Podophyllotoxin Aza-Analogue, A Novel DNA Topoisomerase II Inhibitor

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The pendant E-ring moiety of the podophyllotoxin aza-analogue 1 that is a potent inhibitor of microtubule assembly was modified in order to acquire inhibitory activity of DNA topoisomerase II. The monophenolic analogue 2 did not exhibit human topoisomerase II inhibition, while the *ortho*-quinone 3 that was obtained by oxidation of 2 inhibited its catalytic activity (decatenation) in a dose-dependent manner and stimulated double strand DNA breaks in supercoiled circular plasmid DNA, resulting in the production of linear DNA. These results showed that the topoisomerase II inhibition of the *ortho*-quinone 3 is due to stabilization of the topoisomerase II-DNA covalent binary complex. On the other hand, the *ortho*-quinone 3 did not inhibit the relaxation process of supercoiled DNA by topoisomerase I at concentrations up to 400 μM, nor was intercalation observed in unwinding measurements of 3. Therefore, the *ortho*-quinone 3 was shown to be a novel nonintercalative topoisomerase II specific inhibitor that stabilizes the cleavable complex. The present results suggest that the 4′-free hydroxyl group on the E-ring and the sugar moiety on the C-ring are not a prerequisite for topoisomerase II inhibition by podophyllotoxin derivatives.

Key words DNA topoisomerase II; etoposide; podophyllotoxin; podophyllotoxin aza-analogue; inhibitor; cleavable complex

DNA topoisomerases, mechanistically divided into two main classes of types I and II, are critical enzymes that control the level of DNA supercoiling by catalyzing the passage of individual DNA strands or double strands through one another.[1] DNA topoisomerases are involved in biological processes of DNA metabolism such as replication, transcription, recombination and chromosome segregation at mitosis. Therefore, compounds that inhibit these enzymes as the primary cellular target are of special interest since those are promising candidates for anticancer drugs.[2]

Etoposide,[3] a DNA topoisomerase II specific inhibitor, is a semisynthetic lignan derived from podophyllotoxin,[4] a microtubule assembly inhibitor isolated from roots of *Podophyllum* sp. (Chart 1). Etoposide inhibits topoisomerase II by trapping the topoisomerase II–DNA covalent binary complex termed the cleavable complex,[5] resulting in cell death. Extensive structure–activity relationship studies on podophyllotoxin derivatives have suggested that the phenolic 4′-hydroxyl group of the pendant E-ring plays a crucial role in the DNA cleavage in which topoisomerase II is implicated.[6]

In our continuing studies toward antitumor compounds,[7] we have already reported the design and synthesis of podophyllotoxin aza-analogues **1** and **2**.[8] Both analogues exhibited potent cytotoxicity against KB cells due to the inhibition of microtubule assembly, which resulted in arresting cells in mitosis. This result allowed us to expect that podophyllotoxin aza-analogues are also potential topoisomerase II inhibitors. The trimethoxy analogue **1** did not show topoisomerase II inhibition, probably due to the lack of the structural requirement described above. However, no inhibitory effect of the monophenolic analogue **2** on the enzyme was observed at all. This unexpected result led to further modification of the E-ring moiety of **2** to the corresponding *ortho*-quinone **3** to exert the potential ability of the podophyllotoxin aza-analogues for topoisomerase II inhibition.[9]

We report herein the inhibitory effect of the *ortho*-quinone **3** on human DNA topoisomerase II. Analogue **3** has been shown to inhibit topoisomerase II by stabilizing the DNA–enzyme cleavable complex nonintercalatively as in the case of etoposide.

Experimental

All melting points were measured with a Buchi 510 melting point apparatus and are uncorrected. IR spectra were recorded on a Jasco IR Report-100 IR spectrometer. NMR spectra were measured with a JEOL GSX-400 spectrometer, and mass spectra were taken on a JEOL JMS-DX300 mass spectrometer. Column chromatography was run with BW-200 silica gel (Fuji Silysia Chemical, Ltd.).

Reagents Purified human placenta topoisomerases I (2 U/μl) and IIα (2 U/μl), kinetoplast DNA (kDNA), etoposide and 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA) were purchased from TopoGen, Inc. (Columbus, Ohio). Supercoiled pUC19 and pBR322 plasmid DNA and

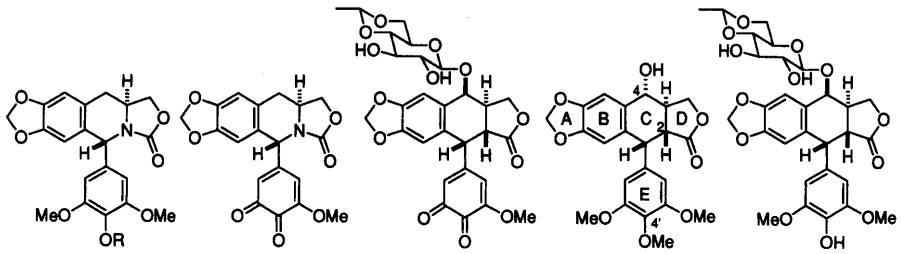

Chart 1

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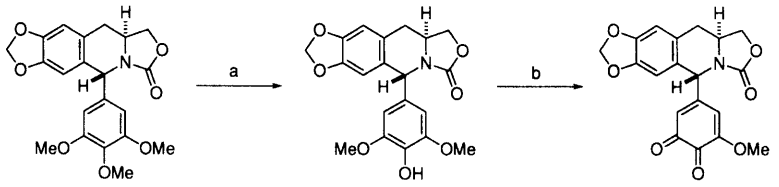

Reagents: a) HBr/Cl(CH$_2$)$_2$Cl, 0°C,14h, 80%; b) 6 M HNO$_3$/CHCl$_3$, r.t., 30s, 99%.

Chart 2

*Hind*III restriction endonuclease were obtained from Toyobo (Osaka).

Synthesis of *Ortho*-Quinone Analogue (3) To a vigorously stirred solution of the racemate of the monophenolic form **2** (24 mg) in $CHCl_3$ (1 ml) was added 6 M HNO_3 at room temperature. The reaction mixture was stirred for 30 s, quenched with H_2O (8 ml) and extracted with $CHCl_3$ (20 ml×3). The organic layer was washed with saturated $NaHCO_3$ (20 ml) and brine (20 ml×2), dried over Na_2SO_4 and concentrated *in vacuo*. The red oily residue was purified by silica gel column chromatography ($CHCl_3$/Me_2CO= 6/1), followed by recrystallization from CH_2Cl_2 and benzene to afford **3** (23 mg, 99%) as red powders, mp 185—188 °C (dec.), IR ($CHCl_3$) cm^{-1}: 1751, 1700, 1667, 1630. ^{1}H-NMR ($CDCl_3$) δ: 2.88 (1H, dd, J=5.9, 9.8 Hz), 2.90 (1H, d, J=9.8 Hz), 3.81 (3H, s), 4.00 (1H, m), 4.21 (1H, dd, J=3.3, 8.8 Hz), 4.61 (1H, dd, J=8.1, 9.8 Hz), 5.55 (1H, s), 5.66 (1H, d, J=1.8 Hz), 5.99 (2H, s), 6.20 (1H, d, J=1.8 Hz), 6.53, 6.63 (each 1H, s). ^{13}C-NMR ($CDCl_3$) δ: 33.7 (t), 48.1 (d), 55.9 (d), 56.2 (q), 68.8 (t), 101.6 (t), 107.7 (d), 109.0 (d), 109.6 (d), 121.0 (d), 122.2 (s), 126.0 (s), 147.3 (s), 148.1 (s), 153.7 (s), 155.7 (s), 157.1 (s), 175.1 (s), 178.9 (s). MS *m/z*: 369 (M^+). *Anal.* Calcd for $C_{19}H_{15}NO_7$: C, 61.79; H, 4.09; N, 3.79. Found: C, 62.07; H, 2.28; N, 3.55.

Kinetoplast DNA Decatenation Reaction mixtures contained 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol (DTT), kDNA (200 ng), 2 μl of a drug solution (10% dimethyl sulfoxide [DMSO]) and 1 U of topoisomerase II in a total volume of 20 μl. Samples were incubated at 37 °C for 30 min and terminated with 2 μl of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol). Reaction products were electrophoresed on a 1% agarose gel in TAE (Tris-acetate-EDTA) running buffer.

Topoisomerase II Mediated DNA Cleavage Reactions (20 μl) containing 30 mM Tris–HCl (pH 7.6), 60 mM NaCl, 8 mM $MgCl_2$, 3 mM ATP, 15 mM mercaptoethanol, 2 μl of a drug solution (10% DMSO), 8 U of topoisomerase II and 250 ng of pBR322 plasmid DNA were incubated at 37 °C for 30 min. Samples were terminated by the addition of 2 μl of a solution containing 5% sodium dodecyl sulfate (SDS) and 2.5 mg/ml of proteinase K and incubated at 37 °C for 60 min. The samples were electrophoresed through a 1.2% agarose gel in TBE (Tris–borate–EDTA) running buffer containing 0.1% SDS. Both agarose gel and running buffer contained 0.5 μg/ml of ethidium bromide.

Topoisomerase I Mediated DNA Relaxation Reactions were carried out in the same manner as described for the decatenation assay except that reaction mixtures contained 10 mM Tris–HCl (pH 7.9), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol and pUC19 plasmid DNA (200 ng).

DNA Unwinding Measurements Relaxed closed circular pUC19 DNA (250 ng) that was obtained by incubation with DNA topoisomerase I, followed by phenol extraction and ethanol precipitation, was incubated with DNA topoisomerase I in the standard reaction mixture for the relaxation assay in the presence of increasing amounts of *ortho*-quinone and *m*-AMSA.

Results and Discussion

DNA Topoisomerase II Inhibition by *Ortho*-Quinone 3 The aza-analogue **3** used for measurements of inhibitory activity of DNA topoisomerases was prepared by HNO_3 oxidation of the racemic monophenolic analogue **2** that was derived through treatment of **1** with HBr (Chart 2).

The inhibitory effect of analogue **3** on human DNA topoisomerase II was examined through the conversion of kDNA, a catenated network of DNA rings, to minicircle monomers

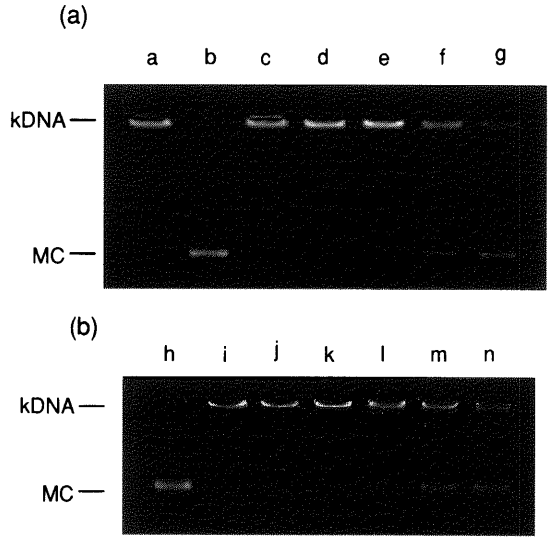

Fig. 1. Effects of *Ortho*-Quinone **3**, *m*-AMSA and Etoposide on the kDNA Decatenation Activity by DNA Topoisomerase II

Decatenation assays were performed in the presence of 1 U of topoisomerase II as described under Experimental. (a) Lane a, kDNA alone (no drug, no enzyme); b, control (no drug); c to g, 100, 50, 25, 12.5 and 6.25 μM of *ortho*-quinone **3**, respectively. (b) Lane h, control; i to k, *m*-AMSA; l to n, etoposide. Drug concentrations were 100 μM (lanes i and l), 50 μM (lanes j and m), 25 μM (lanes k and n). MC: decatenated minicircle DNA.

(decatenation), which is a catalytic process that DNA topoisomerase II mediates. In the presence of 1 U of the enzyme, the *ortho*-quinone **3** inhibited the decatenation process of kDNA in a dose-dependent manner. Figure 1 is a photograph of agarose gels. Aza-analogue **3** completely inhibits the catalytic reaction of topoisomerase II at concentrations above 25 μM (Fig. 1a, lanes c to e), while decatenated minicircle DNA appears with decreasing amounts of **3** (12.5 and 6.25 μM, lane f and g). Its inhibitory activity was compared with those of topoisomerase II inhibitors *m*-AMSA[10] and etoposide (Fig. 1b). Under the same conditions, etoposide is found to require higher amounts for complete inhibition since minicircle DNA appears at all concentrations (lanes l to n) though *m*-AMSA shows comparable activity with that of **3** (lanes i to k). It was suggested that the sugar moiety and the partially aromatic polycyclic array in etoposide interact with the enzyme and DNA, respectively, during topoisomerase II processing,[11] and that the 4′-free hydroxyl group in the E-ring is essential for DNA damage in which the enzyme is implicated. In addition, the catechol analogue corresponding to **3**, which was obtained as a minor product in transformation of **1** to **2**, also exhibited topoisomerase II inhibitory activity that was comparable to that of **3** (not shown). Our data suggest at least that the sugar moiety at C-4 is not a prerequisite for

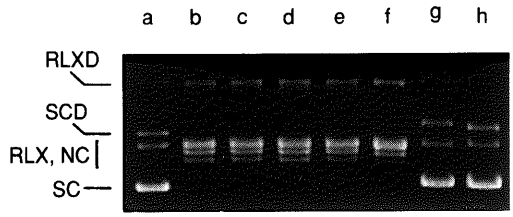

Fig. 2. Topoisomerase I-Mediated DNA Relaxation Reaction in the Presence of *Ortho*-Quinone **3** and *m*-AMSA

Relaxation experiments were carried out in the presence of 1 U of topoisomerase I as described under Experimental. (a) Lane a, supercoiled pUC19 plasmid DNA alone (no drug, no enzyme); b, control (no drug); c to e, 100, 200 and 400 μM of *ortho*-quinone **3**; f to h, 25, 50 and 100 μM of *m*-AMSA. SC: supercoiled DNA. RLX: relaxed DNA topoisomers. NC: nicked circle DNA. SCD: supercoiled DNA dimers. RLXD: relaxed DNA dimers. Under our experimental conditions, *m*-AMSA at high concentrations (over 100 μM) appeared to inhibit relaxation of supercoiled DNA by topoisomerase I due to its strong intercalative ability with DNA. However, it was confirmed that unwinding of relaxed DNA by *m*-AMSA occurred at concentrations over 25 μM. Therefore, the SC band at lane g was unambiguously due to unwinding of relaxed DNA.

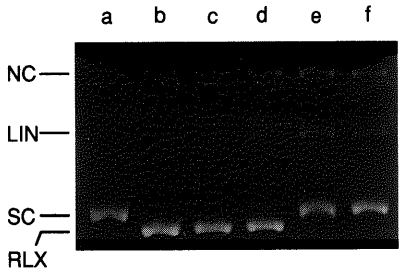

Fig. 3. Effects of *Ortho*-Quinone **3** on Topoisomerase II-Mediated DNA Cleavage Reaction

Cleavage assays were done in the presence of 8 U of topoisomerase II as described under Experimental. (a) Lane a, supercoiled pBR322 plasmid DNA alone (no drug, no enzyme); b, control (no drug); c to f, 1, 10, 50 and 100 μM of *ortho*-quinone **3**. LIN: full-length linear DNA.

topoisomerase II inhibition by podophyllotoxin derivatives, and that the catechol and *ortho*-quinone forms for the E-ring are more essential for the activity than the monophenolic form.

The topoisomerase I mediated relaxation processing of supercoiled pUC19 plasmid DNA, however, was not inhibited by the *ortho*-quinone **3** at all at a concentration range up to 400 μM (Fig. 2, lanes c to e). Supercoiled DNA disappears completely to afford a mixture of relaxed DNA topoisomers. When the relaxation reactions were carried out in the presence of *m*-AMSA which is a topoisomerase II specific inhibitor, unwinding of relaxed DNA was observed at 50 μM (Fig. 2, lanes g and h) due to the strong intercalative ability of *m*-AMSA with DNA.[12] These results reveal that the *ortho*-quinone **3** is a nonintercalative DNA topoisomerase II specific inhibitor. Separately from DNA topoisomerase I mediated DNA relaxation experiments, DNA unwinding assays for **3** were done according to the procedures described in the Experimental to obtain a result that this *ortho*-quinone analogue does not intercalate with DNA (not shown).

Topoisomerase II Mediated DNA Cleavage Many natural and (semi)synthetic compounds have been reported as topoisomerase II inhibitors, most of which can be classified as inhibitors that trap a covalent binary complex consisting of DNA and the enzyme (the cleavable complex) resulting in the formation of the putative noncovalent ternary complex. Those compounds are further divided into two classes, intercalative and nonintercalative inhibitors, both of which afford full-length linear DNA due to double stranded DNA cleavage by treating the ternary complex with a strong protein denaturant and proteinase K. To confirm whether the *ortho*-quinone **3** may stabilize the cleavable complex, DNA cleavage experiments were carried out using supercoiled pBR322 plasmid DNA in the presence of 8 U of topoisomerase II. The agarose gel photograph in Fig. 3 shows effects of the *ortho*-quinone **3** on the DNA processing by topoisomerase II. Formation of full-length linear DNA is enhanced with increasing amounts of **3** and clearly observed at a concentration of 50 μM (lane e). At the same time, nicked DNA that is formed by single stranded DNA breaks accumulates dose-dependently at concentrations up to 50 μM. The levels of formation of both linear and nicked DNA reach steady states between 50 and 100 μM. Another DNA cleavage experiment where the concentration range of **3** was between 10 and 50 μM showed that linear DNA was already present at 25 μM of **3** (not shown). These data indicate that the ortho-quinone **3** interferes with the DNA cleavage/religation equilibrium and stabilizes the covalent binary complex. Therefore, the podophyllotoxin aza-analogue **3** is identified as a nonintercalative DNA topoisomerase II specific inhibitor of which the mode of action is stabilization of the DNA-enzyme cleavable complex.

In this study we succeeded in transformation of microtubule assembly inhibitor **1** to topoisomerase II inhibitor **3** by making slight chemical modifications to the E-ring. This indicates that the E-ring plays a crucial role in affecting biological activities of podophyllotoxin-related lignans. On the other hand, it does not appear that functional groups at C-4 are a prerequisite for activities since the *ortho*-quinone **3** of the aza-analogues inhibits DNA topoisomerase II though it lacks a sugar moiety at C-4. In fact, aza-etoposide, which possesses a sugar moiety at C-4 in addition to the monophenolic E-ring, has been shown to exhibit no DNA topoisomerase II inhibition.[13] Furthermore, Gantchev and Hunting recently reported that the *ortho*-quinone derivative **4** of etoposide traps the cleavable complex more effectively than the parent inhibitor etoposide and suggested that methoxy groups on the E-ring are not a prerequisite for topoisomerase II inhibition.[14] Accordingly, the *ortho*-quinone form for the E-ring of podophyllotoxin-related lignans is most likely to be associated directly with topoisomerase II inhibition though the detailed mechanism of the stabilization of the binary cleavable complex is still unclear.

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