Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 247–250

BIOORGANIC & MEDICINAL CHEMISTRY

Pentacyclic Triterpenoids from *Freziera* sp. that Inhibit DNA Polymerase β

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Received 21 July 1999; accepted 24 September 1999

Abstract—In a survey of crude plant extracts for DNA polymerase β inhibitors, a methyl ethyl ketone extract prepared from *Freziera* sp. exhibited potent inhibition of DNA polymerase β. Bioassay-guided fractionation of the extract, guided by an assay to detect DNA polymerase β inhibition, resulted in the isolation of six active pentacyclic triterpenoids (**1**–**6**). These triterpenoids had IC_{50} values ranging from 7.5 to 16 μM in the presence of bovine serum albumin (BSA) and 2.6–5.8 μM in the absence of BSA, consistent with the possibility that these inhibitors may be of use in vivo.

Introduction

DNA damaging agents comprise an important part of the armamentarium of drugs used for the clinical treatment of cancers. For some DNA damaging agents, tumor cell resistance results from the ability of the cell to effect DNA damage repair, thereby diminishing the efficacy of the applied agents.[1,2] DNA polymerase β, a 39 kDa enzyme whose role is to repair DNA damage, is responsible for DNA repair after exposure to some anticancer drugs such as bleomycin,[3–8] monofunctional DNA alkylating agents,[9–11] cisplatin[12–14] and neocarzinostatin.[8] Due to its central role in DNA repair, DNA polymerase β is a potential target for adjuvant anticancer therapy; selective inhibition of this enzyme by otherwise non-cytotoxic agents could potentiate chemotherapeutic treatment by DNA damaging agents, thus, enhancing the efficacy and permitting lower doses of such anticancer agents to be administered. Consistent with this thesis, a recent study in our laboratory using isolated DNA polymerase β inhibitors revealed that inhibition of enzyme function in cultured cells resulted in potentiation of bleomycin and cisplatin cytotoxicity.[15] DNA polymerase β inhibitors characterized to date have included bile acids,[16] sulfated glycoglycerolipids,[11] triterpenoids,[17,18] flavonoids,[19] as well as fatty acids[20–22] and their derivatives[23] and dideoxythymidine triphosphate.[24]

In our continuing search for DNA polymerase β inhibitors,[15,25–27] we found that a methyl ethyl ketone extract prepared from *Freziera* sp. (Theaceae) exhibited good inhibition of DNA polymerase β (79% inhibition at 100 μg/mL and 65% inhibition at 50 μg/mL, respectively). Bioassay-guided fractionation of this extract, using an assay to detect DNA polymerase β inhibition, resulted in the isolation of six DNA polymerase β inhibitory pentacyclic triterpenoids: oleanolic acid (**1**), ursolic acid (**2**), 3β-*O*-*cis*-*p*-coumaroylmaslinic acid (**3**), pomolic acid (**4**), 3β-*O*-*cis*-*p*-coumaroyl-2α-hydroxy-urs-12-en-28-oic acid (**5**) and 3β-*O*-*trans*-*p*-coumaroyl-2α-hydroxy-urs-12-en-28-oic acid (**6**). Principles **1**–**6** exhibited potent inhibitory activity against DNA polymerase β with IC_{50} values ranging from 7.5 to 16 μM in the presence of bovine serum albumin (BSA) and 2.6–5.8 μM in its absence. It may be noted that pentacyclic triterpenoids have been reported to have many biological and pharmacological activities,[28–31] including anti-inflammatory, anti-tumor, anti-ulcer and anti-HIV effects. We report herein the isolation of compounds **1**–**6** through bioassay-guided fractionation and their potencies as DNA polymerase β inhibitors.

Results and Discussion

Dried leaves and flowers of *Freziera* sp. (Theaceae) were soaked successively with hexanes, methyl ethyl ketone, methanol and water. The methyl ethyl ketone extract was found to inhibit DNA polymerase β (Table 1) and was fractionated initially on a polyamide 6S column which was washed successively with H_2O, 1:1 MeOH:H_2O, 4:1

Keywords: chemotherapy; enzyme inhibitors; nucleic acids; terpenes and terpenoids.

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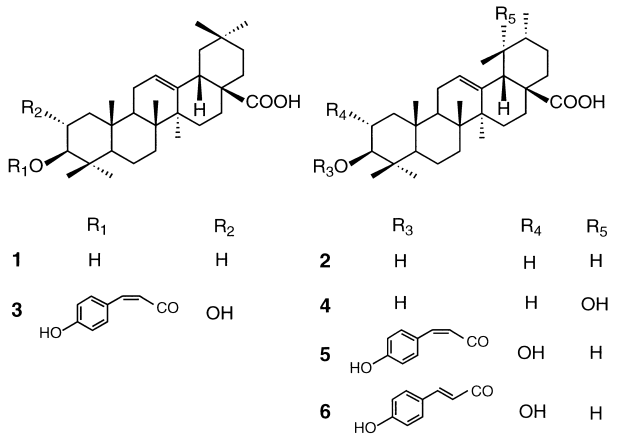
0968-0896/00/$ - see front matter
PII: S0968-0896(99)00276-X



MeOH:CH_2Cl_2, 1:1 MeOH:CH_2Cl_2 and 9:1 MeOH:NH_4OH. The final eluate contained polyphenols which tend to bind DNA strongly and are not specific inhibitors of the enzyme. The 4:1 MeOH:CH_2Cl_2 fraction showed significant DNA polymerase β inhibitory activity (87% inhibition at 100 μg/mL; 75% inhibition at 50 μg/mL) and was applied to a Sephadex LH-20 column for fractionation employing a normal phase elution scheme. The 1:1 CH_2Cl_2:acetone fraction from the Sephadex LH-20 column, which showed the strongest inhibition, was fractionated further using a C_8 reversed phase open column. Two fractions (13:7 and 19:1 MeOH:H_2O) from the C_8 open column had the greatest inhibitory potencies. Further fractionation of the active 19:1 MeOH:H_2O fraction, using a C_{18} reversed phase high performance liquid chromatography (HPLC) column, provided pure inhibitors **1** and **2**. The active 13:7 MeOH:H_2O fraction was fractionated further on a C_{18} reversed phase HPLC column to provide four inhibitory fractions. Purification of these fractions using a C_{18} reversed phase HPLC column afforded purified inhibitory principles **3**–**6**.

Table 1. DNA polymerase β inhibition data from bioassay-guided fractionation of a methyl ethyl ketone crude extract from *Freziera* sp.

Column	% Inhibition of DNA polymerase β		
	100 μg/mL	50 μg/mL	10 μg/mL
Crude extract	79	65	
Polyamide 6S			
4:1 MeOH:CH_2Cl_2	87	75	
Sephadex LH-20			
1:1 CH_2Cl_2:acetone		74	30
C_8 reversed phase			
13:7 MeOH:H_2O		78	34
19:1 MeOH:H_2O		82	45
HPLC (C_{18})			
1[a]		92	58
2[a]		90	55
Fraction 3[b]		80	32
Fraction 4[b]		66	30
Fraction 5[b]		75	20
Fraction 6[b]		89	16
HPLC (C_{18})[c]			
3		88	49
4		92	55
5		83	40
6		89	45

[a]From C_8 reversed phase 19:1 MeOH:H_2O fraction.
[b]From C_8 reversed phase 13:7 MeOH: H_2O fraction.
[c]Purification of fractions 3–6.

The structures of **1** and **2** were identified as oleanolic acid and ursolic acid, respectively, by direct comparison with authentic samples.[32–34] Subsequently, the other isolated inhibitors were determined to be 3β-*O-cis-p*-coumaroylmaslinic acid (**3**),[32] pomolic acid (**4**),[35,36] 3β-*O-cis-p*-coumaroyl-2α-hydroxy-urs-12-en-28-oic acid (**5**),[37] and 3β-*O-trans-p*-coumaroyl-2α-hydroxy-urs-12-en-28-oic acid (**6**)[33,34] through comparison of their ^1H and ^{13}C NMR, and MS spectral data with literature reports.

Natural products serve as one source of novel bioactive compounds and can provide an abundance of specific inhibitors of various target enzymes, such as DNA polymerase β. Compounds **1**–**6** displayed potent inhibitory activity against DNA polymerase β; their IC_{50} values were 7.5, 8.5, 12, 9.4, 13 and 16 μM, respectively, in the presence of bovine serum albumin (BSA), and 3.7, 4.8, 5.8, 2.6, 4.1 and 5.4 μM in the absence of BSA (Table 2), as determined under the assay conditions described in Experimental. That their activity was not greatly affected by the presence of serum albumin, a basic protein known to bind many lipophilic and acidic species, is consistent with the possibility that these inhibitors could be of utility in vivo. As regards their inhibitory potency, oleanolic acid (**1**) (IC_{50} = 7.5 μM, in the oleanolic series) and compounds **2** (IC_{50} = 8.5 μM) and **4** (IC_{50} = 9.4 μM), both in the ursolic acid series, exhibited similar potencies as DNA polymerase β inhibitors in the presence or absence of BSA. The coumaroyl derivatives **3** and **5** (IC_{50}s 12 and 13 μM, respectively), which both contain the same coumaroyloxy substituent, showed virtually identical inhibitory potencies. This suggests that in both the oleanolic and ursolic series, the coumaroyloxy substituents may play similar roles in contributing to the potency of DNA polymerase β inhibition. Further, the similar IC_{50} values for **5** and **6** argues that the geometry of the olefin within the C-3 substituent is not an important determinant of inhibitory potential.

Table 2. DNA polymerase β inhibitory activity for pentacyclic triterpenoids from *Freziera* sp.

Compound	IC_{50} (μM)	
	In the absence of BSA[a]	In the presence of BSA[a]
1	3.7	7.5
2	4.8	8.5
3	5.8	12
4	2.6	9.4
5	4.1	13
6	5.4	16

[a]BSA, bovine serum albumin.

Compound **3** was chosen as a representative inhibitor of DNA polymerase β and tested for its ability to potentiate the action of bleomycin in reducing the numbers of viable cultured mammalian cells. Accordingly, $P388D_1$ cells (derived from a mouse lymphoid neoplasm) were incubated for 6 h in the presence of bleomycin alone, compound **3** alone, and the two in combination. As shown in Table 3, bleomycin and **3** were non-toxic to the cultured cells when employed at 75 nM and 10 μM concentrations, respectively. However, in the presence of both compounds, the number of viable cells was reduced to 82% of that found in the untreated control. Thus, pentacyclic triterpenoid **3** was capable of potentiating the action of bleomycin in a cultured mammalian cell line. This finding underscores the potential utility of such polymerase β inhibitors for functioning as adjuvants in chemotherapeutic regimens. The virtue of this approach is also suggested by the recent finding that the sensitivity of CHO cells to a number of DNA damaging agents was decreased when DNA polymerase β was overexpressed.[2]

Among naturally occurring triterpenoids, only lanostanes[17,18] have thus far been reported to inhibit DNA polymerase β. Nigranoic acid was reported to have an IC_{50} value of 16.6 μM,[17] while several fomitellic acid derivatives exhibited minimum inhibitory concentration (MIC) values of 90–130 μM.[18] As demonstrated here, inhibitors **1**–**6** represent examples of pentacyclic triterpenoids that inhibit DNA polymerase β; their significant potencies of inhibition, and relatively rigid structures, may facilitate the development of agents capable of blocking DNA repair in complex biological systems.

Experimental

General methods

Polyamide 6S (a product of Riedel-de Haen, Germany) was obtained from Crescent Chemical Co. (Hauppauge, NY, USA). Sephadex LH-20 (Pharmacia; 40 μm) was purchased from Sigma Chemicals (St. Louis, MO, USA). C_8 reversed phase resin (~32–60 μm) was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). The Kromasil reversed phase C_{18} HPLC column (250×10 mm, 5 μm) for HPLC was from Higgins Analytical Inc. (Mountain View, CA, USA). ^1H and ^{13}C NMR spectra were obtained on General Electric GN-300 or QE-300 NMR spectrometers. Low resolution chemical ionization (CI) and electron impact (EI) 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 was from ICN Pharmaceuticals. DEAE-cellulose paper (DE-81) was purchased from Whatman (Abingdon, MD, USA). Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

Extraction and isolation of inhibitors 1-6 from *Freziera* sp

The leaves and flowers of *Freziera* sp. (Theaceae) were collected in February 1974 in Colombia. The dried leaves and flowers were soaked successively with hexanes, methyl ethyl ketone, methanol and water. The methyl ethyl ketone extract inhibited DNA polymerase β (79% inhibition at 100 μg/mL). The crude extract also had significant inhibitory activity after passage through a polyamide 6S column to remove polyphenols. Consequently, this crude extract was chosen for bioassay-guided fractionation. A total of 790 mg of the methyl ethyl ketone crude extract was used for the bioassay-guided fractionation; a typical set of experiments is described below. The crude extract (250 mg) was applied to a polyamide 6S column, which was washed successively with H_2O, 1:1 MeOH:H_2O, 4:1 MeOH:CH_2Cl_2, 1:1 MeOH:CH_2Cl_2 and 9:1 MeOH:NH_4OH. The 4:1 MeOH:CH_2Cl_2 fraction (96 mg) strongly inhibited DNA polymerase β (87% inhibition at 100 μg/mL) and was fractionated further on a Sephadex LH-20 column, which was eluted successively with hexanes, 1:1 hexane:CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2:acetone, acetone, and MeOH. The 1:1 CH_2Cl_2:acetone fraction (21 mg), which showed the strongest activity (74% inhibition at 50 μg/mL), was applied to a C_8 reversed phase open column for fractionation, using 11:9, 13:7, 15:5, 17:3, 19:1 and 20:0 MeOH:H_2O as the eluants. The 13:7 and 19:1 MeOH:H_2O fractions displayed the strongest inhibition of DNA polymerase β. Therefore, the 13:7 MeOH:H_2O fraction (5 mg) was applied to a C_{18} reversed phase HPLC column for fractionation; elution was with a linear gradient of 85–95% CH_3CN in H_2O over a period of 50 min at a flow rate of 3.5 mL/min (monitoring at 220 nm). Four active fractions were obtained from the C_{18} HPLC column. Purification of the four active fractions, employing the same C_{18} HPLC column and eluting with 90% CH_3CN in H_2O at a flow rate of 2.0 mL/min (monitoring at 220 nm), afforded active compounds **4** (0.3 mg), **3** (0.2 mg), **5** (0.5 mg) and **6** (0.6 mg) in order of elution. The active 19:1 MeOH:H_2O fraction (6 mg) from the C_8 reversed phase open column was fractionated using the same C_{18} HPLC column with the same isocratic elution system described above to afford **1** (1.3 mg) and **2** (1.1 mg).

Table 3. Potentiation of bleomycin cytotoxicity in P388D, cells by 3β-*O*-*cis*-*p*-coumaroylmaslinic acid (**3**)

Compound	Viable cells (% of control)		
	Compound alone[a]	BLM[b]	Compound[a] + BLM[b]
3β-*O*-*cis*-*p*-coumaroylmaslinic acid (**3**)	100	98	82

[a]Compound **3** was employed at 10 μM concentration.
[b]Bleomycin was employed at 75 nM concentration.

DNA polymerase β inhibition assay

After dissolving the crude extract samples or fractions in 1:1 DMSO:MeOH, 6 μL of the sample and 4 μL of rat DNA polymerase β (6.9 units, 48,000 units/mg) were added 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, 0.1 mg/mL bovine serum albumin, 6.25 μM dNTPs, 0.04 Ci/mmol [^{3}H]dTTP 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 successively with 0.4 M K_2HPO_4, pH 9.4, and 95% ethanol and then used for determination of radioactivity.

Potentiation of the action of bleomycin in cultured cells

Suspension cultures containing $P388D_1$ cells were maintained in 90% (v/v) Dulbecco's modified Eagle's medium containing glucose (4.5 g/L), 10% (v/v) donor horse serum, and 100 IU of penicillin, 0.1 mg of streptomycin and 0.25 μg of amphotericin β per mL at 37 °C in a 5% CO_2 in air atmosphere. Tissue culture flasks (12.5 mL volume) were treated with 5 mL of cell suspension ($\sim 5\times10^5$ cells/mL) and the culture was incubated for 1 h to stabilize the cells. The assays were carried out in a total volume of 6 mL containing the cells and test compounds, the latter of which were added to the cells after dissolution in media. Bleomycin was employed in the form of blenoxane, the clinically used mixture of bleomycins consisting primarily of bleomycin A_2 and B_2. The 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.

Acknowledgements

We thank Dr. Xiangyang Wang and Hongge Wang for the DNA polymerase β preparation employed in this work, and Professor Shou-Xun Zhao, China Pharmaceutical University, Nanjing, China, for the gifts of oleanolic acid and ursolic acid. This work was supported by Research Grant CA50771 from the National Cancer Institute.

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