Biscoumarin Derivatives from *Edgeworthia gardneri* that Inhibit the Lyase Activity of DNA Polymerase β

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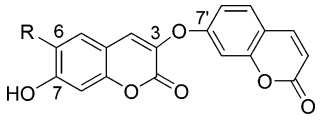
Bioassay-guided fractionation of an active methyl ethyl ketone extract of *Edgeworthia gardneri*, using an assay to monitor DNA polymerase β lyase inhibition, resulted in the isolation of three known biscoumarin derivatives, 7-hydroxy-3,7′-dicoumaryl ether (edgeworin, **1**), 7-hydroxy-6-methoxy-3,7′-dicoumaryl ether (daphnoretin, **2**), and 6,7-dihydroxy-3,7′-dicoumaryl ether (edgeworthin, **3**). Compounds **1**−**3** inhibited the lyase activity of DNA polymerase β with IC_{50} values of 7.3 μg/mL (22.5 μM), 43.0 μg/mL (122.3 μM), and 32.1 μg/mL (94.8 μM), respectively.

Genome stability is a hallmark of the survival and functioning of most organisms.[1] To mitigate the deleterious effects of DNA damage, several cellular mechanisms for repairing damaged DNA have evolved.[2] One of these, the base excision repair pathway (BER), repairs DNA damage to nucleobases arising spontaneously or induced by oxidizing or alkylating agents.[3,4] DNA polymerase β (pol β), a member of the X family of DNA polymerases,[5−8] is the major DNA repair enzyme involved in base excision repair.[9,10] Evidence for the role of pol β in mouse cells was obtained by Sobol et al.,[11] who showed that deletion of polymerase β results in sensitivity to alkylation damage. Base excision repair is initiated by DNA glycosylases, which produce an apurinic/apyrimidinic (AP) site at the location of the damaged nucleobase. Following DNA backbone incision by an AP endonuclease, the resulting 5′ deoxyribose residue is removed by the 5′ deoxyribosephosphatase (dRP lyase) activity of pol β. The polymerase activity of pol β also fills the resulting gap in the DNA, after which ligase I or III seals the nick.[12−14] Pol β is composed of a single 39 kDa polypeptide chain containing 335 amino acid residues; this can be proteolyzed into specialized N- and C-terminal domains.[3] The 31 kDa C-terminal domain is responsible for DNA polymerization (gap-filling polymerization),[15] while the 8 kDa N-terminal domain carries out the dRP lyase function of the enzyme.[4,16−19] There is compelling evidence that both the gap-filling and 5′-dRP lyase activities of polymerase β are essential for base excision repair in vivo;[20] removal of 5′-dRP at incised abasic sites is a key rate-limiting step during short-patch base excision repair.[15,16,21−23]

Not surprisingly, polymerase β is also believed to participate in repair of the DNA damage caused by clinically employed antitumor agents, such as bleomycin, cisplatin, and monofunctional DNA alkylating agents.[11,20,22−34] Logically, transient inhibition of the enzyme concomitant with antitumor therapy by such agents might improve the efficacy of these DNA-damaging agents. Thus, inhibitors of the lyase activity of polymerase β could prove useful for adjuvant cancer therapy, e.g., in reversing the drug resistance of certain tumor cells.

During a survey of plant metabolites for specific inhibitors of the lyase activity of polymerase β, the methyl ethyl ketone extract of *Edgeworthia gardneri* Meisn. (Thymelaeaceae) was found to exhibit good inhibition. Subsequent bioassay-guided fractionation of the extract, using an assay to detect polymerase β lyase inhibition, led to the isolation of three biscoumarins that inhibited the lyase: 7-hydroxy-3,7′-dicoumaryl ether (edgeworin, **1**), 7-hydroxy-6-methoxy-3,7′-dicoumaryl ether (daphnoretin, **2**), and 6,7-dihydroxy-3,7′-dicoumaryl ether (edgeworthin, **3**). Compounds **1**−**3** exhibited inhibitition of polymerase β lyase with IC_{50} values of 7.3 μg/mL (22.5 μM), 43.0 μg/mL (122.3 μM), and 32.1 μg/mL (94.8 μM), respectively. Described herein is the isolation of these three biscoumarins and their in vitro inhibition of polymerase β lyase.



1 R = H
2 R = OCH_3
3 R = OH

The crude plant material was soaked successively with hexanes, methyl ethyl ketone, methanol, and water. The methyl ethyl ketone extract of *E. gardneri* was found to inhibit the dRP lyase activity of polymerase β. The methyl ethyl ketone extract of *E. gardneri* 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 polymerase β. The 9:1 MeOH−NH_4OH fraction contained tannins, which tend to bind DNA and protein nonspecifically[35−37] and thus are not specific inhibitors of the enzyme. Accordingly, the 1:4 CH_2Cl_2−MeOH fraction was chosen for further bioassay-guided fractionation on diol open columns with gradient eluting systems containing hexane−CH_2Cl_2−MeOH (see Experimental Section). Three pure inhibitory components, **1**−**3**, were finally obtained.

Through comparison of their ^1H and ^{13}C NMR and MS spectral data with those reported in the literature, the chemical structures of the isolated inhibitors were determined to be 7-hydroxy-3,7′-dicoumaryl ether (edgeworin, **1**),[38] 7-hydroxy-6-methoxy-3,7′-dicoumaryl ether (daphnoretin, **2**),[39,40] and 6,7-dihydroxy-3,7′-dicoumaryl ether (edgeworthin, **3**).[41,42]

Compounds **1**−**3** inhibited the dRP lyase activity of polymerase β in the in vitro assay in a dose-dependent

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Table 1. DNA Polymerase β Lyase Inhibitory Activities for Compounds **1**−**3** from *Edgeworthia gardneri*

compound	IC_{50} (μM)
1	22.5
2	122.3
3	94.8

Table 2. Potentiation of Bleomycin Cytotoxity in A549 Cells by Edgeworin

treatment	cell growth inhibition (%)
control	0
edgeworin (6 μM)	1.6
blenoxane (0.5 μM)	35.5
edgeworin (6 μM) + blenoxane (0.5 μM)	50.7

manner; their IC_{50} values were 22.5, 122.3, and 94.8 μM, respectively (Table 1). The three biscoumarins from *E. gardneri* possess very similar structures, but exhibit rather different inhibitory potencies toward polymerase β lyase. The dearth of functional groups available for interaction with the enzyme and DNA substrates suggest that some or all of the existing groups must be important for specific interaction. This is reinforced by the negative effect of a substitutent or bulky functionality at the 6-position, which must interfere with this interaction, thus resulting in a lesser inhibitory effect. Also studied was the ability of edgeworin (**1**) to potentiate the cytotoxicity of bleomycin toward cultured A549 cells by blocking the repair of bleomycin-mediated DNA damage. As shown in Table 2, compound **1** clearly increased the cytotoxicity of bleomycin when the two were employed jointly.

We also tested compounds **1**−**3** in an in vitro polymerase β-mediated DNA polymerization assay. In the polymerization inhibition assay (see Experimental Section), none of these compounds exhibited inhibition of the gap-filling polymerization activity of polymerase β at concentrations up to 100 μg/mL. This suggests that compounds **1**−**3** represent a prototype of enzyme inhibitors that may selectively block the dRP lyase activity of polymerase β. These compounds may specifically target the N-terminal domain of polymerase β, thus blocking the lyase activity of the enzyme. The dRP lyase activity of polymerase β is the rate-limiting step in "single-nucleotide" base excision repair of lesions generated by anticancer agents in tumor cells. Therefore, these natural products may constitute valuable leads in defining more potent lyase inhibitors of utility for adjuvant cancer therapy to enhance the efficacy of DNA-damaging agents.

Compound **1** was isolated from *E. gardneri* for the first time. Oligocoumarins, including biscoumarins, are relatively rare natural products; they have been isolated predominantly from plants in the families Thymelaeaceae, Luguminosae, and Rutaceae.[39] Compound **2** (daphnoretin) is the best known biscoumarin derivative and was reported to show in vivo antineoplastic activity against the Ehrlich ascites carcinoma in mice and to inhibit a number of enzymes involved in DNA synthesis in Ehrlich ascites cells.[39] It has also been reported that daphnoretin is a protein kinase C activator and suppresses hepatitis B virus gene expression in human hepatoma cells.[43] Presently, we have demonstrated that daphnoretin inhibits the lyase activity of polymerase β. It is unclear at present whether its inhibition of polymerase β lyase activity is related to its earlier-reported in vivo antineoplastic activity.[39] In this context, it is worth mentioning that polymerase β has been found to be overexpressed in some human tumor tissues, and more recently it has been shown that overexpression of polymerase β results in a mutator and genome instability phenotype.[44,45]

Polymerase β is a small enzyme, but apparently with multiple functions. By using an in vitro primer extension assay, Bergoglio et al.[46] recently discovered that purified human and calf thymus polymerases β can synthesize up to 8-mer RNA, and they proposed that incorporation of ribonucleotides into DNA by polymerase β may contribute to the high frequency of mutagenesis observed in cells containing up-regulated polymerase β. If this occurs physiologically, this new function may constitute another potential new target for the development of novel anticancer agents. Recently results reported by Horton et al.[47] from cell cycle analyses of wild-type and polymerase β null cells following treatment with methyl methanesulfonate and 5-hydroxymethyl-2′-deoxyuridine, respectively, suggested that polymerase β might be involved in a DNA damage-associated checkpoint control pathway. It seems clear that we still have more to learn about the biological functions of this important enzyme.

Experimental Section

General Experimental Procedures. Polyamide 6S (pore density 0.25 g/mL, a product of Riedel-del Haen, Germany) was obtained from Crescent Chemical Co. (Hauppauge, NY). LiChroprep Diol material (40−63 μm) (a product of E. Merck, Germany) was purchased from EM Separations Technology (Gibbstown, NJ). Hexanes, dichloromethane, and methanol of analytical grade used in open column chromatography were purchased from Fisher Scientific. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were obtained on a Varian 500 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. Uracil-DNA glycosylase was purchased from New England Biolabs, Inc. Synthetic oligodeoxyribonucleotides were obtained from Integrated DNA Technologies, Inc. DEAE-cellulose paper (DE-81) was purchased from Whatman.

Plant Material. The stem bark of *Edgeworthia gardneri* Meisner was collected in April 1986 in Bhutan. A voucher specimen (3101299) is stored at the U.S. National Arboretum Herbarium, Washington, DC.

Extraction and Isolation. The crude, dried plant materials were soaked at room temperature successively with hexanes, methyl ethyl ketone, methanol, and water. The concentrated methyl ethyl ketone extract exhibited good polymerase β lyase inhibitory activity and was chosen for further bioassay-guided fractionation. A typical set of experiments is described below. The active methyl ethyl ketone extract (30 mg) was fractionated initially using 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 fraction (10.8 mg) still possessed good inhibitory activity after removal of the polyphenols by the polyamide 6S column and was fractionated further on a Diol column, which was washed successively with 80:20 CH_2Cl_2−hexanes, CH_2Cl_2, 95:5 CH_2Cl_2−MeOH, 90:10 CH_2Cl_2−MeOH, and MeOH. The 80:20 CH_2Cl_2−hexanes (A), CH_2Cl_2 (B), and 95:5 CH_2Cl_2−MeOH (C) fractions retained inhibitory activity. Fraction B (2.8 mg) was fractionated on a diol column, using 90:10 CH_2Cl_2−hexanes, CH_2Cl_2, 98:2 CH_2Cl_2−MeOH, 95:5 CH_2Cl_2−MeOH, and MeOH as eluants; the CH_2Cl_2 fraction exhibited the strongest inhibitory activity and afforded pure compound **1** (0.2 mg). Fraction A (3.4 mg) was fractionated further by employing a diol column, which was washed successively with 50:50 CH_2Cl_2−hexanes, 70:30 CH_2Cl_2−hexanes, 80:20 CH_2-

Cl_2−hexanes, 90:10 CH_2Cl_2−hexanes, CH_2Cl_2, and MeOH; the 70:30 CH_2Cl_2−hexanes and 80:20 CH_2Cl_2−hexanes fractions possessed the strongest inhibitory activity. These were combined, according to their identical ^1H and ^{13}C NMR spectral as well as MS spectral data, to afford pure compound **2** (2.5 mg). Fraction C (2.7 mg) was fractionated further on a diol column, which was eluted successively with CH_2Cl_2, 95:5 CH_2Cl_2−MeOH, 90:10 CH_2Cl_2−MeOH, and MeOH; pure compound **3** (1.4 mg) was obtained from the 95:5 CH_2Cl_2−MeOH fraction, which exhibited the strongest inhibitory activity. The physicochemical and spectral data for compounds **1**, **2**, and **3** were identical to those in previous reports.[38−42]

DNA Polymerase β Lyase Inhibition Assay.[48] (1) 3′-^{32}P-End labeling: A 36-nucleotide oligodeoxyribonucleotide containing a uridine at position 21 on one strand was ^{32}P-end labeled at its 3′-end with terminal deoxynucleotidyltransferase + [α-^{32}P]ddATP. The product was then purified by 20% denaturing polyacrylamide gel electrophoresis. The band of interest was visualized by autoradiography and excised from the gel. After removal by the "crush and soak" method, the oligodeoxyribonucleotide was then annealed to its complementary strand by heating the solution at 70 °C for 3 min, followed by slow cooling to 25 °C. (2) Apurinic/apyrimidinic (AP) site preparation: An 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 a uridine at position 21, 10 mM Hepes-KOH, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10 mg/mL 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 21 was ready for the dRP-excision assay. (3) dRP-excision assay: dRP-excision activity was determined using a reaction mixture (5 μL total volume) that contained 354 nM ^{32}P-end-labeled DNA substrate containing an AP site at position 21, 0.17 unit of rat polymerase β, and the test samples (crude extracts, fractions or compounds **1**−**3**, dissolved in DMSO). After incubation at room temperature for 30 min, the reaction was terminated. The product was stabilized by the addition of 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 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.

DNA Polymerase β Inhibition Assay. Compounds **1**−**3** were dissolved in DMSO. Six microliters of each sample and 4 μL of rat polymerase β (6.9 units, 48,000 units/mg) were added to 50 μL of reaction solution (60 μL total volume), which contained 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 DNase I-treated calf thymus DNA. After incubation for 1 h at 37 °C, 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, then with H_2O, and briefly with 95% ethanol, and used for determination of radioactivity.

Cell Growth Inhibition Assay. For assay of cell growth inhibition, A549 cells were seeded at a density of 40 000 cells/well in six-well plates. The following day, cells were exposed to 0.5 mM blenoxane in growth medium for 1 h in the presence or absence of edgeworin. Cells were then washed with Hank's balanced salt solution, and fresh medium was added with or without edgeworin. Dishes were incubated for 48 h in a 10% CO_2 incubator. Cells (in triplicate for each drug concentration) were counted, and the results were expressed as "percent growth inhibition" according to the formula $[(N_c - N_e)/N_c] \times 100\%$, where N_c is the number of cells in control wells and N_e is the number cells in drug-treated wells.

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