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Novel Mechanisms of DNA Topoisomerase II Inhibition by Pyranonaphthoquinone Derivatives—Eleutherin, α Lapachone, and β Lapachone*

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ABSTRACT. Pyranonaphthoquinones have diverse biological activities against Gram-positive bacteria, fungi, and mycoplasms, and, recently, there has also been an increasing interest in their anti-cancer activity. This study includes three derivatives: eleutherin (compound 1), β lapachone (compound 2), and its structural isomer, α lapachone (compound 3). The mechanism of topoisomerase II inhibition by the three derivatives was examined systematically with respect to the steps of the catalytic cycle of the enzyme. Etoposide, the prototypical enzyme poison, was used as a control and in combination with compounds 1–3 to localize their mechanism of action. The study revealed that eleutherin (1) and β lapachone (2) inhibited topoisomerase II by inducing religation and dissociation of the enzyme from DNA in the presence of ATP. Whereas compound 2 was an "irreversible" inhibitor of topoisomerase II, compound 1 merely slowed the catalytic cycle of the enzyme. α Lapachone (3), on the other hand, inhibited initial non-covalent binding of topoisomerase II to DNA and, in addition, induced religation of DNA breaks (even in pre-established ternary complexes) before dissociating the enzyme from DNA. Compound 3 was an "irreversible" inhibitor of topoisomerase II. The diverse and unique mechanisms of topoisomerase II inhibition by pyranonaphthoquinone derivatives reveal novel ways to target the enzyme with potential for anti-cancer drug design. BIOCHEM PHARMACOL **60**;9:1367–1379, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. naphthoquinones; topoisomerase II; catalytic inhibitors

Topoisomerase II is the target for many structurally diverse and clinically useful anti-tumor agents. During the catalytic cycle the enzyme goes through various conformational changes. After initial non-covalent binding of topoisomerase II to DNA, and in the presence of a divalent cation, the enzyme introduces a transient double-stranded break in the DNA and is attached covalently to the 5′-phosphate of the DNA via tyrosyl residues (pre-strand passage cleavage). Subsequent to ATP binding, a second strand of DNA passes through the gate (post-strand passage cleavage), and the enzyme reseals the break. ATP hydrolysis releases the DNA (with linkage number reduced in steps of two) and results in enzyme turnover [1]. Due to this important catalytic function, topoisomerase II is essential for maintaining the topological state of DNA during replication, transcription, and recombination, which has made it a desirable target for anti-cancer drugs [2].

Compounds that stabilize the covalent intermediates are termed topoisomerase II "poisons." Most of the clinically used topoisomerase II inhibitors belong to this class, for example etoposide, doxorubicin, and amsacrine [3]. Compounds that target any other step during the catalytic cycle are classified as "catalytic inhibitors" of topoisomerase II. Some examples are aclarubicin, merbarone, staurosporine, ICRF-187, and novobiocin [4].

This report is a systematic investigation of mechanisms of topoisomerase II inhibition by three pyranonaphthoquinone derivatives: eleutherin, β lapachone, and α lapachone (Fig. 1). β Lapachone (compound **2**) is of current interest as an anti-cancer agent, even though the intracellular target(s) and mechanism of action remain unknown. Eleutherin (compound **1**) is a *para*-quinone that is isolated from the bulb of *Eleutherine americana* and has been identified recently as a catalytic inhibitor of topoisomerase II [5]. α Lapachone (compound **3**, a *para*-quinone) is structurally related to compound **1** and is a positional isomer of compound **2**.

β Lapachone (**2**) has a wide range of biological activities, including inhibition of trypanosomatid growth, inhibition of DNA, RNA, and protein syntheses, production of strand breaks in parasite DNA [6], inhibition of tumor growth, prevention of oncogenic transformation of CHEF/18A cells, induction of clastogenic chromosomal alterations, and topoisomerase inhibition [7, 8]. The anti-trypanosomal activity has been attributed to free radical production [6]

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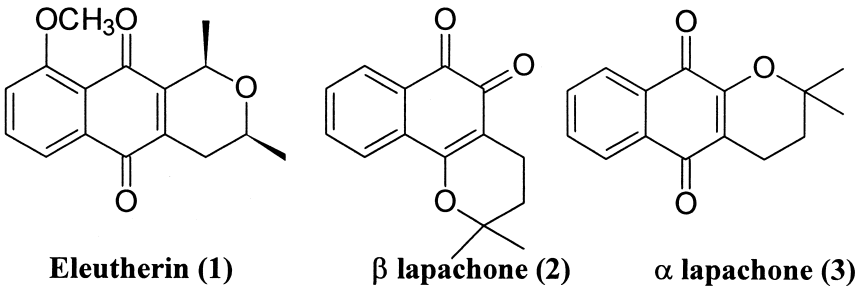

FIG. 1. Structures of pyranonaphthoquinone derivatives: eleutherin (compound 1), β lapachone (compound 2), and α lapachone (compound 3).

and bioreductive alkylation of nucleic acid or protein [9]. α Lapachone (**3**), which does not produce free radicals [10], does not have any anti-trypanosomal activity [6].

Compound **2** was first reported as a topoisomerase I catalytic inhibitor [7]. The compound also has significant anti-neoplastic activity against a variety of human tumor cells, including prostate cancer [11], promyelocytic leukemia cells [12], and the S-180 mouse tumor line. However, growth of yeast lacking topoisomerase I expression is suppressed by **2**, raising doubts about the mechanism of action, and suggesting critical intracellular targets other than topoisomerase I. Frydman *et al.* [8] subsequently reported **2** as a weak topoisomerase II poison that, unlike prototypical poisons, also inactivates the enzyme upon drug/enzyme pre-incubation. The DNA cleavage pattern induced by the drug is similar to the enzyme background and is independent of the presence of ATP. The same group reported that **3** is not a topoisomerase II poison, and the mechanism was not studied further. *Ortho*-quinones such as **2** have a better redox cycling ability than *para*-quinones such as **3**; therefore, it was suggested that cytotoxic actions of naphthoquinones derive, in part, from alkylation of exposed thiol residues on topoisomerase II–DNA complexes [13]. The intracellular mechanisms of both compounds remain unknown.

Studies by Frydman *et al.* [8] were carried out in the presence of dithiothreitol. Since oxidation of dithiothreitol by pyranonaphthoquinones may complicate analysis, all experiments reported herein were carried out using a mono-thiol reducing agent, 2-mercaptoethanol, which is not chemically reactive with these compounds under physiological conditions (data not shown). Results from this work indicate novel and diverse mechanisms of topoisomerase II inhibition by the three pyranonaphthoquinone derivatives.

MATERIALS AND METHODS

Materials

pBR322 was prepared by standard methods. Topoisomerase II (p170 isoform) was purchased from TopoGEN, Inc. *Hin*dIII-digested pBR322 was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, ICN Radiochemicals) using T4 DNA polymerase (GIBCO-BRL). Eleutherin (compound **1**) was isolated from the bulb of *E. americana*, and was a gift from Dr. Y. Imakura (Naruto University of Education) [5]. β Lapachone and α lapachone were prepared according to methods published by Hooker [14]. Etoposide was provided by the Natural Products Laboratory, UNC.

Catalytic Assay and Reversibility Assay

Relaxation of supercoiled pBR322 by topoisomerase II was monitored on gels electrophoresed in the presence of ethidium bromide, to measure inhibition of catalytic activity [15]. All reactions were carried out in a final volume of 10 μL containing 50 mM Tris–HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 2 mM 2-mercaptoethanol, 30 μg/mL of nuclease-free BSA, 2 μg pBR322, and drugs as indicated. Reactions were initiated by the addition of 0.5 U of topoisomerase II. The samples were incubated for 10 min, which is the optimal incubation time for the reaction. Duplicate samples were also incubated for 40 min. To check reversibility, the samples were diluted 6-fold with the assay buffer after 10 min, and incubation was continued for 30 min before termination. All reaction mixtures were terminated by the addition of SDS [1% (w/v) final concentration] and treated with proteinase K (1 mg/mL final concentration) for 1 hr at 37°. The samples were electrophoresed in 0.8% agarose gels in the presence of 0.5 μg/mL of ethidium bromide in TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0). Gels were visualized by transillumination with ultraviolet light and photographed using Polaroid Type 667 film.

Mobility Shift Assay

Topoisomerase II–DNA binding was measured using ^{32}P-labeled linear DNA [16]. The reactions were carried out in a final volume of 20 μL containing 50 mM Tris–HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 30 μg/mL of nuclease-free BSA, 3.5 ng of ^{32}P-labeled pBR322 DNA, and drugs and/or 0.5 mM ATP where indicated. The reactions were initiated by the addition of 0.5 U of topoisomerase II. Antagonism of etoposide by compounds **1** and **2** (in the presence of ATP) was measured under three treatment conditions: co-incubation of com-

pounds **1** and **2** with etoposide, enzyme, and DNA; pre-incubation of compounds **1** and **2** with enzyme and DNA prior to the addition of etoposide; and post-incubation of ternary complexes (enzyme–DNA–etoposide) with compounds **1** and **2**. After incubation for 15 min at 37°, the reactions were adjusted to 3% (w/v) sucrose, 0.0025% (w/v) bromophenol blue, and electrophoresed in 0.7% agarose in TBE buffer at 4°. The gels were dried under vacuum and autoradiographed using phosphor screens. The percent of the DNA bound to the enzyme was quantified based on the relative mobilities using ImageQuant software (Molecular Dynamics).

Topoisomerase II-Mediated DNA Cleavage

These experiments used conditions similar to the mobility shift assay, except that all reactions were carried out in the presence of ATP [17], and the reactions were terminated by the addition of SDS [1% (w/v) final concentration] and proteinase K (1 mg/mL final concentration). Antagonism of etoposide by compounds **1** and **2** was measured under three treatment schedules: co-incubation of compounds **1** and **2** with etoposide, enzyme, and DNA; pre-incubation of compounds **1** and **2** with enzyme and DNA prior to the addition of etoposide; and post-incubation of ternary complexes (enzyme–DNA–etoposide) with compounds **1** and **2**. Following treatments, the samples were electrophoresed in 1% agarose gels in TBE buffer. The gels were dried and autoradiographed using phosphor screens. The percentage of the DNA cleaved was quantified using ImageQuant software (Molecular Dynamics).

Topoisomerase II-Mediated DNA Religation

One part of the experiment was carried out similarly to the cleavage assay conditions, in the presence and absence of compound **3**. The cleavage products were trapped by the addition of SDS [1% (w/v) final concentration] and proteinase K (1 mg/mL final concentration) for 1 hr at 37°. Religation of cleaved DNA in the absence of compound **3** was induced by shifting duplicates of the enzyme control from 37° to 0° for 15 and 30 sec, before trapping the cleavage products with SDS [18]. To duplicates of enzyme control reactions, compound **3** was added for 15 and 30 sec at 37°, and the cleavage products were trapped by the addition of SDS. All samples were electrophoresed in 1% agarose gels in TBE buffer, and the dried gels were autoradiographed using phosphor screens. The percentage of the DNA cleaved was quantified using ImageQuant software (Molecular Dynamics).

Topoisomerase II–DNA Binding under Religation Conditions

The experiments were carried out under normal cleavage conditions as described previously, with religation being induced by transferring reactions onto ice. Compound **3** was added either before initiation of the reaction or after binary complexes were established (in 10 min) for 15 and 30 sec. The samples, however, were terminated without SDS treatment by adjusting the reactions to 3% (w/v) sucrose, 0.0025% (w/v) bromophenol blue, followed by electrophoresis on 0.7% agarose in TBE buffer at 4°. The gels were dried and autoradiographed using phosphor screens. Based on the relative mobilities of bound DNA as compared with free DNA, the percentage of DNA bound to topoisomerase II was quantified using ImageQuant software (Molecular Dynamics).

Statistical Analysis

Results were analyzed with Student's *t*-test using a software package from GraphPad Prism.

RESULTS

Catalytic Inhibition of Topoisomerase II

Relaxation of supercoiled pBR322 by either topoisomerase I or II was measured in the presence of the three pyranonaphthoquinone derivatives to evaluate catalytic inhibition and DNA unwinding activities. Compounds **1**-**3** did not inhibit topoisomerase I, nor did they possess unwinding activities, suggesting that compounds **1**-**3** did not bind to DNA (data not shown). The compounds, however, were selective inhibitors of topoisomerase II. Figure 2 shows inhibition of the enzyme analyzed in a gel electrophoresed in the presence of ethidium bromide. This agent intercalates into covalently closed circular DNA and alters the electrophoretic mobility of the topoisomers such that they all co-migrate as a single band with negatively supercoiled DNA (form I). The manipulation allows for differentiation between linear (form III) and nicked (form II) DNA. As shown in Fig. 2, at the optimal incubation time (10 min) compounds **1**-**3** inhibited the catalytic activity of the enzyme (lanes 6, 8, and 10, respectively). Linear DNA accumulated only in the presence of etoposide, which acts as an enzyme poison and stabilizes ternary complexes (lane 4). Duplicates of each reaction were incubated for another 30 min to measure the effect of compounds on the continuity of the catalytic cycle. Interestingly, compound **1** allowed relaxation of DNA upon extended incubation (lane 7), which suggested that **1** merely slowed the catalytic activity of topoisomerase II, in contrast to the inhibition by the other two derivatives (lanes 9 and 11).

Reversibility of enzyme inhibition upon removal of the compounds was measured by diluting the test compounds 6-fold (to non-inhibitory levels) after 10 min and continuing incubation for another 30 min as shown in Fig. 3. As expected, the activity of the enzyme in the presence of the prototypical poison etoposide was restored completely upon dilution (compare lanes 3 and 4 with lane 12). The inhibition by compounds **2** and **3** was irreversible (compare lanes 7 and 8 with lane 14, and lanes 9 and 10 with lane 15, respectively). Consistent with the results in Fig. 2, inhibi-

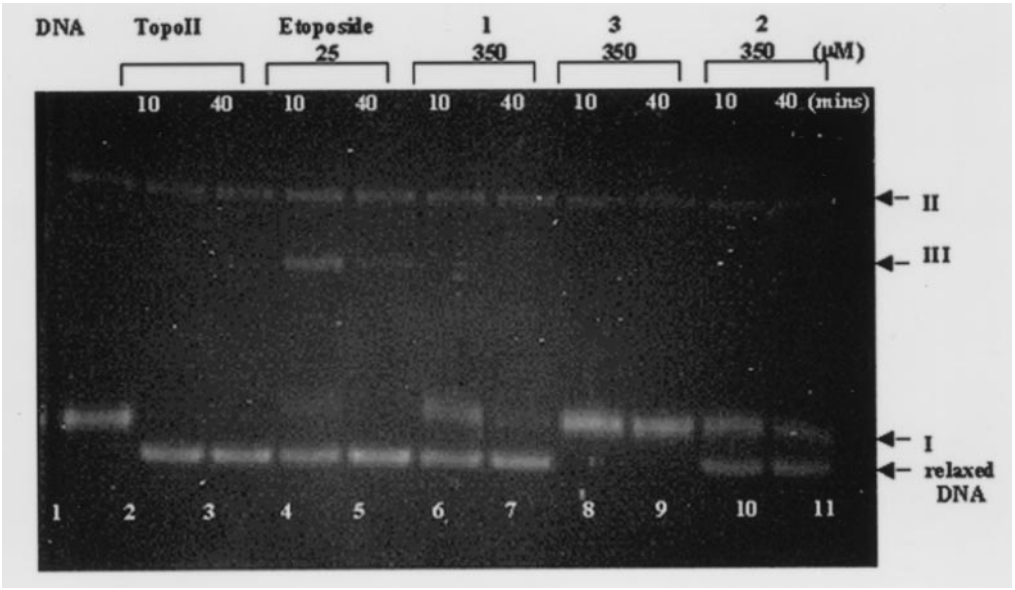

FIG. 2. Catalytic inhibition of topoisomerase II. Supercoiled pBR322 DNA (lane 1) was relaxed with the enzyme in the presence or absence of drugs for 10 min (optimal incubation time) or 40 min as described in Materials and Methods. The figure (a representative result of three independent experiments) shows drug-free control (lanes 2 and 3), etoposide at 25 μM (lanes 4 and 5), and 350 μM concentrations of compounds 1 (lanes 6 and 7), 3 (lanes 8 and 9), and 2 (lanes 10 and 11). The incubation times are marked on the figure.

tion by compound **1** was reversible (compare lanes 5 and 6 with lane 13). These results classify compound **1** as a reversible catalytic inhibitor and compounds **2** and **3** as "irreversible" catalytic inhibitors of topoisomerase II. The mechanisms of inhibition were subsequently studied by identifying sensitive steps of the catalytic cycle of the enzyme using a ^{32}P-labeled linear DNA substrate.

Non-covalent Topoisomerase II–DNA Binding

Topoisomerase II can form at least two different complexes with DNA that are in rapid equilibrium, a non-cleavable complex and a cleavable complex. Even though divalent cations are not required for the initial DNA interaction, the levels of binding are reduced greatly in its absence. Mobilities of enzyme-bound and free DNA were distinguished using native agarose gel electrophoresis. In the presence of Mg^{2+} (but without ATP), the smear detected with lower mobility than linear DNA is a combination of non-covalently complexed (predominant) and 'pre-strand passage' covalently complexed DNA [19].

Figure 4 is a representation of the percentage of DNA bound to topoisomerase II in the absence or presence of compounds **1**-**3**. In the absence of any compound, a

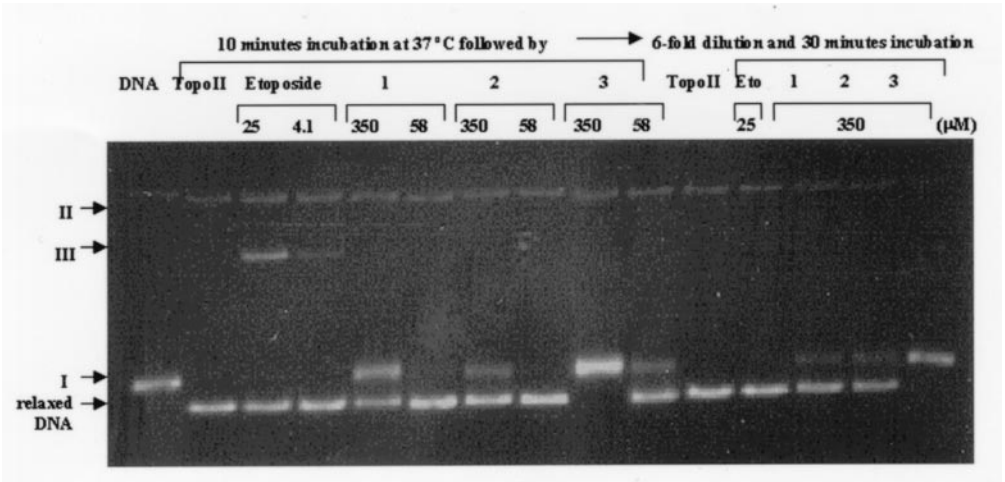

FIG. 3. Reversibility of topoisomerase II catalytic activity. Supercoiled pBR322 DNA (lane 1) was relaxed with drug-free enzyme (lane 2), in the presence of etoposide at 25 and 4.1 μM (lanes 3 and 4) and in the presence of 350 and 58 μM concentrations, respectively, of compounds 1 (lanes 5 and 6), 2 (lanes 7 and 8), and 3 (lanes 9 and 10). Duplicates of reactions in lanes 2, 3, 5, 7, and 9 were diluted 6-fold and incubated further for 30 min before analysis as shown in lanes 11, 12, 13, 14, and 15, respectively. The figure is a representative result from three independent experiments.

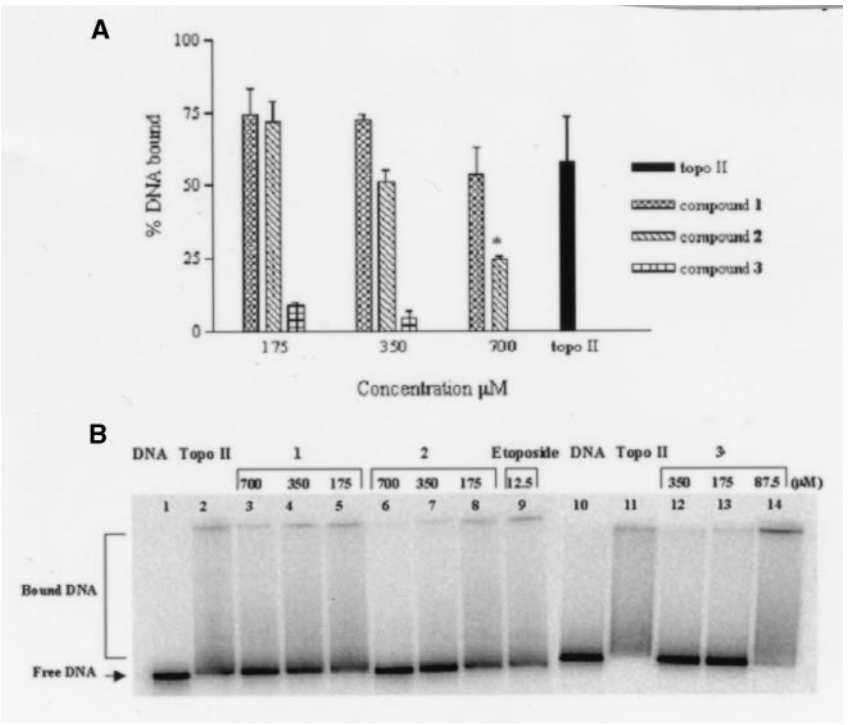

FIG. 4. Topoisomerase II–DNA non-covalent interactions. (A) Percentage of the DNA bound to topoisomerase II was measured in the absence of ATP using linear radiolabeled DNA as a substrate, in the presence and absence of compounds 1-3 as indicated in the figure key. The histogram represents the mean and SD of data obtained from three independent experiments. Key: (*) $P = 0.16$. (B) The representative gel retardation assay shows free DNA (lanes 1 and 10), topoisomerase II-bound DNA (lanes 2 and 11), compounds 1 and 2 at 700, 350, and 175 μM (lanes 3–5 and lanes 6–8, respectively), etoposide at 12.5 μM (lane 9), and compound 3 at 350, 175, and 87.5 μM (lanes 12–14). Two gel autoradiograms were juxtaposed to prepare this figure.

considerable fraction (>60%) of the DNA substrate was bound to the enzyme (topoisomerase II control). Relative to the enzyme control, compounds **1** and **2** did not have any effect on topoisomerase II–DNA binding [$P > 0.1$ even at the highest concentration (700 μM) of compound **2**]. In contrast, compound **3** completely inhibited the initial non-covalent binding of the enzyme to DNA. Based on their behavior as catalytic inhibitors and their effect on non-covalent enzyme–DNA binding, the inhibitory mechanism of each pyranonaphthoquinone derivative appeared to be distinct.

To further localize the mechanisms of action, the effects of compounds **1** and **2** on subsequent steps of the catalytic cycle were studied. In addition, to evaluate whether the mechanism of compound **3** was limited to inhibition of non-covalent enzyme–DNA binding, its effects on pre-established binary complexes also were investigated.

Topoisomerase II–DNA Covalent Complex Formation

Since compounds **1** and **2** did not inhibit the first step of the catalytic cycle, their effects on topoisomerase II-mediated DNA breaks were studied in the presence of ATP using a cleavage assay. Under the assay conditions used, the DNA cleavage measured is a combination of pre-strand passage and post-strand passage complexes. Enzyme-mediated DNA cleavage products were trapped by the addition of SDS. The percentages of DNA cleaved by topoisomerase II in the absence or presence of compounds **1** and **2** or etoposide are shown in panels a and b of Fig. 5 (represented as solid bars). As compared with the topoisomerase II control, compound **2** inhibited DNA cleavage with P values < 0.05 (Fig. 5b), but compound **1** did not have a significant effect on cleavage in the tested concentration range ($P > 0.5$, Fig. 5a). As expected, etoposide, an enzyme poison, stabilized almost all of the DNA in cleavage complexes at a 12.5 μM concentration.

To localize the inhibitory action to steps before and after DNA cleavage, interference with a cleavage complex stabilizing agent, etoposide, was analyzed. Antagonism of etoposide-stabilized ternary complexes was measured under three conditions: (i) compounds **1** and **2** and etoposide were co-incubated with enzyme and DNA; (ii) compounds **1** and **2** were pre-incubated with enzyme and DNA prior to the addition of etoposide; (iii) ternary complexes of etoposide, enzyme, and DNA were incubated with compounds **1** and **2** (post-incubation condition).

Under both pre-incubation and co-incubation conditions, compounds **1** and **2** significantly ($P < 0.05$) inhibited etoposide-stabilized double-stranded breaks (Fig. 5, a and b), which is an expected property of catalytic inhibitors that interfere with cleavage activity. This indicated that compounds **1** and **2** either inactivated the enzyme prior to the addition of etoposide or competed for the etoposide

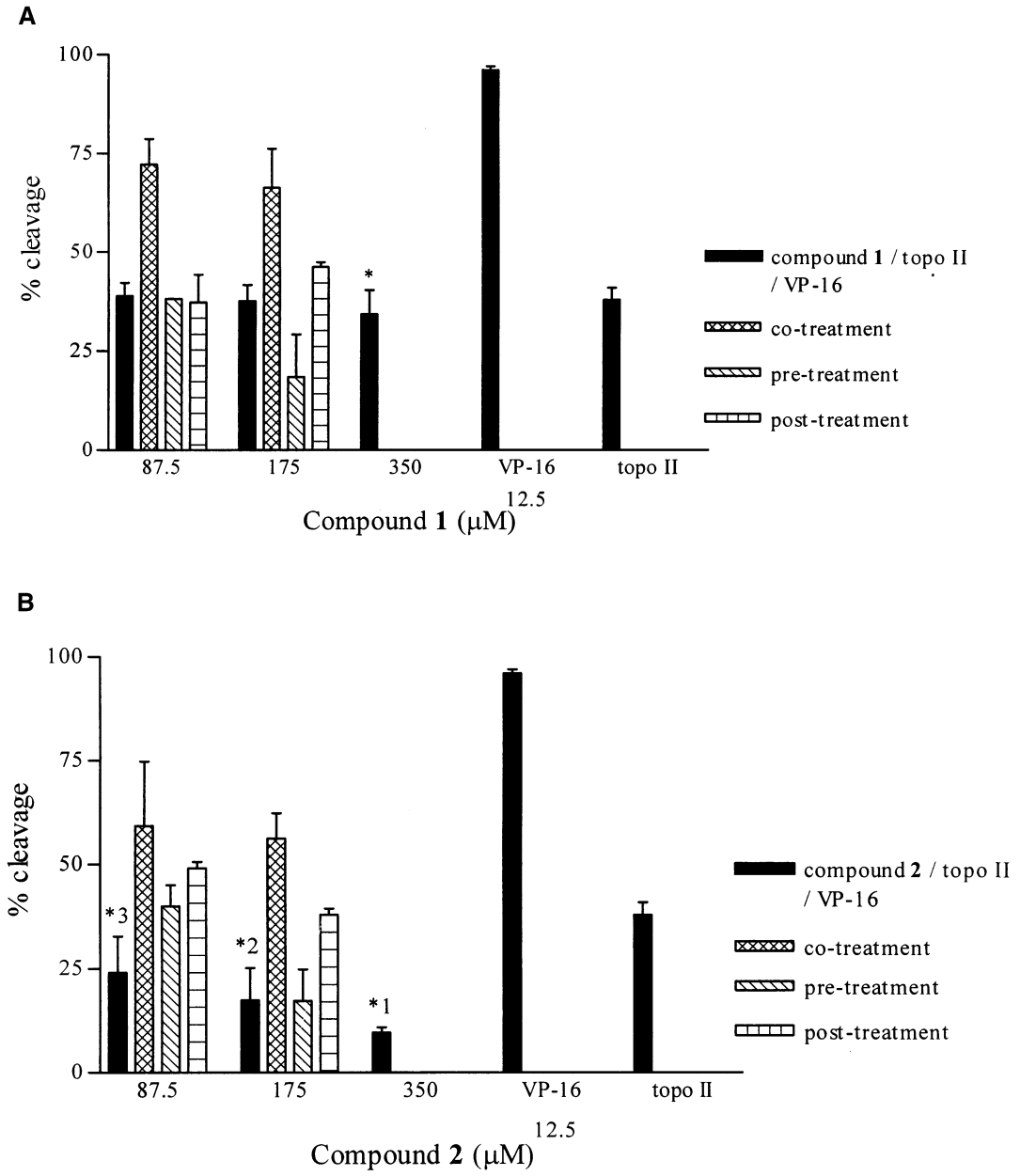

FIG. 5. Topoisomerase II-mediated DNA cleavage. Enzyme-mediated DNA cleavage products, in the presence or absence of compounds 1/2 and etoposide (VP-16), were trapped with SDS. Antagonism of etoposide-stabilized complexes was measured under co-, pre-, and post-incubation conditions as indicated in the figure keys and described in Materials and Methods. (a) Effects of compound 1 on cleavage and interference with etoposide (12.5 μM) stabilized complexes. Key: (*) $P = 0.56$. (b) Effects of compound 2 on cleavage and interference with etoposide (12.5 μM) stabilized complexes. Key: (*1) $P = 0.001$, (*2) $P = 0.043$, and (*3) $P = 0.147$. Each histogram represents means and SD of data obtained from three independent experiments.

binding site on enzyme–DNA complexes. Interestingly, even when ternary complexes were pre-established (post-incubation conditions), both **1** and **2** decreased the levels of double-stranded breaks. This indicated that compounds **1** and **2** induce topoisomerase II to religate DNA breaks stabilized by etoposide in the ternary complexes. The possibility that compounds **1** and **2** also share an overlapping drug-binding domain with etoposide will be discussed. In contrast, compound **3** only interfered with etoposide-stabilized ternary complexes under co-incubation and pre-incubation conditions (data not shown), which is consistent with the effect of the drug on the initial step of the catalytic cycle (Fig. 4).

Other relevant examples of catalytic inhibitors include aclarubicin and acridones [16, 20], which inhibit initial non-covalent interactions; merbarone, which inhibits topoisomerase II-mediated DNA cleavage [21]; and staurosporine, which inhibits strand passage [22]. None of these agents induce topoisomerase II-mediated religation. Therefore, compounds **1** and **2** have a novel mechanism of action, which is distinct from both compound **3** and previously reported catalytic inhibitors.

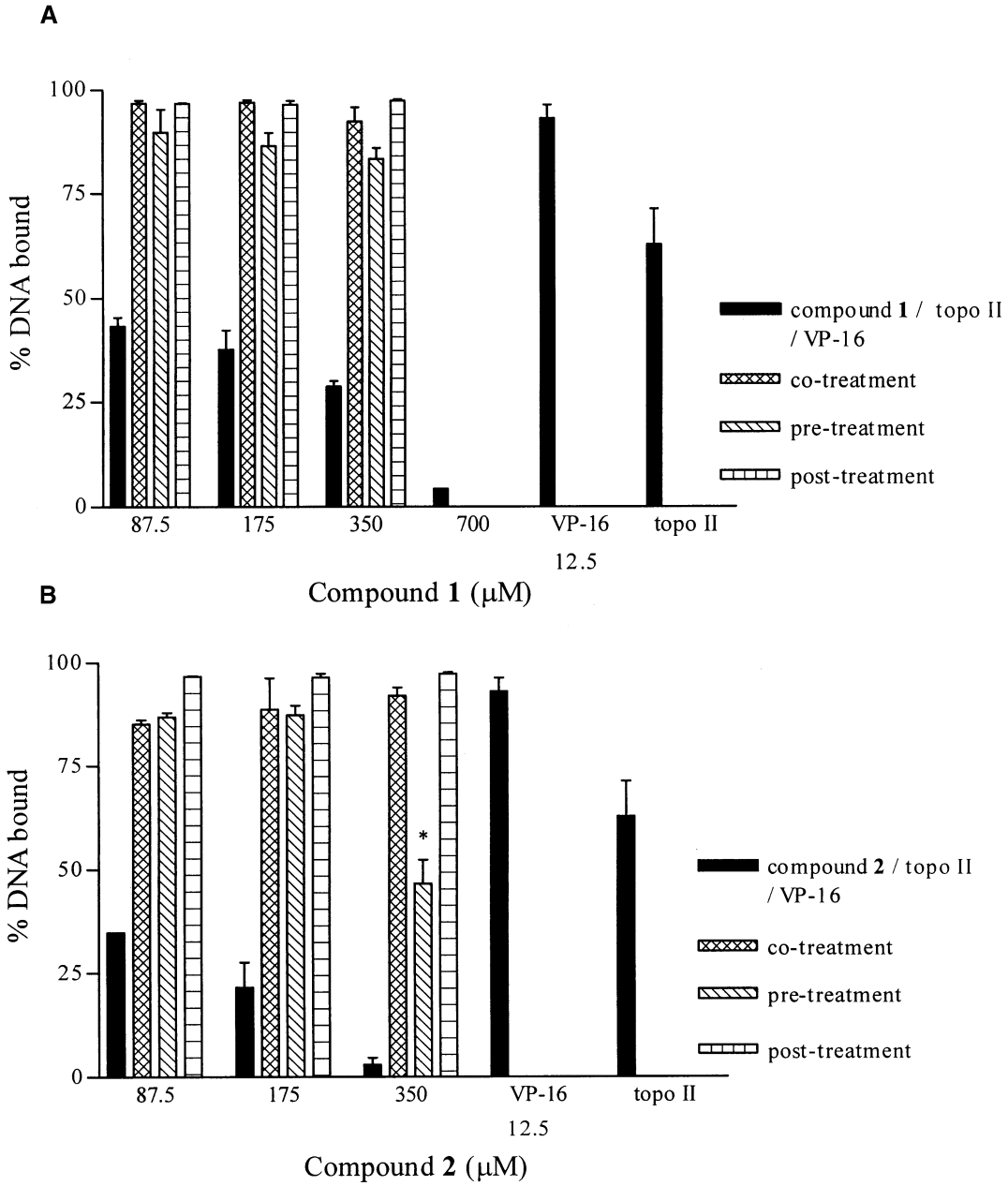

FIG. 6. Topoisomerase II–DNA binding in the presence of ATP. Enzyme and DNA were allowed to interact in the absence or presence of compounds 1/2 and etoposide. Antagonism of etoposide-stabilized complexes was evaluated under three treatment schedules (co-, pre-, and post-incubation) as indicated on the figure keys and described in Materials and Methods. (a) Effects of compound 1 on enzyme–DNA binding in the absence or presence of etoposide (12.5 μM). (b) Effects of compound 2 on enzyme–DNA binding in the absence or presence of etoposide (12.5 μM). Key: (*) $P = 0.0001$. Each histogram represents means and SD of data obtained from three independent experiments.

Topoisomerase II–DNA Binding in the Presence of ATP

The mechanism was studied further by evaluating the interactions between enzyme and DNA in the presence of ATP and the respective compounds. In the presence of ATP, the binding measured is a combination of pre-strand passage and post-strand passage enzyme–DNA interactions. The percentages of DNA bound to topoisomerase II (bands with lower mobility than free DNA) in the absence or presence of compounds **1** and **2** are represented in panels a and b of Fig. 6, respectively. DNA is bound to topoisomerase II in the presence of the enzyme alone and in ternary complexes with etoposide. Both compounds **1** and **2** inhibited enzyme–DNA binding in the presence of ATP (represented as solid bars) with P values < 0.05. Since neither compound affected binding in the presence of divalent cations (Fig. 4), the results indicate that they target the post-strand passage cleavage–religation equilibrium.

Antagonism of etoposide-stabilized cleavage complexes by compounds **1** and **2** was re-analyzed under co-incubation, pre-incubation, and post-incubation treatments as described in the previous section. Interestingly, compound **1** did not affect topoisomerase II–DNA binding in the presence of etoposide (Fig. 6a). Compound **2** also did not affect topoisomerase II–DNA interactions of ternary complexes significantly under any conditions (Fig. 6b). However, upon pre-incubation of **2** (at a high concentration) with enzyme and DNA, there was inhibition of binding, which showed that **2** inhibited topoisomerase II prior to the addition of etoposide. Interpretation of the results indicated that compounds **1** and **2** dissociated topoisomerase II from the DNA in the presence of ATP. In addition, they induced religation of double-stranded DNA without dissociating the enzyme from the ternary complex. However, etoposide could reverse compound **1**- and compound **2**-induced enzyme–DNA dissociation (as observed from results of the pre-incubation treatment schedule).

Taken together, the results suggest that compounds **1** and **2** induced topoisomerase II to religate DNA breaks and dissociated the enzyme from the DNA in the presence of ATP, by acting at the post-strand passage cleavage–religation equilibrium step of the catalytic cycle.

Topoisomerase II-Mediated DNA Religation

Religation of DNA breaks by the enzyme was evaluated to study the effects of compound **3** on pre-established enzyme–DNA complexes and also to investigate the possible effects of **3** upon religation activity of the enzyme (to compare with the mechanisms of compounds **1** and **2**). During the catalytic cycle, the cleavage–religation equilibrium lies heavily towards religation (~99%), so to visualize religation, extreme temperature conditions are utilized. At 0°, the enzyme loses its cleavage activity but continues to religate the DNA, and this activity can be evaluated by measuring the disappearance of cleaved linear fragments [18]. The temperature-dependent religation experiments were carried out in the presence of ATP.

Compound **3** inhibited initial non-covalent binding of topoisomerase II to DNA (Fig. 4), and, therefore, also inhibited enzyme-induced cleavage (Fig. 7a). Religation in the enzyme control was observed as a decrease in the percentage of DNA cleaved on shifting the samples to 0°. To duplicates of the enzyme control (after 15 min at 37°), compound **3** was added for the indicated time periods at 37°. The percentage of DNA cleaved was reduced upon addition of compound **3**, which indicated that **3** induced the enzyme to religate DNA breaks even at 37°, at levels comparable to enzyme-induced religation at 0° as shown in Fig. 7b. Therefore, compound **3**, in addition to inhibition at the initial step in the catalytic cycle, also induces topoisomerase II to religate the DNA breaks even in pre-established binary complexes.

Topoisomerase II–DNA Binding under Religation Conditions

Based on the dual action of compound **3** as an enzyme inhibitor, its mechanism was investigated further by analyzing its effects on pre-established enzyme–DNA complexes using the mobility shift assay. These experiments were carried out in the presence of ATP, under conditions similar to those used in the cleavage–religation assays, described in the previous section. The enzyme–DNA interactions were terminated without the addition of SDS. Based on the decreased mobility of bound DNA as compared with free DNA, the percentage of DNA bound was estimated and is shown in Fig. 8. The enzyme remained bound to the DNA both at 37° and after religation, which was induced at 0° (Fig. 8b). Compound **3** by itself completely inhibited topoisomerase II–DNA binding in the presence of ATP (Fig. 8a). Compound **3** was also added to pre-established binary complexes for 15 and 30 sec at 37°. Under these conditions, compound **3** induced a time-dependent dissociation of topoisomerase II from the DNA. This showed that compound **3** induced topoisomerase II to religate DNA breaks and then dissociated the enzyme from the DNA. Although the mechanism of induction of religation and dissociation is shared by the three derivatives, compound **3** targets the enzyme irrespective of the status of enzyme and DNA in the catalytic cycle.

DISCUSSION

Several structurally diverse natural and synthetic compounds inhibit topoisomerase II at different steps of the catalytic cycle. A few such examples of catalytic inhibitors include aclarubicin and acridones (which inhibit the initial enzyme–DNA interactions [16, 20]), merbarone (which inhibits enzyme-mediated DNA cleavage [21]), staurosporine (which inhibits strand passage by competing at the ATP binding site [22]), novobiocin (which inhibits ATP hydrolysis and prevents enzyme turnover [23]), and bisdioxopiperazines (which hold the enzyme in closed clamp form and also prevent enzyme turnover [24]). Due to the substantial conformational changes during the catalytic cycle of the enzyme, these structurally diverse agents are able to bind to the enzyme and/or the enzyme–DNA complexes. Although compounds **1**-**3** are pyranonaphthoquinone derivatives, the structural differences can affect their mechanism of action significantly, as reported and discussed here.

The *ortho*-quinone compound **2** was reported first as a topoisomerase I inhibitor, then as a weak topoisomerase II poison. These discrepancies perhaps can be explained by considering the redox cycling properties of pyranonaphthoquinones, which are greater for *ortho*-quinones (compound **2**) than for *para*-quinones (compounds **1** and **3**). Thus, these compounds can oxidize certain nucleophiles such as dithiothreitol, which has two thiols (presumably forming a cyclic 6-membered ring), without forming adducts; how-

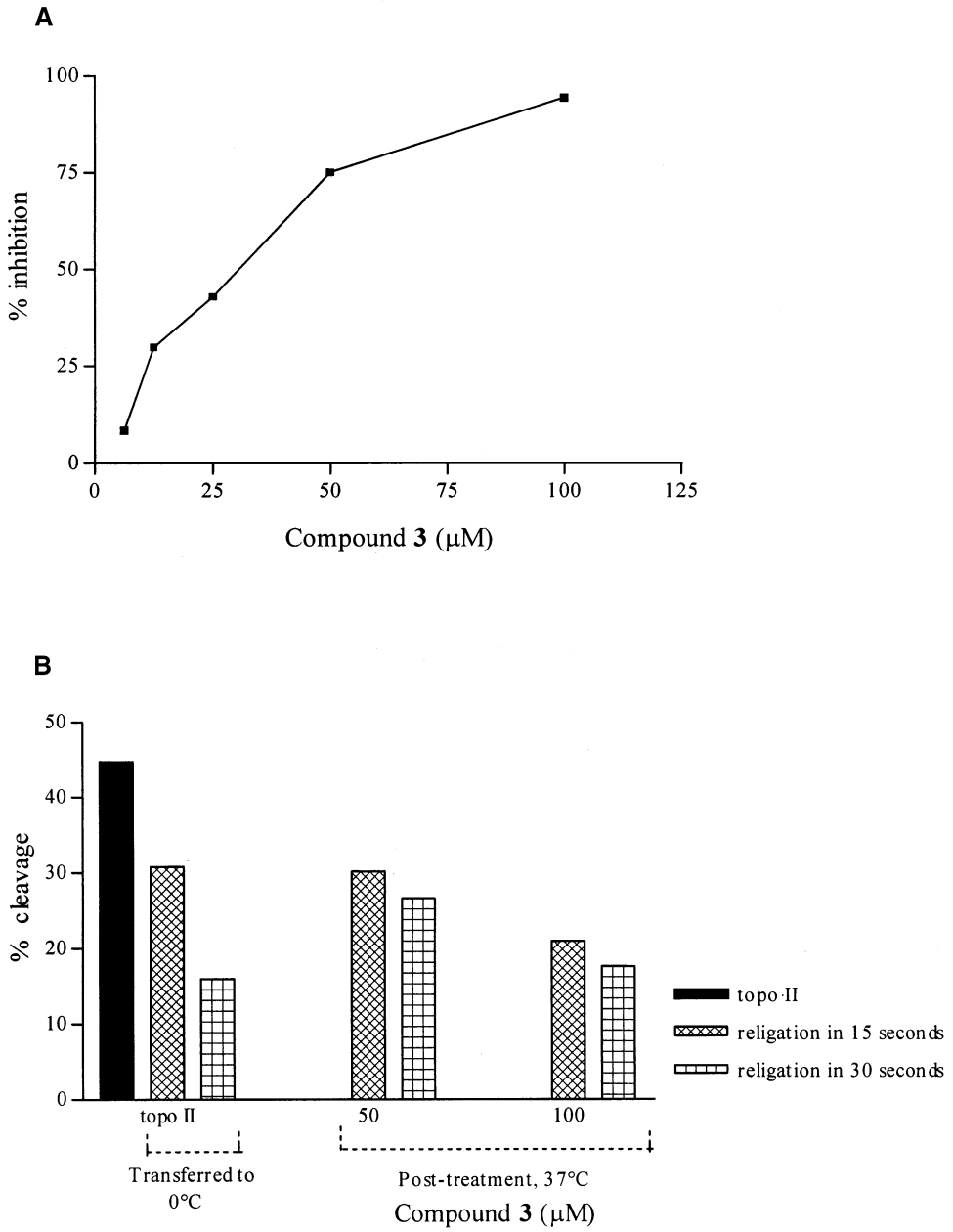

FIG. 7. Topoisomerase II-mediated DNA religation. Enzyme–DNA covalent complexes in the absence and presence of compound 3 (in the presence of ATP) were trapped by the addition of SDS. Panel (a) shows inhibition of enzyme-mediated DNA cleavage by compound 3. Enzyme-mediated religation was induced at 0° for 15 and 30 sec as shown in panel (b). To duplicates of enzyme control at 37°, compound 3 was added for 15 and 30 sec at 37°, also shown in panel (b). Experimental variation is not shown, since control cleavage using enzyme alone was variable between experiments. However, within independent trials using 25, 50, and 100 μM concentrations of compound 3, the effect of treatment on religation was consistent with the results illustrated in panel (b).

ever, under similar "physiological" conditions, they do not react with mono-thiols such as 2-mercaptoethanol (unpublished observations). As seen by utilizing a mono-thiol in the experiments, the three derivatives were selective catalytic inhibitors of topoisomerase II (data for topoisomerase I catalytic activity are not shown). Topoisomerase II has 15 cysteine residues [25], and in the absence of or during rapid depletion of reducing agents, the catalytic activities of both topoisomerase I and II are reduced significantly (unpublished observations). Moreover, the enzyme itself may be susceptible to direct oxidation by the compounds, as was also discussed by Neder *et al.* [13]. However, it is interesting that for yeast DNA topoisomerase II, the 9 naturally occurring cysteines are not required for the enzyme to be catalytically active [26]. Whether the cysteine reactivity of pyranonaphthoquinones can account for the novel mechanism of enzyme inhibition described here is unclear at this time.

Compounds **1**-**3** inhibited the catalytic activity of topoisomerase II. However, compound **1** allowed the enzyme to

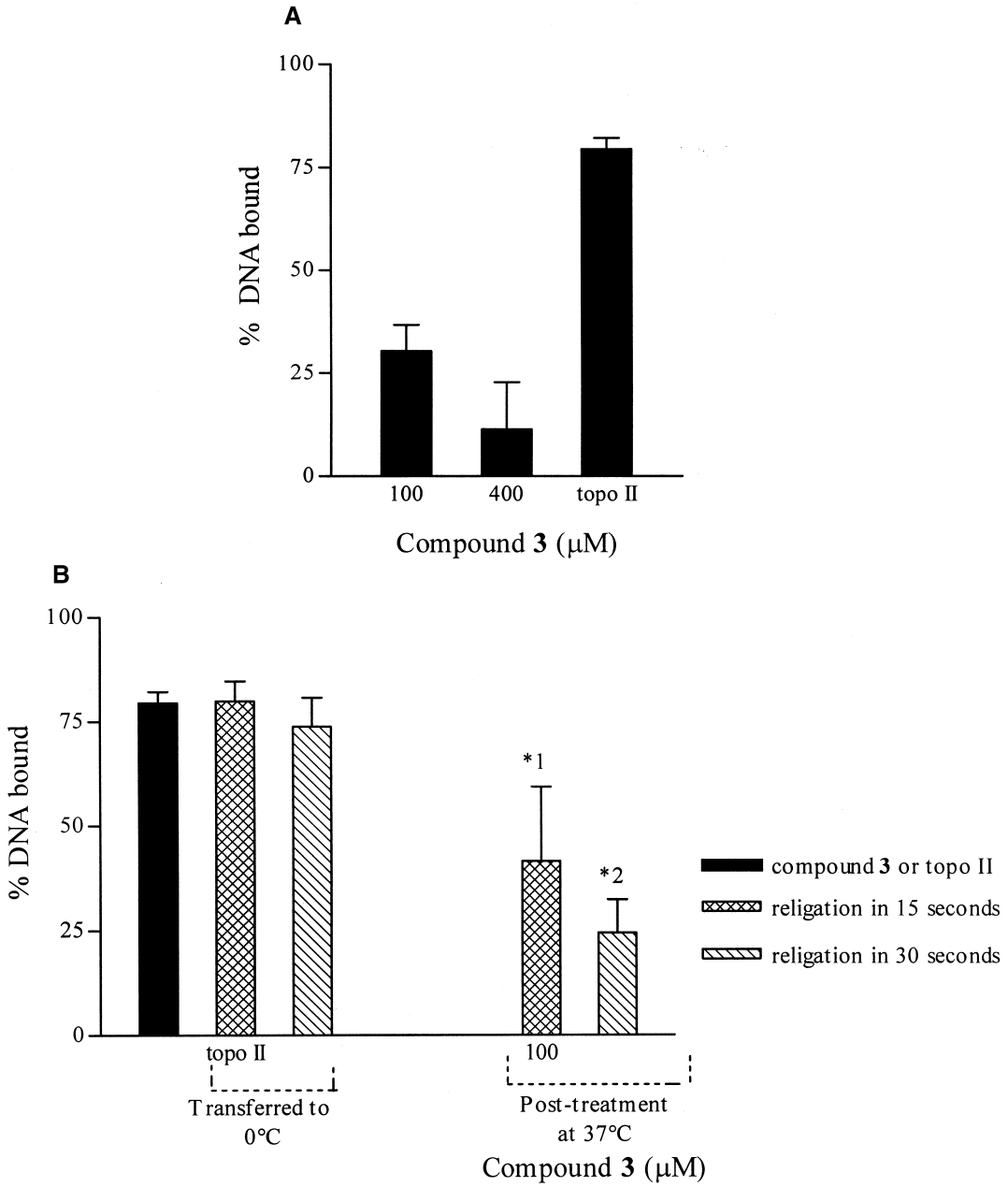

FIG. 8. Topoisomerase II–DNA binding under religation conditions. Enzyme–DNA complexes in the presence of ATP were electrophoresed without addition of SDS. Panel (a) shows inhibition of binding in the presence of compound 3. Panel (b) shows binding after religation of DNA by the enzyme, which was induced at 0° for 15 and 30 sec. To duplicates of enzyme control at 37°, compound 3 was added for 15 and 30 sec at 37°. Key: (*1) $P = 0.06$, and (*2) $P = 0.006$. Each histogram represents means and SD of data obtained from three independent experiments.

continue relaxation activity upon extended incubation, unlike **2** and **3**, which inhibited the enzyme irrespective of the incubation times. Reversibility of the activity of the enzyme was measured by diluting the samples to concentrations below inhibitory levels. This confirmed that **1** was a reversible inhibitor, whereas compounds **2** and **3** were "irreversible" catalytic inhibitors of topoisomerase II.

The mechanisms of compounds **1-3** were studied systematically at each step of the catalytic cycle of the enzyme, using linear radiolabeled DNA as a substrate. Effects on the initial non-covalent binding of topoisomerase II to DNA further differentiated the compounds with respect to the mechanisms of action. Only compound **3** inhibited the initial enzyme–DNA interaction (in the absence of ATP), which indicated that the activity of compounds **1** and **2** targeted the enzyme–DNA complex. The second step of the catalytic cycle is topoisomerase II-mediated DNA breakage and strand passage in the presence of ATP, where the isolated cleaved fragments analyzed *in vitro* are derived from pre-strand passage and post-strand passage covalent com-

plexes. Compound **3** inhibited cleavage as expected. Compound **2** also inhibited cleavage, but compound **1** had no significant effect up to a 350 μM concentration. The observed inhibition of topoisomerase II-mediated DNA cleavage (by compound **2**) could have occurred before or during covalent-complex formation.

To localize the mechanism to steps before or after DNA cleavage, antagonism of etoposide-stabilized complexes was evaluated using three treatment schedules. Under co-incubation of compounds **1** and **2** with etoposide, enzyme, and DNA, there was significant ($P < 0.05$) interference with cleavage complex formation. When compounds **1** and **2** were pre-incubated with enzyme and DNA prior to the addition of etoposide, there was greater antagonism as compared with the co-incubation condition. This implied inactivation of topoisomerase II prior to the addition of etoposide, or competition for the etoposide binding site on the enzyme–DNA complex. Interestingly, post-incubation of ternary complexes with compounds **1** and **2** also decreased the percentage of DNA cleaved. The overall results suggested that both compounds induced the enzyme to religate DNA breaks in ternary complexes, which limited the mechanism to pre-strand passage or post-strand passage cleavage–religation equilibrium.

As compared with a catalytic inhibitor such as merbarone, which inhibits topoisomerase II-mediated DNA cleavage, both compounds **1** and **2** share a novel mechanism of action. To explain inhibition of catalytic activity based on induction of enzyme-mediated religation, the effects of compounds **1** and **2** on topoisomerase II–DNA complexes in the presence of ATP were subsequently evaluated. Under the assay conditions used, the DNA cleavage measured was a combination of pre-strand passage and post-strand passage complexes.

The complexes formed in the presence of ATP were electrophoresed without trapping the cleavage products. Compounds **1** and **2** inhibited topoisomerase II–DNA binding in the presence of ATP with P values < 0.05. Antagonism experiments showed that a high concentration of compound **2** inactivated the enzyme prior to the addition of etoposide. Surprisingly, at other concentrations of compounds **1** and **2**, irrespective of the treatment schedule with etoposide, there was no significant decrease in binding. These findings are important since they suggest that the drugs are not typically competitive with etoposide for an overlapping drug-binding domain on the enzyme. The overall results demonstrated that **1** and **2** targeted enzyme–DNA complexes in the presence of ATP and induced the enzyme to religate DNA breaks before dissociating the enzyme from the DNA. Although the breaks stabilized by etoposide were religated in the presence of compounds **1** and **2**, etoposide could reverse the drug-induced dissociation of topoisomerase II from DNA.

Since the primary mechanism of compounds **1** and **2** was induction of religation followed by enzyme–DNA dissociation, the effect of compound **3** on pre-established enzyme–DNA complexes was also evaluated. Interestingly, compound **3** induced the topoisomerase II-mediated DNA religation of pre-established ternary complexes even at 37°. This religation at 37° was comparable to the enzyme-induced religation at 0°. Simultaneous investigation of the status of enzyme–DNA interaction during religation showed that the enzyme did not dissociate from the DNA after religating the breaks (induced at 0°). However, compound **3** dissociated the enzyme from DNA even in pre-established binary complexes. This indicated that compound **3** induced topoisomerase II to religate DNA breaks and dissociated the enzyme from DNA.

In conclusion, the three pyranonaphthoquinone derivatives have distinct and novel mechanisms of action. Compounds **1** and **2** induce topoisomerase II to religate DNA breaks in the presence of ATP and dissociate the enzyme from DNA. Compound **3** has a more complex mechanism. The drug inhibits initial non-covalent binding of topoisomerase II to DNA, and also induces the active enzyme to religate DNA breaks independent of the catalytic stage before dissociating the enzyme from the DNA. Since compounds **2** and **3** are "irreversible" inhibitors, the catalytic activity probably halts after dissociation from DNA. In the presence of compound **1**, however, the enzyme can interact with the DNA again, so the catalytic cycle is merely slowed. The scheme in Fig. 9 summarizes the established mechanism of action of each compound.

Based on earlier studies by Neder *et al.* [13] and observations regarding the redox cycling property of pyranonaphthoquinone derivatives including compounds **2** and **3**, it is possible that this property plays a role in the inhibitory mechanisms. Topoisomerase II has 15 essential cysteine residues, any of which, if located favorably, may be susceptible to oxidation by this class of compounds. This may explain, in part, the common mechanisms of drug-induced religation despite the structural differences among the three derivatives. Synthesis of other derivatives based on the redox cycling potential and intracellular mechanistic studies will be required to elaborate and define the importance of drug–enzyme chemical interactions.

Development of drug resistance has limited the efficacy of clinically useful anti-tumor agents like etoposide, doxorubicin, and amsacrine. One form of multi-drug resistant (MDR) phenotype, atypical MDR, is associated with alterations in expression or activity of topoisomerase II [27]. Elevated levels of topoisomerase II confer increased sensitivity to enzyme poisons, whereas acquisition of drug resistance is usually associated with a reduction in nuclear topoisomerase II levels [28]. Catalytic inhibitors of the enzyme might be expected to be more toxic to MDR cell lines expressing reduced levels of topoisomerase II, as was reported recently for the ICRF compounds [29, 30]. Since the inhibitory mechanisms of compounds **1**-**3** are novel and distinct, the mechanistic study has revealed novel ways to target the enzyme. Although the pyranonaphthoquinone derivatives are not potent inhibitors of DNA topoisomerase II *in vitro*, their effective concentrations are lower in cell-based assay systems (greater than 20-fold for compound

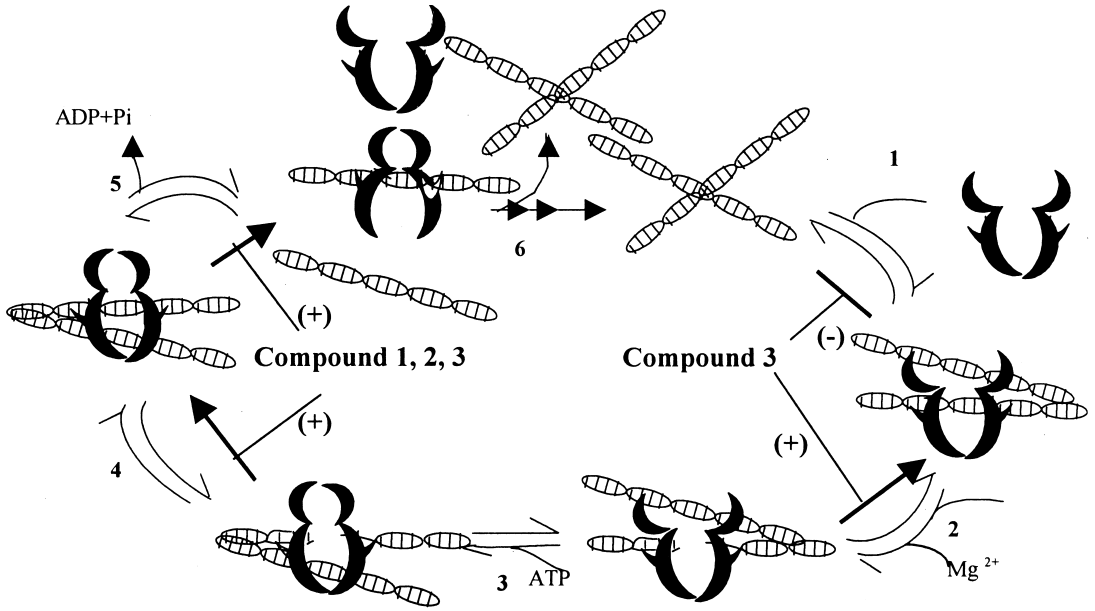

FIG. 9. Schematic diagram of the topoisomerase II catalytic cycle showing the steps inhibited by compounds 1-3. The scheme was adapted from Ref. 21.

2). The intracellular mechanisms of compounds **1-3** are under current investigation and will be reported in due course.

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