J = 9.6 Hz), 6.97 (s, 1H), 7.00 (s, 1H), 7.66 (d, 1H, J = 9.0 Hz), 8.02 (d, 1H, J = 9.6 Hz) and 13.19 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 65.6, 102.2, 111.3, 113.3, 113.5, 130.2, 145.0, 155.9, 161.0, 161.6 and 170.3.

4.2.4. 7-(2-Methoxyethoxymethoxy)-3-(2-oxo-2*H*-chromen-7-yloxy)chromen-2-one (10). To a solution containing 0.480 g (2.18 mmol) of **9** were added 0.61 mL (0.440 g, 4.36 mmol) of triethylamine and 0.668 g (6.54 mmol) of acetic anhydride in 10 mL of dry DMF. The reaction mixture was stirred at 110 °C under nitrogen for 18 h. The cooled reaction mixture was poured into 150 mL of ice water, and extracted with three 100-mL portions of ethyl acetate. The combined organic layer was washed with two 100-mL portions of brine and dried over $MgSO_4$. The solvent was concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (28 × 3 cm), elution with 10:1 chloroform–methanol, gave the desired product **10** as a brown solid: yield 492 mg (55%); silica gel TLC R_f 0.51 (10:1 chloroform–methanol); ^{1}H NMR (DMSO-d_6) δ 3.28 (s, 3H), 3.56 (m, 2H), 3.80 (m, 2H), 5.43 (s, 2H), 6.43 (d, 1H, J = 9.6 Hz), 7.13 (dd, 1H, J = 8.4 and 2.1 Hz), 7.19 (d, 1H, J = 2.7 Hz), 7.21 (d, 1H, J = 2.1 Hz), 7.30 (d, 1H, J = 2.7 Hz), 7.68 (d, 1H, J = 8.7 Hz), 7.78 (d, 1H, J = 8.4 Hz), 8.00 (s, 1H) and 8.11 (d, 1H, J = 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 59.3, 68.4, 71.7, 92.6, 107.3, 109.0, 116.3, 118.8, 119.0, 119.3, 119.8, 134.9, 135.3, 136.5, 140.7, 149.6, 158.1, 159.3, 162.4, 165.1, 165.5 and 166.1; mass spectrum (electrospray) m/z 410.8 $(M)^+$, theoretical m/z 410.1 $(M)^+$.

4.2.5. 7-Hydroxy-3-(2-oxo-2*H*-chromen-7-yloxy)chromen-2-one (edgeworin) (2). To a stirred solution containing 125 mg (0.304 mmol) of **10** in 10 mL of dry CH_2Cl_2 was added 61 mg (0.30 mmol) of bromocatechol borane. The reaction mixture was stirred at room temperature under nitrogen for 3 h. The reaction mixture was then filtered, and the precipitate was washed with three 30-mL portions of 1 N HCl, and dried to yield **2** as a grayish white solid: yield 91 mg (83%); silica gel TLC R_f 0.45 (10:1 methanol–chloroform); ^{1}H NMR (DMSO-d_6) δ 6.35 (d, 1H, J = 9.0 Hz), 6.80 (s, 1H), 6.83 (d, 1H, J = 8.0 Hz), 7.11 (dd, 1H, J = 8.5 and 1.5 Hz), 7.18 (d, 1H, J = 1.5 Hz), 7.51 (d, 1H, J = 8.5 Hz), 7.70 (d, 1H, J = 9.5 Hz), 7.92 (s, 1H), 8.02 (d, 1H, J = 9.5 Hz) and 10.56 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 107.6, 109.3, 116.3, 118.8, 119.0, 119.3, 119.8, 134.9, 135.3, 136.5, 140.7, 149.6, 158.9, 160.5, 162.4, 165.2, 165.4 and 166.1; mass spectrum (FAB), m/z 323.0557 $(M+H)^+$ ($C_{18}H_{11}O_6$ requires 323.0553).

4.3. Preparation of gapped DNA substrates

The 34-mer oligodeoxyribonucleotide (9 nmol) containing a uridine at position 16 was labeled at its 3′-end with terminal deoxynucleotidyltransferase using 50 μCi of [α-^{32}P]ddATP (5000 Ci/mmol). 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 recovery from the gel matrix by the 'crush and soak' method, the oligodeoxyribonucleotide was annealed to its complementary strand by heating the solution at 70 °C for 3 min, followed by slow cooling to 25 °C.

An AP site was created in a reaction mixture (200 μL total volume) that contained 40 pmol [α-^{32}P]-labeled double-stranded oligodeoxynucleotide having a deoxyuridine at position 16 in 10 mM Hepes–KOH, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10 mg/mL BSA, 3 units of AP endonuclease, and 2.4 U of uracil–DNA glycosylase. After incubation at 37 °C for 2 min, the [α-^{32}P]-labeled double-stranded oligodeoxynucleotide was ready for the DNA pol β lyase–polymerase assay and DNA binding mobility shift assay.

4.4. Pol β lyase–polymerase assay

The lyase–polymerase reactions were performed as follows. The reaction mixture (10 μL total volume) contained 50 mM Hepes–KOH, pH 7.4, 20 mM KCl, 2 mM DTT, 4 mM ATP, 5 mM $MgCl_2$, 20 μM each of dGTP, dTTP, dATP and 67 nM [α-^{32}P]dCTP (6000 Ci/mmol), 200 nM 3′-labeled DNA, 3 nM pol β and pol β inhibitors (15–250 μM). After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 1 μL of 0.5 M EDTA and the product was stabilized by the addition of $NaBH_4$ to a final concentration of 50 mM and incubation at room temperature for 10 min. After additional incubation at 75 °C for 5 min the reaction products were separated on a 20% denaturing polyacrylamide gel and visualized by autoradiography. Lyase and polymerase products were quantified using ImageQuant software, after scanning the gel by the use of a Molecular Dynamics phosphorimager.

4.5. DNA binding mobility shift assay

The affinity of pol β for a DNA substrate containing an AP site at position 16 was studied using a gel mobility assay[18,61] in the presence and in the absence of pol β inhibitors. Pol β (30 nM) was incubated with 200 nM radiolabeled DNA substrate, and pol β inhibitors (30–500 μM) in buffer (10 μL total volume) containing 10 mM Hepes–KOH, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 10 mg/mL BSA at room temperature for 5 min. The product was stabilized by the addition of 0.5 M $NaBH_4$ to a final concentration of 50 mM. After 10 min, the samples were loaded onto a 12% non-denaturing polyacrylamide gel, run for 2 h at 30 W and visualized by autoradiography. The ^{32}P-labeled substrate bound to pol β exhibited an electrophoretic mobility distinguishable from that without pol β. Bound protein was

quantified using ImageQuant software, after scanning the gel using a phosphorimager.

4.6. Cell culture conditions

Human lung carcinoma A549 cells were routinely grown at 37 °C in a 5% CO_2 incubator in Kaighn's modification of Ham's F12 medium with 2 mM L-glutamine, supplemented with 1.5 g/L sodium bicarbonate and 10% fetal calf serum.

4.7. Cell growth inhibition assay

An MTT assay[62] was performed to monitor cell growth inhibition. Two hundred microliter aliquots of cell culture medium, containing approximately 1.0×10^4 A549 cells, were placed in 96-well culture plates. The following day, the cells were exposed to the desired concentration of bleomycin, with or without pol β inhibitors, for 1 h. The cells were then washed with Hank's balanced salt solution. Fresh medium with or without pol β inhibitors was added to the cells, which were incubated for another 48 h at 37 °C under a 5% CO_2 atmosphere. The culture medium was removed and 10 μL of MTT reagent was added to each well. Following incubation at 37 °C for 4 h, 200 μL DMSO was added to each well. The contents were mixed thoroughly and the OD_{570} value was measured using a microplate reader. The results were expressed as '% control growth' according to the formula $[(N_c - N_e)/N_c] \times 100\%$, where N_c is the OD_{570} value obtained in the control cultures and N_e is the OD_{570} value in the treated cultures.

4.8. Preparation of whole cell extracts

For the Western blot assay, A549 cells were cultured in 150 cm^2 flasks until nearly confluent, then harvested by scraping. Approximately 5×10^7 cells were suspended in 1 mL of buffer (5 mM $NaPO_4$, pH 7.1, 150 mM NaCl, 2.5 mM KCl) containing 10 mg/mL of the protease inhibitors aprotinin, 1 mM dithiothreitol, and 1 mM PMSF. The suspension was subjected to five freeze/thaw cycles. The resulting homogenate was centrifuged at $15{,}000 \times g$ for 15 min at 4 °C. The supernatant fraction was removed, aliquoted and stored at −80 °C. Protein concentration was measured using the Bio-Rad protein assay dye reagent.

4.9. Western blot assay

Whole cell extracts derived from cells that had been exposed or not exposed to pol β inhibitors were resolved by electrophoresis in a 15% SDS–polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked in 3% gelatin–TBS (2 mM Tris, 50 mM NaCl, pH 7.5) and then incubated with anti-human DNA–polymerase β antibody and anti-β-actin antibody, which was used to assure the equivalence of protein loading. The membranes were incubated with horseradish peroxidase-conjugated anti-goat IgG, and protein binding was detected using an Immun-Blot assay kit following the manufacturer's instructions. The membranes were scanned, processed on a phosporimager, and quantified.

4.10. Unscheduled DNA synthesis assay

The unscheduled DNA synthesis assay was based on that described by Barra[63] with some modifications. A549 cells ($\sim 5 \times 10^5$), were placed into 25 mL flasks and treated with 10 mM bleomycin. After incubation at 37 °C under a humidified 5% CO_2 atmosphere for 30 min, the appropriate amount of dimethylsulfoxide, 10 mM hydroxyurea, 2 mM nicotinamide, and 5 μCi of [^{3}H]thymidine (80 Ci/mmol) was added to each flask, and pol β inhibitors **1**–**4** were added to the flasks (triplicates were used for each drug concentration). The samples were incubated for 3 h. Three milliliters of ice cold 0.9% saline was added to each sample to terminate the reaction. For determination of radioactivity, the cells were collected by centrifugation and the media were removed by aspiration. Three milliliters of ice cold 5% trichloroacetic acid was added and the samples were incubated on ice for 30 min. Cell pellets were resuspended by brief sonication; the samples were collected on Whatman GF/C filters and washed 5 times with ice cold 5% trichloroacetic acid and once with ice cold 95% ethanol. Filters were dried and used for determination of radioactivity.

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