Emodin Triggers DNA Double-Strand Breaks by Stabilizing Topoisomerase II-DNA Cleavage Complexes and by Inhibiting ATP Hydrolysis of Topoisomerase II

Yan Li, Yang Luan, Xinming Qi, Ming Li, Likun Gong, Xiang Xue, Xiongfei Wu, Yuanfeng Wu, Min Chen, Guozhen Xing, Jun Yao, and Jin Ren

Center for Drug Safety Evaluation and Research, State Key Laboratory of New Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China

Received July 6, 2010; accepted September 10, 2010

Emodin, an anthraquinone derived from a plant and fungi, has been reported to possess potential genotoxicity, but the mechanism is not entirely clear. Here, we report that emodin causes DNA double-strand breaks (DSBs) through stabilization of topoisomerase (Topo) II-DNA cleavage complexes and inhibition of ATP hydrolysis. In our study, emodin did not induce mutagenicity in the salmonella mutation test but caused genotoxicity in the thymidine kinase gene mutation assay and in the micronucleus test. Moreover, emodin induced DNA DSBs demonstrated by induction of comet tails, the expression of phosphorylated histone H2AX, and phosphorylation of ataxia telangiectasia mutated. Our studies also revealed that emodin exerted strong inhibitory activity against Topo II in the supercoiled pBR322 relaxation assay and in Topo II–mediated kinetoplast DNA decatenation, similar to the previous report. We also showed that the inhibitory effect of emodin on Topo II was because of its ability to stabilize Topo II-DNA complexes and to inhibit the ATP hydrolysis of Topo II. Furthermore, emodin was found to trigger DNA DSBs in a Topo II–dependent manner using the Topo II catalytic inhibitor aclacinomycin and in Topo II–deficient mitoxantrone-resistant variant HL-60/MX2 cells. Together, these results suggest that in emodin-induced DNA DSBs and genotoxicity, stabilization of Topo II-DNA cleavage complexes and inhibition of ATP hydrolysis play an important role.

Key Words: emodin; DNA double-strand breaks; topoisomerase II; cleavage complexes; ATP hydrolysis.
Topo II-DNA cleavage complexes and generate DNA DSBs. The accumulation of DNA DSBs can induce chromosomal translocations linked to specific types of acute leukemia (Felix, 2001; Harker et al., 1991), implying that Topo II poisons may be potentially converted to potent genotoxic toxins and thus possess genotoxicity (Gupta et al., 1987; Lähdetie et al., 2006).

In this report, we found that emodin induced DNA DSBs likely through stabilization of Topo II-DNA complexes and inhibition of ATP hydrolysis. Our results suggest that emodin is genotoxic.

MATERIALS AND METHODS

Drug and Chemicals

Emodin (purity 98%) was purchased from Feida Biotech Co., Ltd (Xi’an, China). The compound was solubilized to 16 mg/ml in dimethyl sulfoxide (DMSO). Aliquots were thawed immediately before each experiment and diluted to the indicated concentrations (1% DMSO). Purified human Topo IIβ (TopoGEM, Inc., Columbus, OH) and Topo I from Takara (Dalian, China). Supercoiled plasmid pBR322 from Sangon (Shanghai, China) and ([γ-32P]ATP (~3000 mCi/mmol) from Perkin Elmer (Little Chalfont, Buckinghamshire, UK). Cell Counting Kit-8 was bought from Dojindo Laboratories (Tokyo, Japan) and etoposide (VP16), aclarentin (Acla), adriamycin (ADR), m-AMSA, camptothecin, and 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St Louis, MO). All other chemicals were analytical grade reagents.

Cell Lines and Cell Culture

The TK6 cell line has been described previously (Zhan et al., 2004). Human hepatoma HepG2, human promyelocytic leukemia HL-60, and the mitoxantrone-resistant variant HL-60/MX2 were purchased from the American Type Culture Collection (Manassas, VA). TK6 cells, HL-60 cells, and HL-60/MX2 cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technology Inc., Grand Island, NY) supplemented with 200 μg/ml sodium pyruvate, 100 μU/ml penicillin, and 100 μg/ml streptomycin. For the TK6 cells, 1% horse serum (Minhai Bio-Engineering, Co., Ltd, Lanzhou, China) was added, and for the HL-60 and HL-60/MX2 cells, 10% fetal bovine serum (Gibco-BRL, Life Technology Inc.) was used. HepG2 cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM l-glutamine, and 100 μg/ml streptomycin. All the cultures were incubated at 37°C in a 5% CO2 atmosphere with 100% humidity.

DNA DSBs-Related Assays

Neutral single-cell gel electrophoresis assay. We used the neutral comet assay reported earlier (Singh et al., 1988; Wada et al., 2003) with slight modifications. Briefly, the slides were electrophoresed in chilled neutral solution (pH 8.0) containing 90mM Tris, 2mM Na-EDTA, and 90mM boric acid. The slides were stained with DAPI and observed using an Olympus model BX51 fluorescence microscope. At least 1000 cells were pictured, and the comet tail moment was measured using Comet 5.5 software (Kinetic Imaging Ltd, Nottingham, U.K.).

Western blotting. Standard Western blotting analysis was performed using phosphorylated histone H2AX (γ-H2AX) rabbit antibody (Cell Signaling Technology, Beverly, MA). Immunofluorescence analyses. For immunofluorescence analyses, treated or untreated cells growing on coverslips were rinsed with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The samples were blocked with PBS/5% bovine serum albumin for 60 min and incubated with the γ-H2AX rabbit antibody (diluted 1:100; Cell Signaling Technology) and the phosphorylation of ataxia telangiectasia mutated (p-ATM, Ser 1981) mouse antibody (diluted 1:100; Rockland Immunotechnology Inc., Gilbertsville, PA) overnight at 4°C. After three washes with Tris-buffered saline, the samples were incubated with fluorescent secondary Alexa Fluor 488 anti-rabbit and Alexa Fluor 555 anti-mouse serum immunoglobulin G (diluted 1:100; Molecular Probes, Eugene, OR) for 45 min, then washed with PBS, and incubated with DAPI for 15 min. Images were photographed using a laser-scanning confocal microscopy (Fluoview1000, Olympus Optical Co., Tokyo, Japan).

Topo-Related Assays

Topo I– and Topo IIβ–mediated supercoiled pBR322 relaxation. DNA relaxation assays were conducted according to the procedure described previously (Osheroff et al., 1993). Relaxation was initiated at 37°C for 6 min in 20 μl reaction buffer containing 1mM ATP (ATP was omitted in Topo I–mediated DNA relaxation), 0.15 μg supercoiled pBR322, and 1 μl of Topo I or 2 μl of Topo II and stopped by the addition of 3 μl of stop solution (100mM EDTA, 0.5% SDS, 50% glycerol, and 0.05% bromophenol blue). Electrophoresis was carried out in a 1% agarose gel. DNA bands were stained with 0.5 mg/ml ethidium bromide (EB) solution and visualized under UV light.

Detection of cellular Topo-DNA cleavage complexes. The ability of emodin to intercalate into plasmid DNA was investigated using an unwinding assay described previously (Gupta et al., 1987). After treating for 4 h with 40 and 80 μg/ml of emodin, the collected TK6 cells were lysed by the rapid addition of 1 ml lysis buffer (1% Sarkosyl). CsCl step gradient centrifugation at 165,000 × g for 20 h and fractionation was carried out, and immunodetection of Topo I and IIβ complexes was performed using Topo I and Topo IIβ antibodies as required (Santa Cruz Biotechnology Inc., California).

Molecular Docking

DOCK 4.0 software (Ewing and Kuntz, 1997) was used in our study. Emodin was docked to the ATPase domain of hTopo IIβ (PDB code: 1ZXM; Wei et al., 2005; resolution 1.87 Å). Using a probe radius of 1.4 Å, the Connolly molecular surface was calculated for the target. The residues within 7 Å of the co-crystallized ligand were defined as being part of the binding site using Sybyl6.8 (Tripos, 6.8 ed.; Tripos Inc., St Louis, MO). Allowing for ligand flexibility, the ligand was docked into the ATPase domain. The scoring grid was defined so that all the spheres were enclosed within a box with an extra 7 Å added in each dimension. The bump check eliminated conformers with steric contacts that were closer than 75 percent of the van der Waals radii of the interacting atoms. A 6–12 Lennard-Jones van der Waals potential and a Coulomb potential with a distance-dependent dielectric coefficient of 4 were used to simulate salvation effects. The energy cutoff was set to 10 Å. Ligand atoms were matched to receptor spheres using the anchor first search with the anchor size set to 10 atoms.

EMODIN TRIGGERS DNA DSBs

ATP Hydrolysis

ATP hydrolysis was assessed using two independent assays. In the first assay [γ-32P], ATP was employed to detect hydrolysis using a modification of the method described previously (Kingama et al., 2001). The ATP hydrolysis reaction mixtures contained 2 U Topo II, 1mM [γ-32P] ATP (3000 mCi/mmol stock), and 0, 20, 40, or 80 μg/ml emodin in a total volume of 20 μl reaction buffer. The concentrations of ATP used were 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0mM. The reaction was initiated by the addition of Topo II followed by incubation at 37°C. Samples (2.5 μl) were removed at intervals up to 30 min and spotted on polyethylenimine-impregnated cellulose thin layer chromatography plates (Merck, Germany). Plates were developed by chromatography in freshly prepared 400mM NH4HCO3 and analyzed by Storm (GE healthcare). The radioactivity of free inorganic phosphate released by ATP hydrolysis was determined by ImageQuant 5.2 software (GE healthcare).

The second assay was performed via 31P nuclear magnetic resonance (NMR) spectroscopy. Reaction solution of 500 μl was centrifuged at 11,000 × g for 10 min at 4°C to remove any precipitates. Aliquots of 450 μl of the resulting supernatant were moved into 5 mm NMR tubes containing 50 μl D2O to turn on the field frequency lock. One-dimensional 31P NMR spectra of the solution systems were recorded at 25°C on a Varian Unity INOVA 600 NMR spectrometer equipped with a broadband probe and operating at 242.747 MHz 31P frequency. Inverse-gated 1H decoupling (waltz 16) was applied to eliminate 31P-1H coupling and to avoid build-up of the nuclear Overhauser effect. Other acquisition parameters were as follows: relaxation delay, 8 s; pulse length, 6 s (60°); acquisition time, 0.675 s; data points, 13 K; spectral width, 10,000 Hz, and transients, 4504. After zero-filling to 32 K data points and multiplied by 10-Hz line broadening, the free induced delays were Fourier transformed. All spectra were corrected for phase and baseline and referenced relative to inorganic phosphate at 2.5 ppm. The peaks were assigned by comparison with the chemical shifts reported in the literature (Raghunand et al., 1999; Singer et al., 1995). Quantification was performed by measuring relative integrals of the peaks. The decomposition rate of ATP was determined from the ratio between the integrals of Pi and Pi + ATP.

Statistical Analysis

Data are presented as mean ± SD, and their significance was assessed using Student’s t-test. Differences were considered significant at p < 0.05.

RESULTS

DNA DSBs Induced by Emodin

DNA DSBs induced by emodin were examined in mammalian cells. The neutral comet assay is known for its sensitivity to detect DNA DSBs (Olive, 1999). As shown in Figure 1A, emodin caused an obvious induction of comet tails in TK6 cells, and quantitative analysis revealed that the induction was dose dependent (Fig. 1B). To confirm that emodin causes DNA DSBs, we detected the phosphorylated level of histone H2AX, a reliable protein recognizing DNA DSBs (Kuo and Yang, 2008; Takahashi et al., 2010). The results showed that the cellular levels of γ-H2AX were enhanced in TK6 cells (Fig. 1C), and the foci of γ-H2AX and p-ATM were formed at DNA DSBs sites in HepG2 cells (Fig. 1D). Thus, our data clearly indicated that emodin triggered DNA DSBs.

Topo II Catalytic Activity Inhibited by Emodin

Emodin has been reported to target Topo II (Müller et al., 1996). To verify the interaction between emodin and Topos, we investigated the effect of emodin on the catalytic activity of Topo I and Topo II. In cell-free systems, we found that emodin had no effect on the Topo II–mediated relaxation of supercoiled pBR322 (Fig. 2A). In contrast, emodin markedly inhibited Topo II activity as indicated by the Topo II–mediated relaxation of supercoiled pBR322 and by the kDNA decatenation assay (Figs. 2B and 2C). Topo II–catalyzed decatenation of double-stranded catenated kDNA in the presence of ATP is a specific assay used to characterize inhibitors of Topo II. As shown in Figure 2D, 40 and 80 μg/ml emodin inhibited the activity of Topo II when measured by a loss in its capacity to decatenate kDNA.

To further study the effects of emodin on Topo II, we used the ICE assay to detect the formation of Topo I- and Topo II-DNA cleavage complexes at the cellular level. Using a specific Topo I antibody revealed that emodin was unable to trap the Topo I-DNA cleavage complexes (Fig. 2E) in agreement with the earlier result in a cell-free system that showed that emodin had no effect on Topo I activity (Fig. 2A). However, using a specific Topo II antibody revealed that emodin trapped the stable Topo II-DNA cleavage complexes (Fig. 2F).

To show that the inhibition of Topo II activity and the formation of Topo II-DNA cleavage complexes are crucial for emodin-induced DNA damage, we did the subsequent experiments using Acla, a Topo II catalytic inhibitor, and HL-60/MX2 cells, Topo II–deficient cell line.

The catalytic inhibitor Acla abrogates Topo II poison–induced DSBs by disrupting the formation of the cleavage complexes. As shown in Figure 1C, emodin-induced DNA DSBs in TK6 cells were dramatically attenuated by pretreatment with Acla. These results indicated that it may be the ability of emodin to poison the activity of Topo II and to induce stable Topo II-DNA cleavage complexes that contributes to its effect on DNA damage.

Topo II–deficient HL-60/MX2 cells have a lowered nuclear Topo IIα content and no Topo Iβ. They exhibit resistance to mitoxantrone and cross-resistance to VP16 and other Topo II inhibitors (Harker et al., 1991). The 50% inhibition of growth (IC50) value for emodin was measured in both HL-60 and HL-60/MX2 cell lines. As expected, emodin exhibited less cytotoxicity against HL-60/MX2 cells with an 50% inhibitory concentration in HL-60/MX2 three times higher than that in HL-60 cells (Fig. 2G). Moreover, γ-H2AX expression in emodin-treated HL-60/MX2 cells was lower than that in HL-60 cells (Fig. 2H). These results suggested that Topo II played an important role in cytotoxicity and DNA DSBs induced by emodin.

Topo II–Catalyzed ATP Hydrolysis Inhibited by Emodin

The ATPase domain of hTopo IIα has been shown to be a target for Topo IIα inhibitor (Hu et al., 2006). To investigate how emodin affects Topo IIα, we used molecular docking simulations to identify the possible interaction with hTopo IIα for emodin. The simulation studies suggest that emodin may bind to the ATPase domain of hTopo IIα (Fig. 3A) by forming strong hydrogen bonds with the enzyme. Specifically,
Emodin caused DNA DSBs. (A) Emodin-induced DSBs in TK6 cells. Cells were exposed to emodin (20, 40, or 80 μg/ml) or etoposide (50μM) for 4 h. Neutral comet assay was performed to evaluate DSBs. Representative comet images are shown (×200). (B) Semiquantitative analysis of the results presented in (A), expressed as the Olive tail moment (tail moment = tail length × % tail DNA, mean ± SD, n = 3), \( *p < 0.05, **p < 0.01 \). (C) Acla abated \( \gamma \)-H2AX production in TK6 cells treated with emodin (40 and 80 μg/ml) or VP16 (50μM) for 4 h. The data are representative of three independent experiments. (D) Emodin (40 and 80 μg/ml) induced formation of \( \gamma \)-H2AX and p-ATM foci in HepG2 cells detected by immunofluorescence (IF) assay. Representative IF images are shown (×400).
FIG. 2. Emodin was a Topo II inhibitor. (A) Emodin did not inhibit Topo I–mediated relaxation of supercoiled plasmid pBR322 (RLC, relaxed circle; SC, supercoiled circle). (B) Emodin inhibited Topo IIα–mediated relaxation of supercoiled plasmid pBR322. (C) Emodin inhibited Topo IIα–mediated kDNA decatenation in the cell-free system. The positions of kDNA and minicircles are indicated. (D) Semiquantitative analysis of the results shown in (C). (E) ICE bioassays showing that emodin did not trap Topo I-DNA cleavage complexes; Topo I inhibitor camptothecin was used as the positive control. Fractions were collected and numbered 1–8 from top to bottom. (F) Emodin-induced stable Topo IIα-DNA cleavage complexes detected by ICE assay; Topo II inhibitor VP16 was used as the positive control. Fractions were collected and numbered 1–8 from top to bottom. (G) Topo II–deficient subline (HL-60/MX2) is resistant to emodin and VP16. IC_{50} was measured using the Cell Counting Kit-8. Three separate experiments were carried out. The results are expressed as mean ± SD, *p < 0.05, **p < 0.01. (H) γ-H2AX upregulation induced by emodin and VP16 in the HL-60/MX2 cells was not as obvious in HL-60 cells.
the 1, 3-hydroxyl groups of emodin can form three hydrogen bonds with Asn150, Lys168, and Ser148 (Fig. 3B) in ATPase domain of hTopo IIα, indicating that emodin may interact with residues in the ATPase domain of hTopo IIα.

To test this possibility, we investigated whether emodin could inhibit Topo IIα–catalyzed ATP hydrolysis. The data collected from both the thin layer chromatography assay and the NMR assay showed that ATP hydrolysis was inhibited in the presence of emodin (Figs. 3C and 3D). Eighty micrograms per milliliter of emodin in 1mM ATP was enough to reduce ATP hydrolysis by more than 60% compared with the control (Fig. 3C). The effects of various concentrations of ATP on the emodin-mediated inhibition of hTopo IIα–catalyzed ATP hydrolysis in the absence of DNA were also investigated (Fig. 3D). Our analysis using a double-reciprocal Lineweaver-Burk plot suggested that emodin was an ATP-competitive inhibitor of hTopo IIα. Together, these results suggested that emodin may interact with the ATPase domain of Topo II and competitively inhibited ATP hydrolysis.

Emodin May Not Intercalate into DNA Double Strands
Among the Topo II inhibitors, some, such as ADR and m-AMSA, are DNA intercalators (Yamashita et al., 1992) and others, like VP16, are not (Harker et al., 1991).

To find out whether emodin is an intercalator, an unwinding assay was performed using linearized pBR322 and T4 DNA ligase. This assay can detect changes in the twist of the duplex helix. The drugs, including emodin and ADR, were preincubated with linearized pBR322. Upon drug removal, the twist returns to normal while the linking number of the unwound DNA, frozen by circulation of drug-bound DNA by T4 DNA ligase, remains constant leading to negative superhelicity of the DNA. Because of its strong intercalation ability (Harker et al., 1991), ADR clearly inhibited the activity of the T4 ligase. On the other hand, emodin behaved in a similar way to VP16 having no influence on T4 DNA ligase–mediated ligation (Fig. 4). These results indicated that emodin was not a DNA intercalator.

DISCUSSION
Previous evidence suggested that the genotoxicity of emodin may be mediated by Topo II (Müller et al., 1996). The mechanisms as to how emodin targets Topo II to induce genotoxicity remained to be fully elucidated. In the present study, we have further specified that emodin facilitated its inhibition on Topo II through stabilizing of Topo II–DNA cleavage complexes and inhibiting ATP hydrolysis of Topo II. Most importantly, we have established a possible mechanistic link between DNA DSBs and Topo II inhibition induced by emodin.

Molecular evidence from Topo II–mediated pBR322 relaxation and kDNA decatenation assays has substantiated that emodin is
a Topo II inhibitor (Figs. 2C and 2D). These studies have also shown that emodin can inhibit Topo II in concentrations that are substantially less than those previously (40 μg/ml compared with the 1mM [≈ 270 μg/ml] used by Müller et al., 1996).

Topo II inhibitors, such as VP16, are strongly mutagenic in mammalian cells but show no significant mutagenic effects in TA 98, TA 100, or TA 1537 salmonella strains (Ferguson and Baguley, 2006; Gupta et al., 1987). In the present study, emodin showed no mutagenic activity in any of the six strains of salmonella without metabolic activation by rat liver S9 (Supplementary table 1), but it increased micronucleus induction of salmonella without metabolic activation by rat liver S9 (Supplementary fig. 1), agreeing with the results reported earlier in L5178Y cells (Müller et al., 1996).

DNA DSBs were induced in TK6 cells by emodin, evidenced by the enhancement of cellular levels of γ-H2AX and the generation of comet tails by emodin (Figs. 1A and 1C). The ATM, known as a DNA damage sensor in response to DSBs, was also activated at the sites of γ-H2AX foci in HepG2 cells (Fig. 1D), further confirming that emodin did induce the DNA DSBs. The above data suggest that emodin-induced DNA DSBs may, at least partially, contribute to the genotoxicity of emodin.

The formation of Topo II-DNA cleavage complexes is essential for Topo II to perform its cellular functions (Champoux, 2001; Velez-Cruz and Osheroff, 2004; Wilstermann and Osheroff, 2003). Increasing the concentration of these complexes resulted in the induction of DSBs (Cao et al., 1990; Resnick, 1976). Using an ICE assay, we were able to show that emodin can increase the formation of Topo II-DNA cleavage complexes, thus clarifying that emodin could act as a Topo II inhibitor (Fig. 2F), same as previous findings (Kong et al., 1992). DNA DSBs were substantially suppressed both in Aclapretreatment TK6 cells and in Topo II–deficient HL-60/MX2 cells (Figs. 1C and 2H), suggesting that inhibition of Topo II activity and stabilization of Topo II-DNA complexes play a critical role in the induction of DNA DSBs by emodin.

Topo II facilitates its catalytic activity in an ATP-dependent manner. The enzyme cuts both strands of one DNA double helix (termed the G segment), and upon ATP binding, its ATPase domains are dimerized into a closed-clamp form to capture the transported DNA (termed the T segment). The T segment passes through the break in the G segment followed by breakage reunion G segment and T segment released with the clamp transiting to open form; then Topo II goes into the next cycle. The energy from ATP hydrolysis helps the T segment passage and Topo II turnover (Harkins et al., 1998; Wilstermann and Osheroff, 2003). Several anticancer drugs like ICRF-187 and ICRF-193 have been shown to be effective in targeting ATP binding or ATP hydrolysis (Classen et al., 2003; Huang et al., 2001; Roca et al., 1994). Emodin, like most anthraquinones, possesses hydroxyl groups, which are argued to be necessary for their genotoxicities (Tikkanen et al., 1983; Westendorf et al., 1990). Our analysis of the crystal structure of the hTopo IIz ATPase domain led us to predict that emodin bounds to the ATPase domain by forming polar hydroxyl bonds between its 1, 3 positions of hydroxy groups and residues of ATPase domain (Fig. 3B). It is implied that anthraquinones with hydroxyl groups may have interaction with ATPase domain. We have found that emodin might interact with ATPase domain through competitively inhibiting ATP hydrolysis of Topo II. However, the inhibitory effect of ATP hydrolysis was not specific to Topo II as a similar effect was seen for some tyrosine kinases like fibroblast growth factor receptor 1 and fibroblast growth factor receptor 3, and serine/threonine protein kinase like cyclin-dependent kinase 2, which transferred a phosphate group from ATP to a tyrosine residue or serine/threonine residue in a substrate, respectively (Supplementary table 2).

As a Topo II inhibitor, emodin was found to intercalate into DNA before (Kong et al., 1992), but in our findings, emodin turned out to be a non-DNA intercalator same as VP16 (Fig. 4). It was indicated that inhibition of Topo II but not direct interaction with DNA contributed to DNA damages induced by emodin.

In summary, we have found that the genotoxicity and DNA-damaging properties of emodin are because of its role in stabilizing Topo II-DNA cleavage complexes and in inhibiting ATP hydrolysis. We believe that it is important to pay more attention to safety issues when laxative drugs containing emodin and other anthraquinones are used or prescribed.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**FUNDING**

The National Basic Research Program (also called 973 Program, 2006CB504701); National Key Technologies R&D Program (2008ZX09305-007, 2009ZX09501-033).

**ACKNOWLEDGMENTS**

We show our great thanks to Dr Zhi Chen for assistance with molecular docking; Miss Xia Liu for assistance with Nuclear
Magnetic Resonance assay; and Mr Yong Xi, Miss Yi Wang, and Dr Hua Xie for assistance with the Topo II experiment. The TK6 cell line used in this study is a kind gift of Dr Honma from Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan.

REFERENCES


