

SHORT COMMUNICATION

L-PROLINE ANALOGUES OF ANTHRAQUINONE-2-CARBOXYLIC ACID: CYTOTOXIC ACTIVITY IN BREAST CANCER MCF-7 CELLS AND INHIBITORY ACTIVITY AGAINST TOPOISOMERASE I AND II

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A series of proline analogues of anthraquinone-2-carboxylic acid (**1–3**) were synthesized and evaluated for cytotoxic activity in the cultured breast cancer MCF-7 cells. The concentrations of **1**, **2** and **3** needed to inhibit [³H]thymidine incorporation into DNA by 50% (IC₅₀) were found to be 107 ± 6 nM, 185 ± 5 nM and 87 ± 6 nM, respectively. To test whether cytotoxic properties were related to topoisomerase action, the most potent compounds **1** and **3** were evaluated in a cell-free system. Compound **3**, which contains a basic substituent at C terminus of the amino acid such as (dimethylamino)propyl inhibited the catalytic activity of both topoisomerases I and II at a concentration of 30 and 60 nM, respectively. However, compound **1** containing an electrostatically neutral moiety, such as methyl ester did not inhibit topoisomerase I or topoisomerase II. In summary, compound **3** is a promising lead compound for a further structural variation in the design of new antitumour drugs.

Key words: breast cancer MCF-7, cytotoxicity, DNA topoisomerase, proline analogues of anthraquinone-2-carboxylic acid

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INTRODUCTION

Recently, certain synthetic peptidyl anthraquinones have been found to exhibit anticancer activity *in vivo* [2, 3, 6, 12]. Previous studies suggest that the potent cytotoxicity of peptidyl anthraquinones may result from the interference with topoisomerase I and II action and/or possible intercalation of the drug at the defined GC-rich sequences [2, 3, 6]. DNA topoisomerases are nuclear enzymes that alter DNA conformation through a concerted breaking and rejoining of DNA strands, thereby controlling the DNA topology, required for replication, transcription and recombination [1, 11]. Topoisomerases are classified as type I and type II on the basis of their distinct differences in sequence and function. Type II enzymes are dimeric, break both strands of the duplex through this transient double-stranded break in an ATP-dependent manner. In contrast, the type I topoisomerases are monomeric and manage DNA superhelical tension in cells by cleaving one strand of duplex DNA. Interference with DNA religation by the topoisomerase inhibitors results in the production of transient DNA strand breaks and DNA-protein complexes

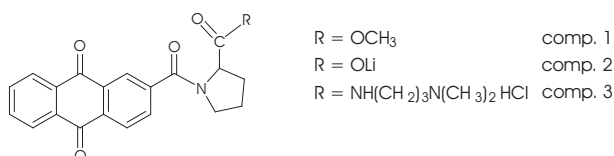


Fig. 1. Chemical structure of the investigated compounds

that appear to be critical for the cytotoxic effects of these drugs [7].

We synthesized three L-proline analogues of anthraquinone-2-carboxylic acid **1–3** (Fig. 1) and evaluated their cytotoxicity against breast cancer MCF-7 cells. The C terminus of the amino acid is the only structural difference between these compounds. The amino acid C-terminus, either methyl ester (**1**) or (dimethyl)aminopropyl amide (**3**), differ in two respects. The methyl ester has a neutral function, while dimethylamino moiety would be protonated at physiological pH of 7.4 to provide favorable electrostatic potential of the DNA. On the other hand, compounds with a neutral side chain can penetrate the cellular membrane more easily.

This paper describes the synthesis, cytotoxicity in breast cancer MCF-7 cells and the effects in topoisomerase I/II inhibition assay of the compounds **1–3**.

MATERIALS and METHODS

Chemistry

N-(anthraquinone-2-carbonyl)-L-proline methyl ester (**1**) was synthesized by condensation of L-proline methyl ester and anthraquinone-2-carboxylic acid by using the carbodiimide coupling method [9]. The mild saponification of **1** in the presence of LiOH gave N-(anthraquinone-2-carbonyl)-L-proline (**2**). Compound **2** was isolated as a lithium salt. Aminolysis of **1** with 3-dimethylamino-1-propylamine and acidification with HCl yielded N-[1-(an-

Table 1. Physicochemical properties of **1–3**

Compound	Mol. formula	M.p. °C*	¹ H NMR [ppm] (Me ₂ SO-d ₆)	¹³ C NMR [ppm] (Me ₂ SO-d ₆)
1	C ₂₁ H ₁₇ NO ₅ (363.4)	85–87	7.95–8.65 (m, 7H), 4.41 (m, 1H), 3.90 (s, 3H), 3.50 (m, 2H), 1.64 (m, 4H)	181.9, 181.8, 169.0, 159.3, 135.4, 134.7, 134.6, 134.3, 133.2, 132.9, 127.3, 127.3, 126.8, 52.0, 50.4, 47.1, 27.8, 23.5
2	C ₂₀ H ₁₄ NO ₅ Li · H ₂ O (373.3)	192–194	7.95–8.65 (m, 7H), 4.41 (m, 1H), 3.48 (m, 2H), 1.68 (m, 4H)	182.0, 181.8, 168.8, 159.3, 135.7, 134.7, 134.6, 134.3, 133.1, 132.9, 127.3, 127.3, 126.8, 50.4, 47.1, 27.7, 23.3
3	C ₂₅ H ₂₇ N ₃ O ₄ · HCl (470.0)	174–176	7.95–8.65 (m, 7H), 4.41 (m, 1H), 3.48 (m, 2H), 3.24 (m, 2H), 3.02 (t, 2H), 2.72 (s, 6H), 1.87 (m, 2H), 1.68 (m, 4H)	182.0, 181.8, 161.0, 159.3, 135.7, 134.7, 134.6, 134.3, 133.1, 132.9, 127.3, 127.3, 126.8, 56.3, 50.4, 47.1, 43.2, 37.2, 27.7, 25.6, 23.3

* Melting points are uncorrected

thraquinone-2-carbonyl)-L-prolyl]-N',N'-dimethyl-1,3-propanediamine hydrochloride (**3**). Purity of **1–3** was verified by NMR and elemental analysis. The physicochemical data for compounds **1–3** are given in Table 1. All compounds were subjected to elemental analysis (C,H,N) and the measured values agreed within $\pm 0.4\%$ with the calculated ones. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 200F spectrometer, using TMS as an internal standard. Multiplicity of resonance peaks is indicated as singlet (s), triplet (t) or multiplet (m).

Materials

Anthraquinone-2-carboxylic acid, L-proline methyl ester, Hoechst 33258, ethidium bromide, pBR322 plasmid DNA, proteinase K and topoisomerase I were purchased from Sigma Chemical Co. (USA). Topoisomerase II was purchased from Amersham Pharmacia Biotech. Stock cultures of breast cancer MCF-7 were purchased from the American Type Culture Collection, Rockville, MD. Dulbecco's minimal essential medium (DMEM) and foetal bovine serum (FBS) used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). [^3H]Thymidine (6.7 Ci/mmol) was the product of NEN (USA).

MCF-7 cell culture

Stock cultures of breast cancer MCF-7 cells [10] were maintained in continuous exponential growth by weekly passage in DMEM supplemented with 10% FBS, 50 g/ml penicillin, 50 g/ml streptomycin at 37°C in humid atmosphere containing 5% CO₂ in an incubator. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin, 0.02% EDTA in calcium-free phosphate-buffered saline. The study was carried out using cells from passages 3 to 7, growing as monolayer in 6-well plates (Nunc) (5×10^5 cells per well). Cells reached about 80% of confluency at day 4 after inoculation and in most cases such cells were used for the assays.

Cytotoxic assay

To examine the effect of compounds **1–3** on MCF-7 proliferation, the cells were seeded in 6-well tissue culture dishes at 5×10^5 cells per well with 2 ml of growth medium. The plates were incubated at varying concentrations of compounds **1–3**, Hoechst 33258 and 0.5 Ci of [^3H]thymidine for

24 h at 37°C. Compound **1** was solubilized in the presence of 1% DMSO. The cells were rinsed 3 times with phosphate buffered saline, solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% sodium dodecyl sulfate, scintillation liquid (9 ml) was added and radioactivity incorporated into DNA was determined by liquid scintillation counting. Radioactive [^3H]thymidine uptake was expressed as dpm/well. The results were calculated in dpm per well and expressed as percentage of control.

Relaxation assay of topoisomerases I and II

PBR322 plasmid DNA (0.25 g) was incubated with 3 units of topoisomerase I (reaction buffer: 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5 M NaCl, 1 mM dithiothreitol) or topoisomerase II (reaction buffer: 10 mM Tris-HCl (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, and 15 g/ml bovine serum albumin) in the presence of varying concentrations of the test compound. The mixture was incubated at 37°C for 1 h and the reaction was terminated by addition of 2 l of 10% SDS and 2 l of proteinase K (1 mg/ml). The reaction mixture was subjected to electrophoresis on 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light.

Statistical analysis

In all experiments, the mean values of three assays \pm standard deviations (SD) were calculated. The results were submitted to statistical analysis using Student's *t*-test. Differences were considered significant when $p < 0.05$. Mean values \pm SD of 3 independent experiments ($n = 4$) done in duplicates are presented.

RESULTS and DISCUSSION

We studied the effect of compounds **1–3** and Hoechst 33258 on DNA synthesis in the cultured breast cancer MCF-7 cells (Fig. 2). Hoechst 33258 is a cytological DNA staining agent and is also used as a therapeutic agent for solid tumors [5]. All studied compounds inhibited DNA synthesis in breast cancer MCF-7 cells, in a dose-dependent manner, however with different potency. The concentrations of **1**, **2** and **3** needed to inhibit [^3H]thy-

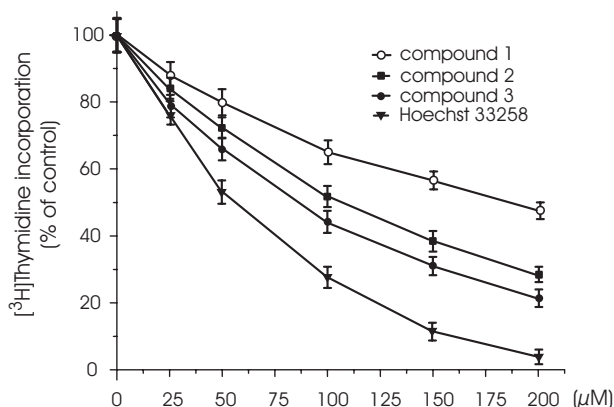


Fig. 2. Cytotoxic effects of **1–3** on the cultured breast cancer MCF-7 cells as measured by the inhibition of [³H]thymidine incorporation into DNA. Mean values \pm SD of 3 independent experiments ($n = 4$) done in duplicates are presented

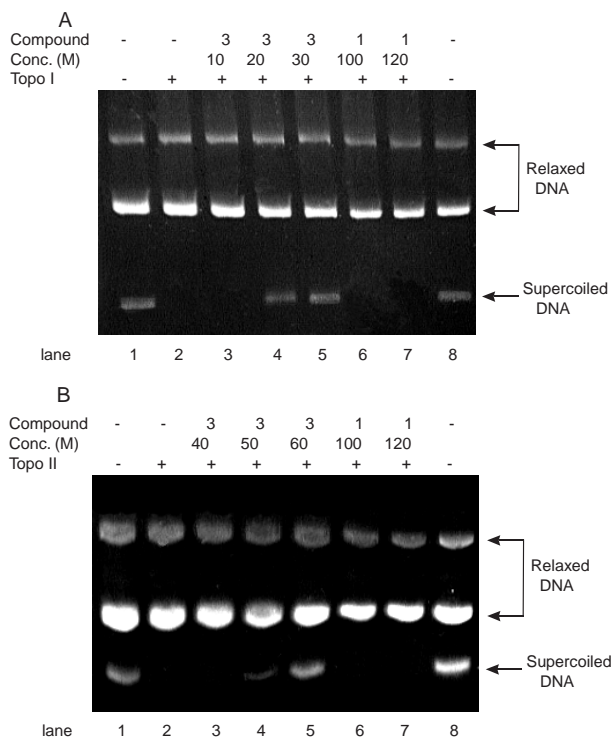


Fig. 3. Inhibition of topoisomerase-mediated DNA supercoiling in the presence of **1** and **3**. (A) Topoisomerase I and (B) topoisomerase II. Native pBR322 plasmid DNA (0.25 μ g) (lanes 1 and 8) was incubated with 3 units of topoisomerase in the absence (lane 2) or in the presence of **1** and **3** at the indicated concentration. After 1 h incubation at 37°C, the reaction mixture was treated as described in Materials and Methods, and DNA was analyzed by 0.8% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed under UV light

midine incorporation into DNA by 50% (IC_{50}) were found to be 185 ± 6 μ M, 107 ± 5 μ M and 87 ± 6 μ M, respectively, suggesting lower cytotoxic potency of these compounds compared to Hoechst 33228 ($IC_{50} = 55 \pm 6$ μ M).

To identify a biochemical target for these compounds, we investigate their action on topoisomerases [1, 11]. Previous studies with both the topoisomerase I inhibitor camptothecin and topoisomerase II inhibitors etoposide and doxorubicin demonstrated a correspondence between the inhibition of DNA synthesis and of growth in MCF-7 breast tumor cells [4]. Purified topoisomerases I and II were incubated with increasing concentrations of **1** and **3** in the presence of supercoiled pBR322 plasmid DNA, and the products were subjected to electrophoresis in the presence of ethidium bromide to separate closed and open circular DNA. Figure 3A shows the result of our electrophoretic analysis of topoisomers of DNA caused by topoisomerase I action. As seen there, the superhelical plasmid (lane 1 and lane 8) was relaxed by topoisomerase I (lane 2). The coexistence of the compound **3** caused some changes in topoisomer distribution. Particularly, the addition of **3** in an amount as high as 30 μ M caused superhelices that looked similar to the intact plasmid (see lane 5 as well as lane 1).

Lanes 3, 4 and 5 of Figure 3B are the result of similar examination of the action of compound **3** and topoisomerase II. If the concentration of **3** was as low as 40 μ M, it did not inhibit topoisomerase II (see lane 3), although it was definitely inhibitory when concentration was as high as 60 μ M (see lane 5). Most likely compound **3** inhibits the binding of topoisomerase I or II to DNA. The inhibitory effects of **3** on topoisomerase I may be in part related to its DNA binding rather than primarily to stabilization of topoisomerase I intermediates with DNA through specific binding to the enzymes. Further investigation is required to clarify if **3**, like camptothecin, may form a ternary complex with topoisomerase I by 3 different mechanisms: binding to adenine-thymine rich regions in DNA, binding to topoisomerase I or binding to the topoisomerase I-DNA complex [8].

Compound **1** containing an electrostatically neutral moiety, such as methyl ester, did not affect topoisomerase I or II activity at the doses used (5–120 μ M). The inability of **1** to interfere with the action of topoisomerase I or II at the doses used re-

sulted in complete relaxation of the supercoiled DNA by these enzymes (see line 6 and 7 Fig. 3A; line 6 and 7 Fig. 3B). This is consistent with the likelihood that the mechanism other than inhibition of topoisomerase I or II is associated with its cytotoxic activity. The situation is complicated by such factors as cellular uptake and conversion of parent compounds to metabolites which may have increased activity towards topoisomerases I and II.

Compound **3** is a promising lead compound for a further structural variation in the design of new antitumour drugs.

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