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A Novel DNA Topoisomerase I Inhibitor with Different Mechanism from Camptothecin Induces G2/M Phase Cell Cycle Arrest to K562 Cells

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Abstract

DNA topoisomerase I (Top1) is an essential nuclear enzyme and a validated target for anticancer agent screening. In a previous study, we found that indolizinoquinoline-5,12-dione derivatives show significant biological activity against several human cancer cell lines. To understand their mechanism of inhibition of cancer cell growth, one indolizinoquinoline-5,12-dione derivative, CY13II, was further studied as lead. Our present results indicate that CY13II shows more potent antiproliferative activity against K562 cells than camptothecin. Additionally, K562 cells were arrested in G2/M and their growth rate decreased after treatment with CY13II at micromolar concentration. Biochemical Top1 assays indicate that CY13II exhibits a different inhibitory mechanism from camptothecin. Unlike camptothecin, CY13II specifically inhibits the catalytic cleavage activity of Top1 instead of forming drug-enzyme-DNA covalent ternary complex.

DNA topoisomerase I (Top1), an essential nuclear enzyme that controls and modifies the topological state of DNA in many cellular metabolic processes (1–3), can be used as a target to screen anticancer agents (3–5). Camptothecin (CPT), the well-known Top1 inhibitor, has been found to target Top1 as its cellular sole antiproliferative target (6), and three CPT derivatives, topotecan (7,8), irinotecan (9) and belotecan (10,11), have been approved for clinical treatment of cancer. Therefore, Top1 is a validated target for anticancer agent screening. In the past years, the research on non-camptothecin Top1 inhibitor has attracted many medicinal chemists because of the limitation of CPT derivatives (12,13). In our preliminary effort to find non-camptothecin Top1 inhibitor, indolizinoquinoline-5,12-dione derivatives have been synthesized and found to show strong Top1 inhibitory activity and significant cytotoxicity against four tumor cell lines at micromolar concentrations (14). In the present study, one lead compound, ethyl 7-fluoro-5,12-dioxo-5,12-dihydroindolizino[2,3-*g*]quinoline 6-carboxylate (CY13II, as shown in Figure 1), was further examined for its effect on Top1 and cell growth *in vitro*.

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EXPERIMENTAL PROCEDURES

General procedures

Plasmid pBR322 DNA, purified calf thymus DNA topoisomerase I and DNase I were purchased from TakaRa Biotechnology (Dalian) Co., Ltd, unless otherwise mentioned. One unit of Top1 was defined as the amount that relaxes 0.5 μg pBR322 DNA at 37°C for 30 min. CY13II was synthesized in our laboratory. The HPLC analysis was carried out on a SHIMADZU LC-20AT system controller with SPD-20A detector.

Relaxation Assay

The relaxation assay was carried out as described with slight modifications (15). Briefly, reaction (20 μl) mixture containing 0.1 μg plasmid pBR322 DNA in relaxation buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl, 50 μg/ml acetylated BSA), was incubated with 0.2 U calf thymus Top1 in the absence or in the presence of compound, previously dissolved in DMSO solution, for 30 min at 37°C. The reaction was started by adding Top1 enzyme. For time-course assay I, Top1 was preincubated with CY13II for 1, 2, 5, 15 or 30 min prior to the addition of plasmid pBR322 DNA (16). For time-course assay II, the plasmid pBR322 DNA was preincubated with CY13II for 1, 2, 5, 15 and 30 min prior to the addition of Top1. The reaction was terminated by the addition of 4 μl loading buffer (30% sucrose, 0.5% bromophenol blue and 0.5% xylene cyanole FF in 10 mM Tris-HCl, pH 7.9). Then the sample was analyzed using a 1% agarose gel in 40 mM Tris-acetate (pH 8.0), 1 mM EDTA (TAE buffer) at 5 V/cm (17). Gel was stained with ethidium bromide (EB) and visualized with a UV transilluminator. Image was acquired and quantified through AlphaEaseFC software.

Unwinding Assay

Unwinding reaction was performed in 40 μl reaction volume containing 0.5 μg supercoiled pBR322 DNA and excess Top1 (20 U) in relaxation buffer (18). The DNA was incubated with compound at room temperature for 10 min prior to the addition of Top1. Reaction was terminated by the addition of 5 μl solution containing 5% of SDS and 5 mg/ml of proteinase K (prewarmed at 37°C for 30 min), and then analyzed using a 1% agarose gel in TAE buffer at 5 V/cm. Gel was stained with EB and visualized with a UV transilluminator.

Cleavage Assay

Human recombinant Top1 was purified from Baculovirus as previously described (19). DNA cleavage assays were performed as follows. A 117-bp DNA oligonucleotide from Integrated DNA Technologies (Coralville, Iowa) encompassing the previously identified Top1 cleavage sites identified in the 161-bp fragment from pBluescript SK(−) phagemid DNA was employed. This 117-bp oligonucleotide contains a single 5′-cytosine overhang, which was 3′-end labeled by fill-in reaction with [α-^{32}P]-dGTP in reaction 2 buffer (50 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$, 50 mM NaCl) with 0.5 units of DNA polymerase I (Klenow fragment, New England BioLabs). Unincorporated ^{32}P-dGTP was removed using mini Quick Spin DNA columns (Roche, Indianapolis, IN), and the eluate containing the 3′-end-labeled DNA substrate was collected. Approximately 2 nM of radiolabeled DNA substrate was incubated with recombinant Top1 in 10 μL of reaction buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL BSA] at 25°C for 30 min in the presence of various drug concentrations. Recombinant Top1 in reaction buffer was pre-incubated with CY13II for 5, 10 or 15 min, followed by the addition of radiolabeled DNA and CPT. The reactions were terminated by adding SDS (0.5% final concentration) followed by the addition of two volumes of loading dye (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue).

Aliquots of each reaction were subjected to 20% denaturing PAGE. Gels were dried and visualized by using a Phosphoimager from Molecular Dynamics (Sunnyvale, CA) and ImageQuant software. For simplicity, cleavage sites were numbered as previously described in the 161-bp fragment (19,20).

EMSA

DNA mobility shift assay was performed as described with slight modifications (21). Briefly, 0.1 μg pBR322 DNA was incubated in 20 μl reaction buffer A with Top1 (4 U) in the absence or in the presence of compound at the 37°C for 30 min. The sample was immediately analyzed using a 1% agarose gel in TAE buffer with 1% EB at 1 V/cm for 6 h.

DNase I

Bovine DNase I was incubated with 0.5 μg pBR322 DNA in 20 μl of buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 50 μg/ml BSA) in the absence or in the presence of CY13II for 30 min at 37°C. The sample was mixed with loading buffer followed by agarose gel electrophoresis as described in the relaxation assay section (22).

Cell Culture and Antiproliferative Activity

K562 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum under standard tissue culture condition, and was maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were exposed to compound for 48 h. The antiproliferative activity of compounds was measured using the MTT assay (14), and expressed as the concentration inhibiting 50% of K562 cell growth (IC_{50}).

Cell Cycle Analysis

Exponentially growing K562 cells were plated onto dishes with fresh medium containing CY13II at various concentrations. The density of the cells was 1×10^5 per milliliter. After 48 h exposure to CY13II, cells were fixed in 70% ethanol, washed with phosphate-buffered saline (20 mM sodium phosphate, 150 mM NaCl, pH 7.2) and incubated for 30 min at 37°C with RNase I (100 μg/ml) followed by PI (50 μg/ml) prior to suspension and flow cytometry (Beckman Coulter) (23). Cell cycle was analyzed through EXPO32 ADC software.

Cell Synchronization Analysis

K562 cells were synchronized by double-thymidine block (23,24). Exponentially growing cells were incubated for 24 h at 37°C in medium containing 2.5 mM thymidine, followed by incubation in thymidine-free medium for 14 h. Synchronization of cells at the G1/S boundary was then achieved by a further 12 h incubation in medium containing 2.5 mM thymidine. Following two washes with thymidine-free medium, the synchronized cells were suspended in fresh medium containing different concentration CY13II, cultivated and cell cycle was analyzed by flow cytometry at different times.

Western Blot Analysis

K562 cells were incubated with CY13II for different times (0, 6, 12, 18 or 24 h), washed with PBS and resuspended in lysis buffer (50 mM Tris, 5 mM EDTA, 150 μM sodium chloride, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinin, 170 μg/ml leupeptin, and 100 μg/ml phenylsulfonyl fluoride, pH 7.5). After 30 min of mixing at 4°C, the mixture was centrifuged at 13,000 × g for 10 min, and the supernatant was collected as whole-cell extract (25). Protein concentration in cell lysates was determined by the BCA assay. For Western blot analysis, equal amounts of proteins was loaded and separated by 10% SDS-PAGE electrophoresis, and transferred to PVDF membrane. After transfer, the membrane was blocked in 5% non-fat milk in TBS buffer (10

mM Tris/Hcl, 150 mM NaCl, pH 8.0) and washed in TBST buffer (TBS buffer containing 0.05% Tween-20). The membrane was incubated with primary antibodies diluted in TBST containing 0.5% BSA at 1:200 (anti-cyclin A, cyclin B1, cyclin D1 and cyclin E, Thermo Fisher Scientific Inc.) or 1:2000 (anti-actin, Proteintech Group, Inc.) for 12 to 16 h at 4°C. After three washes in TBST, the membrane was incubated with secondary antibodies diluted at 1:1000 in TBST for 1 h at room temperature. Result was revealed by the ECL kit (Sangon Biotech, Shanghai) (26).

RESULTS

DNA topoisomerase I may be the target of CY13II

The Top1 inhibitory activity of CY13II was studied by the DNA relaxation assay (Figure 2A). CPT, a well-known Top1 inhibitor, was used as positive control. CY13II exhibited equipotent inhibitory activity to CPT at 1.0 μM, and dose-dependent inhibition of Top1 activity. After treatment with 125 μM of CY13II, 100% of the DNA remained supercoiled.

It is well-established that intercalating agents are able to produce positively supercoiled DNA in the presence of Top1 (18). Therefore, the intercalating ability of CY13II into DNA was assessed using the DNA unwinding assay (Figure 2C), in which EB was used as a positive control (18). The results indicated that, unlike EB, CY13II showed no unwinding effect on DNA up to 50 μM.

The absorption titration was performed according to our previous method (27). CY13II was titrated with calf thymus DNA at various concentrations. No obviously change, such as red shift or hypochromicity, was found on the spectrometric curves (data not shown), which indicates that CY13II has low DNA binding affinity.

To find the possible binding target of CY13II, time-course assays were performed. For time-course assay I, Top1 was preincubated with 25 μM of CY13II for various times prior to the addition of plasmid pBR322 DNA (Figure 2B Upper panel). The result indicated that the Top1 inhibitory activity of CY13II was enhanced in a time-dependent manner. After being preincubated with Top1 for 15 min, CY13II almost completely inhibited the catalytic activity of Top1. It should be noted, comparing with the relaxation assay, in which the reaction was started by the addition of Top1, that CY13II at 25 μM exhibited higher inhibitory activity even after being preincubated with Top1 for 1 min (see Figure 2A Upper panel, Lane 5 and Figure 2B Upper panel, Lane 2). For time-course assay II, plasmid pBR322 DNA was preincubated with CY13II for various times prior to the addition of Top1 (Figure 2B Bottom panel). The results indicate that the inhibitory activity of CY13II slightly increases after preincubation with DNA for 15 min. Comparing with the relaxation assay, CY13II exhibited similar inhibitory activity at 25 μM after being preincubated with DNA for 1 min. Together, the time-course assays suggested that Top1, but not DNA, is the binding target of CY13II.

CY13II can selectively inhibit the cleavage activity of Top1 without affecting the binding of Top1 to DNA

To identify whether CY13II could induce Top1-mediated DNA cleavage, biochemical assays were performed using CPT as positive control (Figure 3). As expected, CPT induced significant Top1-mediated DNA cleavage (19,20). On the contrary, CY13II could not induce Top1-mediated DNA cleavage up to 100 μM concentration.

If Top1 was pre-incubated with CY13II for 5, 10 or 15 min prior to addition of CPT (Figure 3A, Lane 6–11 and panel **B**), CPT-induced DNA cleavage decreased in concentration- and time-dependent manners with differential response at different Top1 sites, which indicates

that CY13II can suppress the ability of CPT to trap and stabilize the covalent complexes of Top1 and DNA (Top1cc). When CY13II and CPT were added to the reaction mixture at the same time, CPT-induced cleaved DNA did not decrease (data not shown). These results indicated that the pretreatment of Top1 with CY13II can inhibit the formation of CPT-induced DNA cleavage at specific sites.

Because the cleavage assays suggested that CY13II might exert its inhibition of Top1 before Top1-mediated DNA cleavage, we studied whether CY13II could interfere with the binding of Top1 to DNA using the DNA mobility shift assay. CPT, which inhibits the religation activity of Top1 without interfering with the binding step of Top1 to DNA, was used as positive control. The results (Figure 2D) clearly indicate that CY13II does not hamper the binding of Top1 to DNA.

To assess the inhibitory specificity of CY13II on the DNA cleavage activity of Top1, we studied to the effects of CY13II on DNase I activity. The results showed that CY13II did not show inhibitory activity on DNase I up to 125 μM (data not shown), which indicated the specificity of CY13II for Top1.

CY13II induce G2/M phase cell cycle arrest

The study of antiproliferative activity indicated that the IC_{50} values of CY13II and CPT for K562 cells were 0.85 μM and 2.56 μM, respectively. To study the drug effects on cell cycle progression, flow cytometry was performed. K562 cells were treated with CY13II at different concentrations for 48 h (Figure 4A). The cell number at the G2/M phase significantly increased from 22.8% to 51.2% or 65.4% after treatment with 0.25 μM or 0.5 μM of CY13II (Figure 4B). Meanwhile, significantly decrease in cells at G0/G1 phase and a slight increase of the subG1 phase were observed. These results show that CY13II induces cell cycle arrest at the G2/M phase.

In order to verify that CY13II could induce G2/M arrest, cell synchronization analyses were carried out. Synchronized cells at the G1/S boundary were released, equally divided and treated with CY13II at 0.25 μM. At various times after treatment, flow cytometry was carried out. Control cells (treated with DMSO) progressed synchronously through the cell cycle, and were normally distributed by 12 h post-release (Figure 4C). Cells treated with CY13II normally progressed into the G2/M phase by 8 h post-release, but were arrested at G2/M phase by 12 h, which indicates that CY13II induces G2/M phase cell cycle arrest as it decreases the growth rate of K562 cells.

CY13II affect K562 cells on the expression of cyclins

To determine whether the cell cycle arrest was associated with modified expression of cyclins, Western blot assays were performed. As shown in Figure 5, the expression of cyclin A and cyclin B1 were increased in response to treatment with CY13II in a time-dependent manner. It should be noted that CY13II showed various effects on the expression of cyclin D1 and cyclin E at various time points. The expression of cyclin D1 was not affected at 6 h, and increased at 12 h and 18 h, and then decreased to almost normal level at 24 h post-CY13II treatment. The expression of cyclin E was increased early after treatment by CY13II and decreased at 24 h post-CY13II treatment.

DISCUSSION

In this work, CY13II was investigated because it has shown significant biological activity in our preliminary study (14). CY13II shows more potent ability than CPT to inhibit the growth of K562 cells. Its IC_{50} value was three times less than CPT. In order to elucidate the mechanism of the antiproliferative activity, the effects of CY13II on Top1 were

investigated. We found that CY13II exhibits significant and dose-dependent Top1 inhibitory activity. Some drugs, such as EB (28) and m-AMSA (29), inhibit Top1 through DNA intercalation. However, DNA-unwinding assay using supercoiled DNA as a substrate indicates that CY13II has no Top1-mediated unwinding effect on DNA, which indicates that CY13II has no significant DNA intercalating activity.

Top1 generates DNA single-strand breaks by reversible transesterification. The mechanism by which it controls the topological state of DNA has been described as a controlled rotation process (Figure 6 Pathway a): i) Top1 bind to the superhelical DNA substrate to form a Top1-DNA noncovalent complex (Top1nc); ii) Top1 catalyzes the cleavage of one of two DNA strands to form a transient Top1cc; iii) controlled rotation releases the superhelical tension of DNA; iv) the cleaved DNA strand is religated; v) Top1 is released from the relaxed DNA, and undergo another cycle of DNA relaxation (12,30). The prototypical Top1 poison, CPT, intercalates between the DNA base pairs at the Top1 cleavage site and stabilizes the Top1cc (Figure 6 Pathway b). This intercalation inhibits the religation of DNA and the release of Top1, and induces an accumulation of cleaved DNA, which results in chromosomal damage (3,12,31–33).

In contrast with the mechanism of action of CPT, CY13II does not induce Top1-mediated DNA cleavage, and acts upstream of catalytic cleavage activity of Top1. Moreover, DNA mobility shift assay indicated that CY13II does not block the formation of Top1-DNA noncovalent complex. Preincubation of CY13II with enzyme prior to addition of DNA increased the inhibitory activity in a time-dependent manner. However, preincubation of CY13II with DNA prior to the addition of Top1 did not affect the inhibitory activity. Based on our results, we hypothesize that CY13II's inhibitory activity operates through a mechanism shown in Figure 6 Pathway c. Firstly, CY13II bind to Top1, without affecting the binding of the enzyme to DNA; Secondly, Top1 bound with CY13II forms a drug-enzyme-DNA ternary noncovalent complex, which is catalytically inactive, and prevents the cleavage activity of Top1 (34). The formation of such ternary complexes may depend on the DNA sequence as suggested by the differential suppressive activity of CY13II at different cleavage sites (see Figure 3). These observations indicate that CY13II acts as a novel kind of Top1 catalytic inhibitor.

Preincubation of CY13II with Top1 for various times prior to the addition of CPT decreased the CPT-induced DNA cleavage (Figure 3), which can be explained because of an inhibition of formation of Top1cc in the presence of CY13II. When CY13II and CPT were added together to Top1, CPT-induced DNA cleavage did not decrease (data not shown), which suggests that the binding of CY13II to Top1 might be a slow process compared with CPT. We also showed that CY13II does not inhibit the catalytic activity of DNase I, which suggests the specificity of CY13II as a catalytic Top1 inhibitor.

In eukaryotes, G2/M phase, a highly complex multi-stage process, is a critical phase before cell division during the cell cycle. Cell cycle arrest at G2/M phase is a common cellular response to a variety of DNA-damaging agents (35,36). Top1 poisons, including CPT, indenoisoquinolines and indolocarbazole derivatives induce replication-mediated double-stranded DNA breaks (DSB) by replication-fork collision mechanism (12), and induce cell cycle arrest of cancer cells at the G2/M phase (22,37–39). Although CY13II showed a different mechanism of Top1 inhibition from CPT, its effect on cell cycle were similar to CPT as a cell cycle arrest at the G2/M phase. This effect can be due to the fact that Top1 is critical for DNA replication and transcription and that lack of Top1 activity leads to replication damage (40), which can then lead to cell cycle arrest in G2. Cell synchronization analysis verified that cells released from the G1/S boundary were arrested at the G2/M phase after being treated with CY13II. Furthermore, CY13II affected the expression of cyclins,

such as cyclin A, cyclin B1, cyclin D1 and cyclin E, which provides molecular determinants for the cell cycle.

In summary, the inhibition to Top1 and the cell cycle effects of CY13II reported in the present study indicate that CY13II is a novel Top1 catalytic inhibitor. We propose that CY13II binds to Top1 to form drug-enzyme-DNA noncovalent ternary complex and inhibits Top1 DNA nicking activity, which leads to DNA damage and cell cycle arrest at the G2/M phase. The biological properties of CY13II suggest its potential as a novel cancer chemotherapeutic agent.

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Abbreviations and Textual Footnotes

CY13II	ethyl 7-fluoro-5,12-dioxo-5,12-dihydroindolizino[2,3-*g*]quinoline 6-carboxylate
Top1	DNA topoisomerase I
CPT	camptothecin
Top1cc	covalent complex of Top1 and DNA
Top1nc	Top1-DNA noncovalent complex

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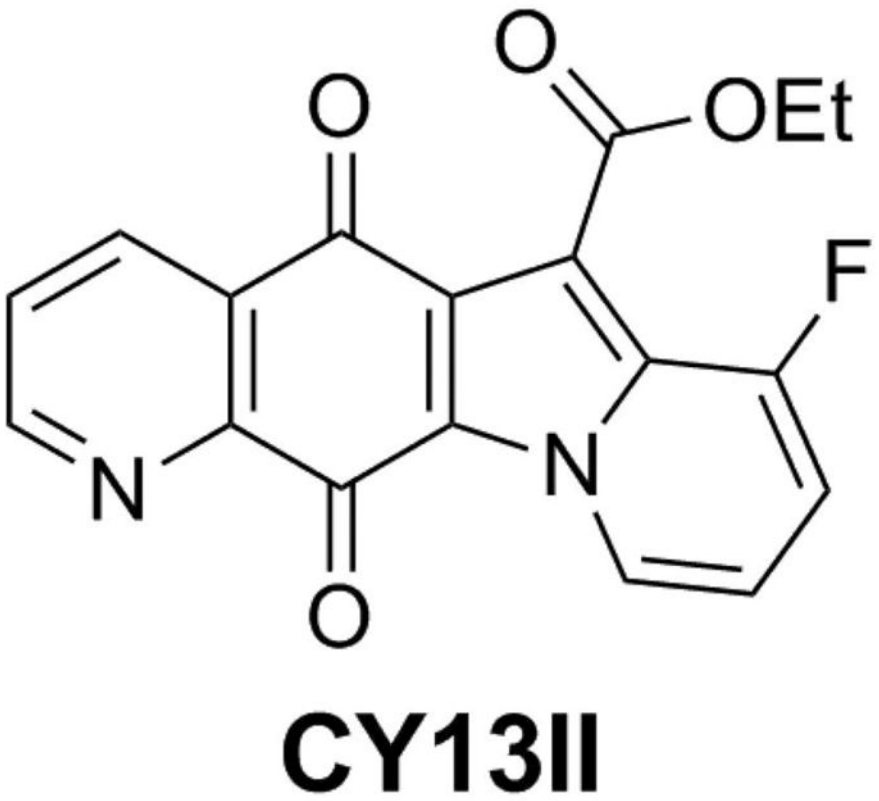

Figure 1.
Structure of CY13II.

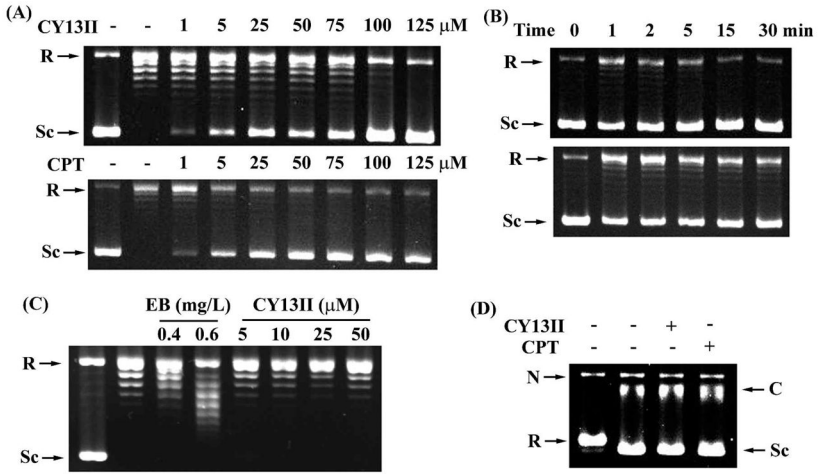

Figure 2.

(A) Inhibition of Top1-catalyzed DNA relaxation by CY13II and CPT. Lane 1, pBR322 DNA only. Lane 2, pBR322 DNA and Top1. Lane 3–9, pBR322 DNA, Top1 and various drug concentrations. (B), Upper panel: Effects of preincubation of Top1 with CY13II on Top1-catalyzed relaxation of pBR322 DNA. Lane 1, pBR322 DNA only; Lane 2–6, Top1 was pretreated with CY13II (25 μM) for 1, 2, 5, 15, 30 min, respectively, prior to addition of pBR322 DNA. Bottom panel: Effects of preincubation of pBR322 DNA with CY13II on Top1-catalyzed relaxation of pBR322 DNA. Lane 1, pBR322 DNA only; Lane 2–6, DNA was pretreated with CY13II (25 μM) for 1, 2, 5, 15, 30 min, respectively, prior to addition of Top1. (C), Effects of CY13II on the DNA unwinding assay with Top1. Lane 1, pBR322 DNA only. Lane 2, pBR322 DNA and Top1. Lane 3–8, pBR322 DNA, Top1 and various concentrations of compound. (D), DNA mobility shift analysis of Top1-DNA binding. Lane 1, pBR322 DNA only. Lane 2, pBR322 DNA and Top1. Lane 3–4, pBR322 DNA, Top1 with CY13II or CPT at 5 μM. Sc, supercoiled DNA; R, relaxed DNA; N, nicked DNA; C, Top1-bound DNA.

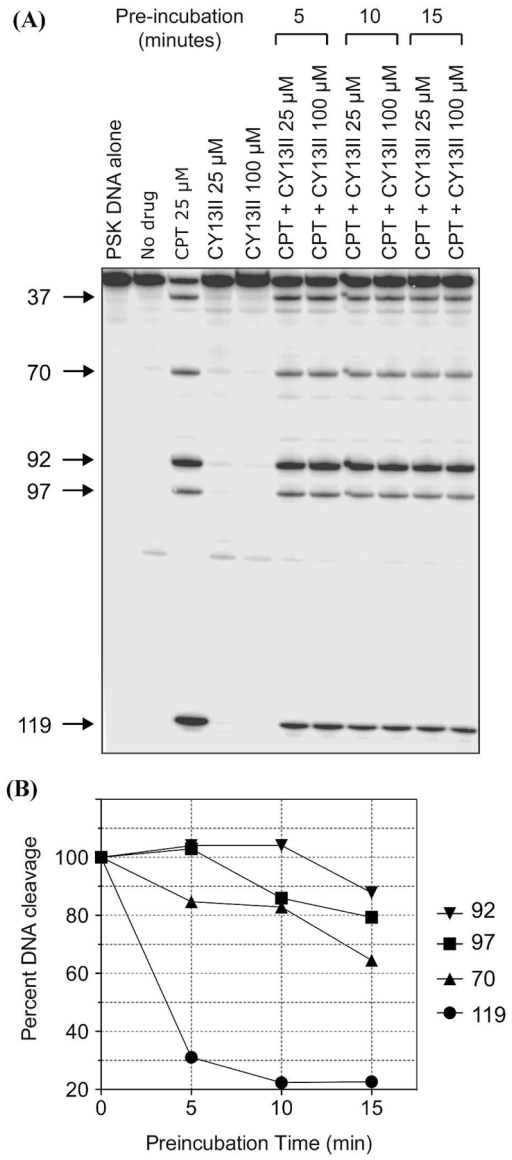

Figure 3.

Top1-mediated DNA cleavage assay. (A) Representative DNA gel. Lane 1, PSK DNA alone. Lane 2, Top1 alone. Lane 3, Top1 and CPT at 25 μM. Lane 4 and 5, Top1 and CY13II at 25 and 100 μM, respectively. Lane 6–11, Top1 was pre-incubated with 25 or 100 μM CY13II for 5 min (Lane 6 and 7), 10 min (Lane 8 and 9) or 15 min (Lane 10 and 11) followed with addition of 25 μM CPT. Numbers and arrows on the left indicate cleavage site positions. (B) Quantitation of the differential effects of CY13II (25 μM) on CPT-induced DNA cleavage at different cleavage sites.

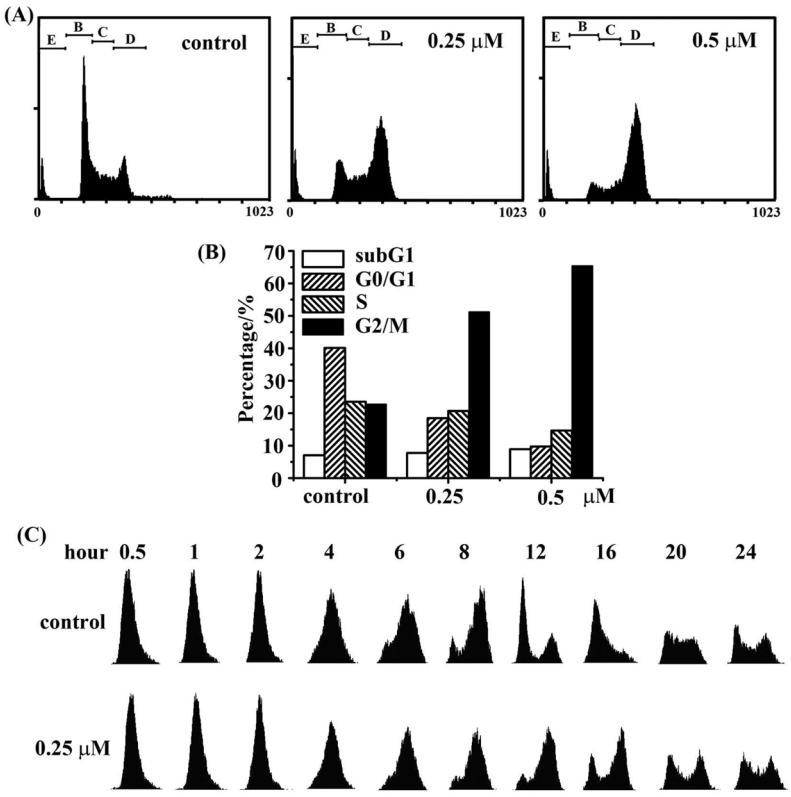

Figure 4.

(A), Flow cytometry analysis of K562 cells treated with CY13II. *x*-axis represents the DNA content, and *y*-axis the cell numbers. K562 cells were treated with CY13II at various concentrations [0 (control), 0.25 and 0.5 μM] for 48 h followed by staining with propidium iodide prior to FACS analysis for DNA content. (B), Quantitation of flow cytometry analysis from panel A. (C), Cell synchronization analysis. After release from a double thymidine block, G1/S boundary cells were treated with CY13II at 0.25 μM. Control cells were treated with free-compound medium. Aliquots of cells were sampled for flow cytometric analysis at various times after treatment.

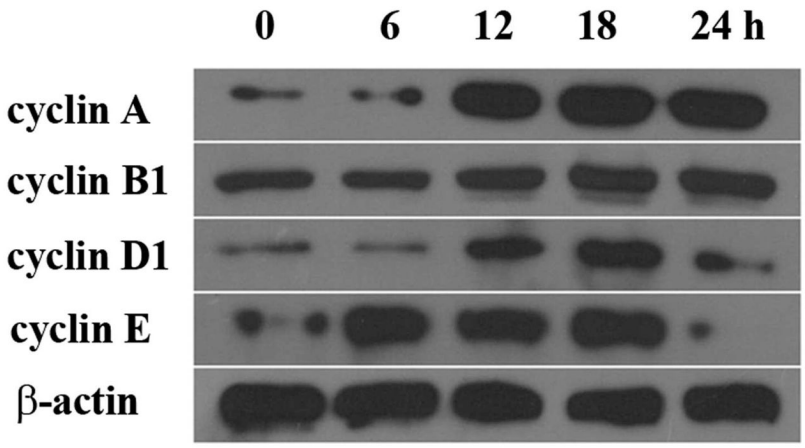

Figure 5.
Effects of CY13II on the expression of cyclins.

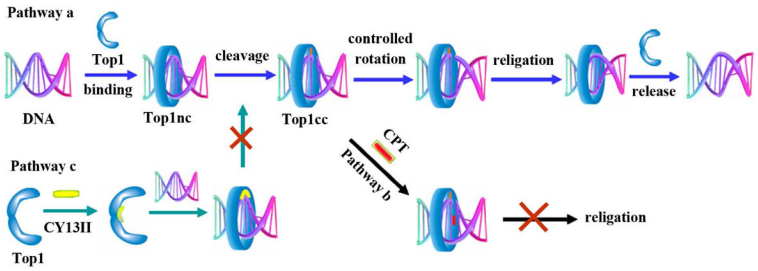

Figure 6.
Proposed mechanisms of the catalytic cycle of Top1 (Pathway a) and the inhibition of Top1 by CPT (Pathway b) and CY13II (Pathway c).