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Supplementary Information

Novel indeno[1,2-*b*]pyridinone derivative, a DNA intercalative human topoisomerase IIα catalytic inhibitor, for caspase 3-independent anticancer activity

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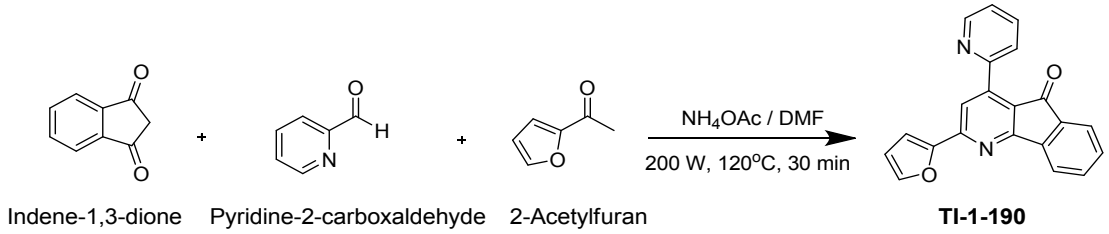
Synthesis and characterization of 2-(furan-2-yl)-4-(pyridin-2-yl)-5H-indeno[1,2-b]pyridin-5-one (**TI-1-190**)



Scheme S1. Synthetic scheme of TI-1-190

1,3-Indandione (0.29 g, 2.0 mmol), pyridine-2-carboxaldehyde (0.19 mL, 2.0 mmol), 2-acetylfuran (0.22 g, 2.0 mmol), ammonium acetate (1.54 g, 5.0 mmol), and DMF (2.0 mL) were mixed together in a 5 mL microwave reaction vial that was then capped. The mixture was irradiated for 30 min at a power of 200 W at 120 °C. The reaction mixture was cooled to room temperature and then poured into cold water. It was then extracted with ethyl acetate and washed with water and brine solution. The organic layer was dried with magnesium sulfate and filtered. The filtrate was evaporated at reduced pressure, then purified with Flash column chromatography (Isolera) using a gradient elution of 5-35% ethyl acetate in *n*-hexane. Rotary evaporation of the pure eluate fractions afforded **TI-1-190** as a yellow solid (122 mg, 0.38 mmol, 18.8% yield).

TLC (ethyl acetate/*n*-hexane = 1:2) R_f = 0.18, mp: 218.2-219.3°C, **HPLC**: Retention time: 6.58 min, purity: 95.1%; **ESI LC/MS**: *m/z* calcd for $C_{21}H_{12}N_2O_2$ $[MH]^+$ 325.09; found 325.0.
^{1}H NMR (250 MHz, $CDCl_3$) δ 8.77 (s, 1H, indenopyridine H-3), 8.02 (d, *J* = 7.85 Hz, 1H, 4-pyridine H-6), 7.98-7.95 (m, 2H, indenopyridine H-6, H-9), 7.83 (t, *J* = 7.25 Hz, 1H, 4-pyridine H-4), 7.67 (d, *J* = 7.3 Hz, 1H, 2-furan H-5), 7.63-7.57 (m, 2H, indenopyridine H-7, H-8), 7.46-7.32 (m, 3H, 4-pyridine H-3, H-5, 2-furan H-3), 6.59 (br, 1H, 2-furan H-4).
^{13}C NMR (62.5 MHz, $CDCl_3$) δ 190.92, 166.47, 153.17, 152.72, 152.52, 149.84, 147.64, 144.76. 142.60, 135.93, 135.37, 134.93, 131.12, 125.71, 124.19, 123.71, 122.16, 121.07, 118.69, 112.57, 112.02.

The compounds used as starting materials and reagents were obtained from from Sigma-Aldrich (USA) and Junsei Chemical Co., Ltd (Japan), and used without further purification.

HPLC-grade acetonitrile and methanol were purchased from Burdick and Jackson, USA. Microwave-assisted synthesis was done using a Biotage Initiator microwave synthesizer. Thin-layer chromatography (TLC) was performed with Kieselgel 60 F_{254} (Merck) and silica gel (Kieselgel 60, 230 - 400 mesh, Merck), respectively. Since the prepared compound contained aromatic rings, it was visualized and detected on TLC plates with UV light (short wave, long wave, or both). In addition, a Biotage Isolera One Flash System (Flash Chromatography) equipped with an internal variable dual wavelength diode array detector (200 − 400 nm) was used for compound purification. SNAP cartridges were used for normal phase purification with KP-Sil (25 g, flow rate of 25 mL/min). Dry sample loading was done by self-packing samplet cartridges using silica. Gradient elution with 5-35% ethyl acetate in *n*-hexane was used for purification.

NMR spectra were recorded on a Bruker AMX 250 (250 MHz, FT for ^{1}H NMR and 62.5 MHz for ^{13}C NMR), and chemical shifts were calibrated according to TMS. Chemical shifts (δ) were recorded in ppm and coupling constants (J) were recorded in hertz (Hz). Melting points were determined in open capillary tubes on an electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

HPLC analysis was performed using a two Shimadzu LC-10AT pumps gradient-controlled HPLC system equipped with a Shimadzu system controller (SCL-10A VP) and a photo diode array detector (SPD-M10A VP) using the Shimadzu Lab Solution program. Sample volume of 10 µL was injected into a Waters X-Terra® 5 µM reverse-phase C_{18} column (4.6 x 250 mm) with a gradient elution of 80% to 100% of solution B in solution A for 10 min followed by 100% to 80% of B in A for 20 min at a flow rate of 0.9 mL/min at 254 nm UV detection, where mobile phase A was a solution (20 mM) of ammonium formate (AF) in doubly distilled water and B was 100% acetonitrile. The purity of compounds is described as a percentage (%). ESI LC/MS analyses were performed with a Finnigan LCQ Advantage® LC/MS/MS spectrometry utilizing the Xcalibur® program.

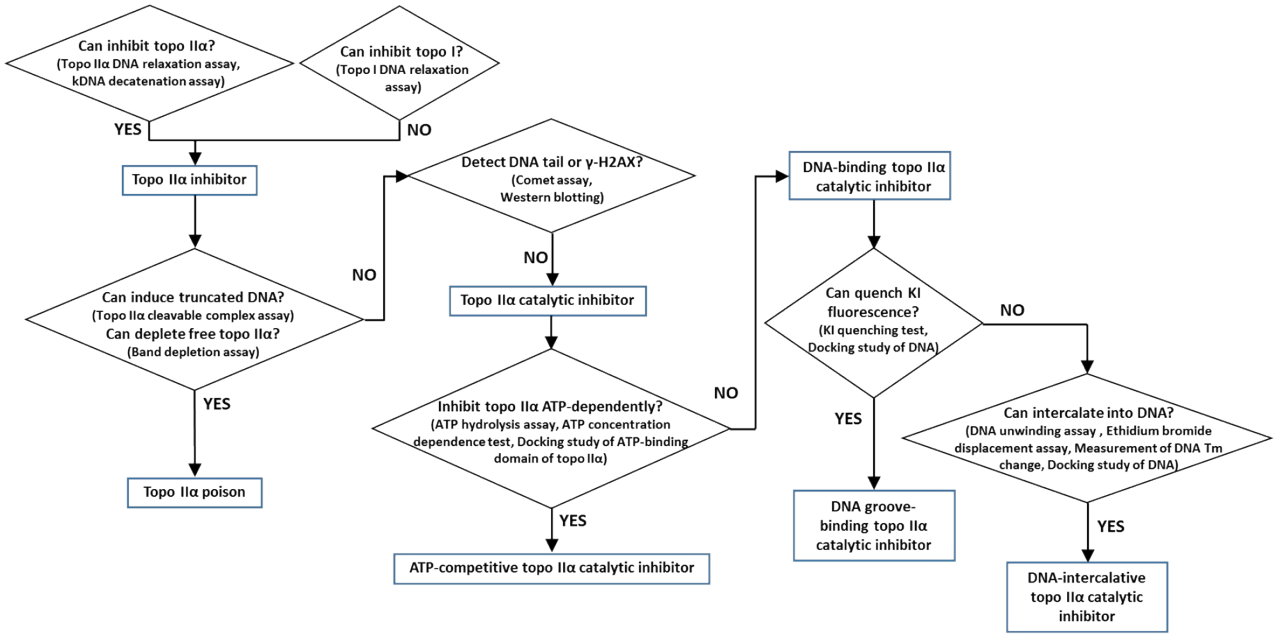


Fig. S1 Systematic evaluation flow chart for the development of topo IIα-specific catalytic inhibitors. We proposed this evaluation system to characterize which step of the topo IIα catalytic cycle is inhibited as well as to ultimately clarify the mode of action of the tested compound.

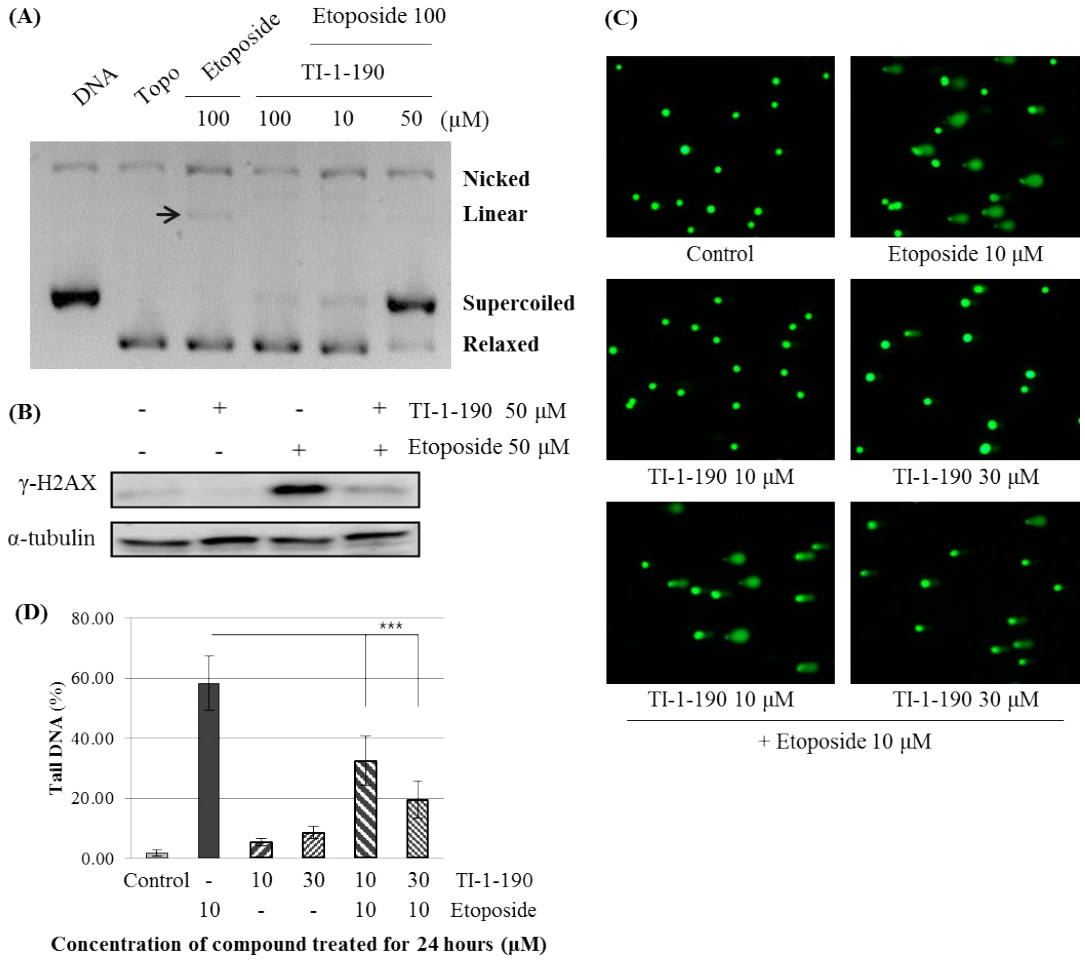


Fig. S2 Topo IIα inhibitory activity of TI-1-190 without causing DNA damage. (**A**) Cleavage complex assay. 250 ng of supercoiled pBR322 DNA was pre-incubated with 3 units of DNA topoisomerase IIα for 10 min, and each compound was added for an additional 20 min at 37 °C. (**B**) DNA damage as detected by γ-H_2AX levels. DNA damage was measured by the γ-H_2AX level, a marker of DNA repair. MCF7 cells were treated with 50 μM of TI-1-190 or etoposide or both compounds simultaneously for 24 hr. (**C**) DNA damage detected by alkaline comet assay. MCF7 cells at 70% confluency were treated with the indicated concentrations of etoposide and TI-1-190 for 24 hr followed by processing of the comet assay. (**D**) Quantification of comet tails in (C). Graphical representation of the selected comet lengths of untreated and treated MCF7 cells in pixels corresponding to concentration. Analysis of images was performed with Komet™ software. Columns and error bars indicate mean ± SD (n = 50). *** $P < 0.001$ for significant differences from the vehicle control.

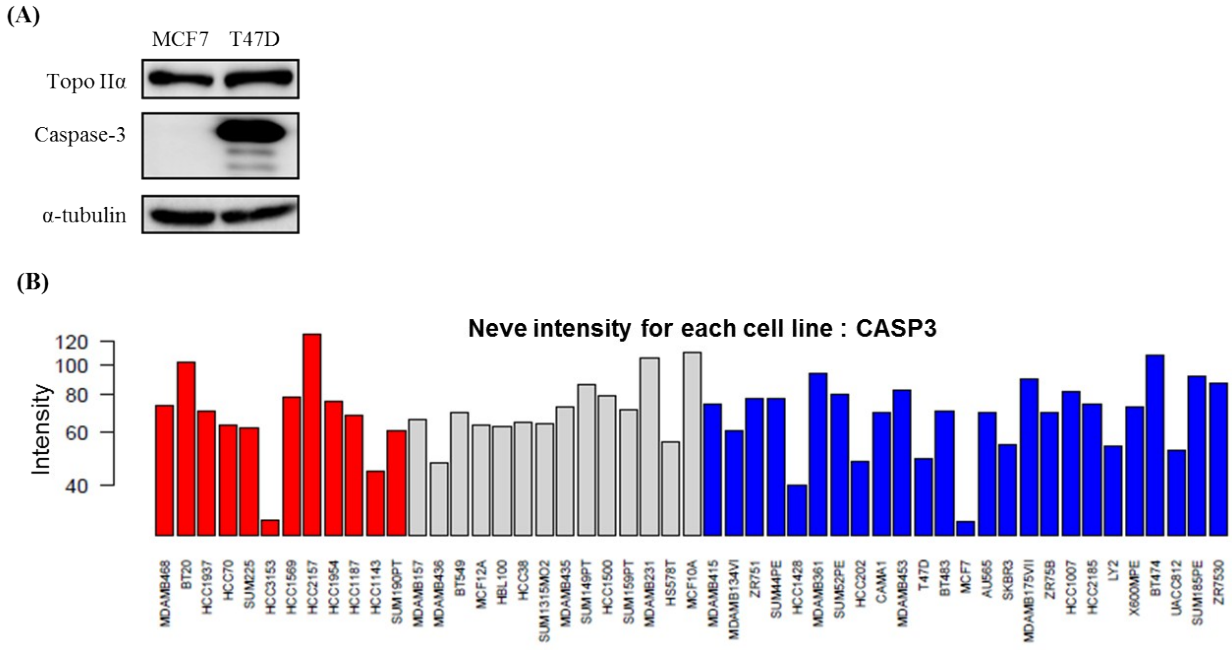


Fig. S3 (**A**) Basal expression levels of topo IIα and caspase-3 in MCF7 and T47D cells. (**B**) Expression level of caspase 3 in various types of cancer cell lines as extracted from the GOBO data set.

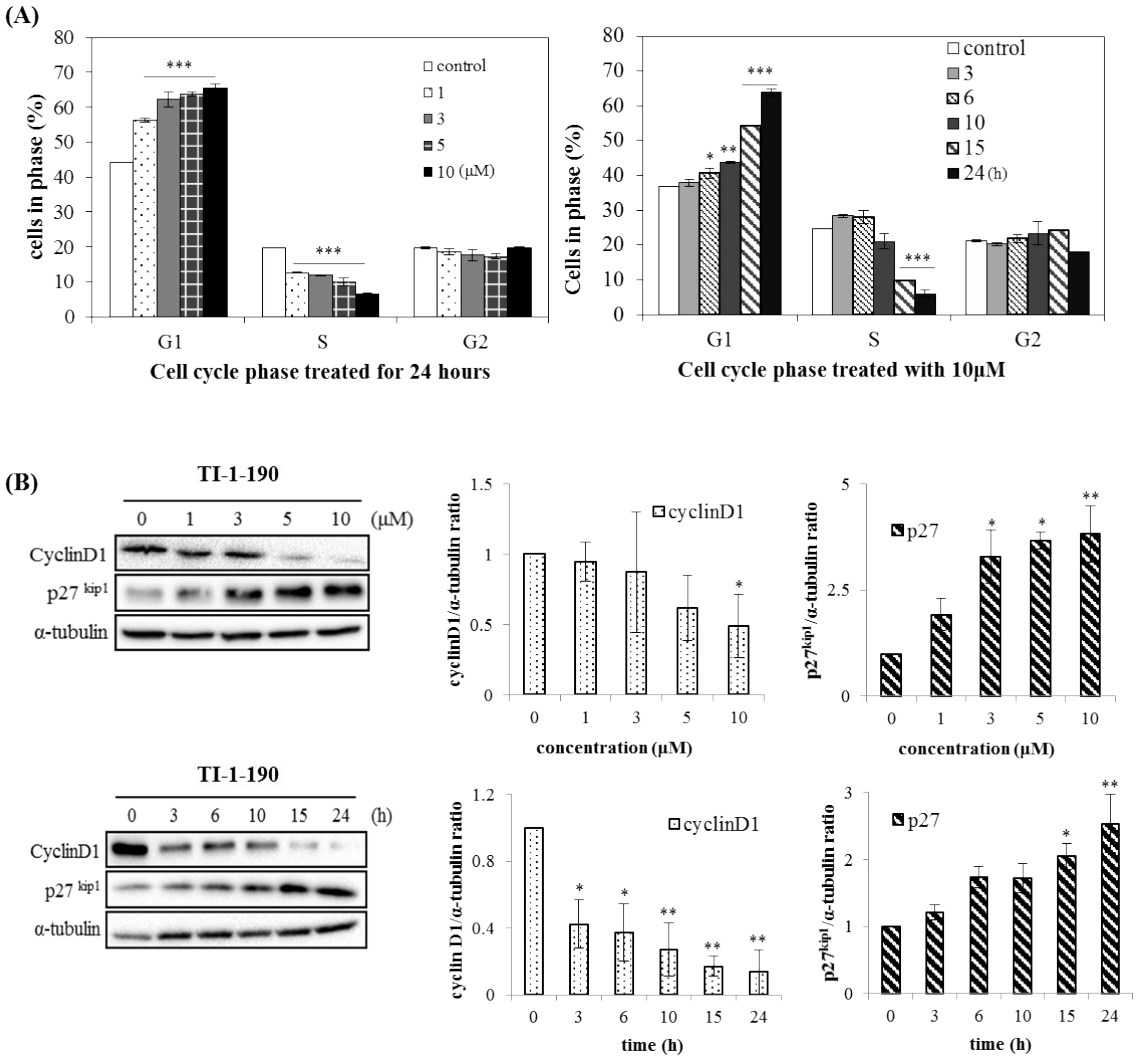


Fig. S4 Cell cycle analysis of TI-1-190 in MCF7 cells. MCF7 cells were treated with 0, 1, 3, 5, and 10 μM of TI-1-190 for 24 hr, or treated with 10 μM of TI-1-190 for 0, 3, 6, 10, 15, and 24 hr. (**A**) Cell cycle distribution was evaluated using flow cytometric DNA content analysis. TI-1-190 clearly increased the G1 fraction in a time- and dose-dependent manner. (**B**) Cyclin D1 and $p27^{kip1}$ expression levels were detected in MCF7 cells using western blot analysis. The levels of proteins of interest were normalized to the expression of α-tubulin. Columns and error bars indicate the mean ± SD of triplicate experiments. *$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ significantly different from the control.

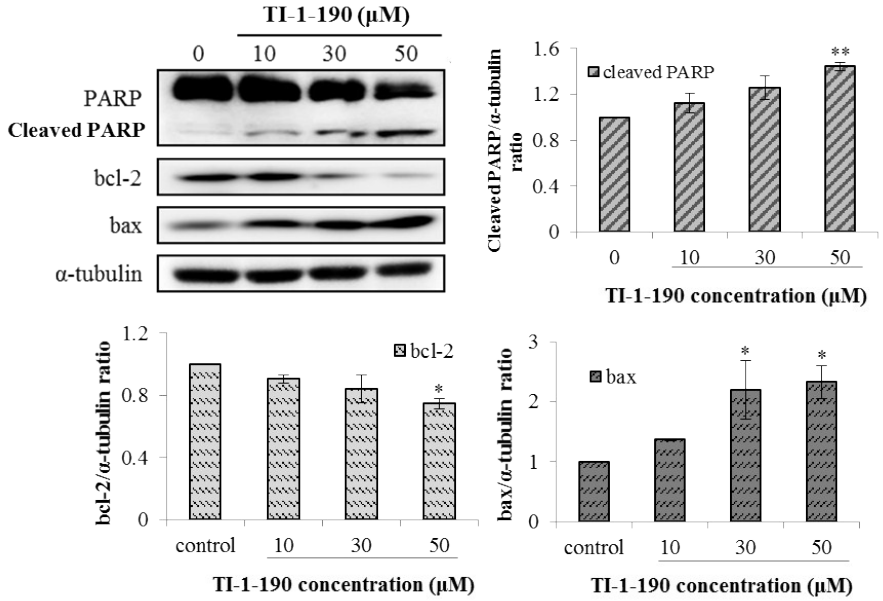


Fig. S5 TI-1-190-induced apoptosis of MCF7 cells as confirmed by an increase in the level of the pro-apoptotic proteins, cleaved PARP and bax, as well as a decrease in the level of the anti-apoptotic protein, bcl-2. MCF7 cells were treated with 0, 10, 30, and 50 μM of TI-1-190 for 24 hr. Bcl-2, bax, and cleaved PARP were detected in MCF7 cells using western blot analysis. The levels of the proteins were normalized to α-tubulin levels and graphed. Values represent the mean ± SD of triplicate experiments. *$p < 0.05$ and ** $p < 0.01$ significantly different from control.

Table S1. Topoisomerase I and II inhibitory activities of TI-1-190.

compound	% Inhibition				
	Topoisomerase I		Topoisomerase II		
	100 μM	20 μM	100 μM	20 μM	10 μM
Camptothecin	70.9	44.7	NT	NT	NT
Etoposide	NT	NT	83	29	19
TI-1-190	41	0	100	100	100

NT: not tested.

Table S2. Cell proliferation inhibitory activities of the tested compounds.

compound	IC_{50} (μM)				
	DU145	HCT15	T47D	MCF10A	MCF7
Adriamycin	0.95 ± 1.05	1.08 ± 0.10	1.88 ± 1.41	1.23 ± 0.20	18.98 ± 0.38
Etoposide	13.1 ± 1.71	2.60 ± 1.68	0.94 ± 0.06	10.14 ± 0.19	73.89 ± 2.07
TI-1-190	> 50	8.50 ± 0.04	3.08 ± 0.07	25.35 ± 0.18	9.38 ± 0.13

Each data point represents the mean ± SD of three different experiments performed in triplicate. Cancer cell lines used: human prostate cancer cell line (DU145); human colon cancer cell line (HCT15); human breast cancer cell line (T47D); human normal breast epithelial cell line (MCF10A); and human breast cancer cell line (MCF7). Positive controls used: adriamycin for cytotoxicity and etoposide for topo IIα and cytotoxicity assays.

Table S3. Non-inhibition of TI-1-190 on topo IIα-catalyzed ATP hydrolysis.

Compounds	Concentration (μM)	% Inhibition
Novobiocin	200	29.78 ± 0.28
	300	35.37 ± 0.21
	400	49.78 ± 3.33
TI-1-190	400	0

Each data point represents the mean ± SD of three different experiments performed in triplicate.

Experimental Procedures

Materials. All compounds were dissolved in DMSO (Sigma Aldrich, USA) at a concentration of 10 mM as a stock solution and stored under -20 °C. HCT15 (human colon cancer cell line), T47D (human breast cancer cell line), DU145 (human prostate cancer cell line), and MCF10A (human breast epithelial cell line) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). RPMI 1640, fetal bovine serum (FBS), penicillin streptomycin, and 0.25% Trypsin were purchased from Hyclone Laboratories Inc. (USA). Phosphate buffered saline (PBS), bovine serum albumin (BSA), and Xpert pre-stained protein marker were purchased from GenDEPOT (USA). PAGE-Mark Plus™ 3-Color Broad Range Protein Marker was purchased from BIOMAX (Korea). NaCl, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and glycine were purchased from Duchefa (Netherlands) and Tween 20 was from Junsei Chemical Co., Ltd (Japan). The BCA™ protein assay kit was purchased from Pierce (USA). Primary antibodies against cyclin D, $p27^{kip1}$, α-tubulin, topoisomerase IIα, caspase-3, bcl-2, bax, γH2AX, and PARP were purchased from Cell Signaling Technology, Inc. (USA) and anti-IgG secondary antibody was purchased from Santa Cruz Biotechnology. Recombinant human topoisomerase I, kinetoplast DNA, and pHOT1 DNA were purchased from TopoGEN (USA) and human recombinant topoisomerase IIα was from USB (USA). pBR322 was purchased from Fermentas (USA). Propidium iodide, RNase, camptothecin, etoposide, adriamycin, and novobiocin were purchased from Sigma-Aldrich (USA).

Cell cultures. HCT15, T47D, DU145, and MCF7 cells were grown in RPMI1640 supplemented with 10% FBS and 1% penicillin streptomycin. The MCF10A cell line was grown in Dulbecco's Modified Eagle's Medium supplemented with Nutrient Mixture F-12 (DMEM/F-12, Invitrogen, CA), 10% horse serum, 1% penicillin, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. All media were changed every 2 - 3 days and cells were subcultured at a ratio of 1:10.

DNA topoisomerase-mediated relaxation assay. The relaxation assay was performed as follows. A mixture containing 100 ng of supercoiled pBR322 DNA and the appropriate amount of human topoisomerase I or IIα was incubated for 30 minutes at 37 °C with or without the prepared compounds in the reaction buffer (topoisomerase I: 10 mM tris-HCl, pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% bovine serum albumin, 0.1 mM spermidine, 5% glycerol; topoisomerase IIα: 10 mM tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL bovine serum albumin, 1 mM ATP). The appropriate amounts of human recombinant topoisomerases were determined before each experiment because their activities are severely affected by freeze/thaw cycles. The minimum amount

of recombinant human topoisomerases which could fully relax 100 ng of supercoiled pBR322 DNA under the condition used for the relaxation assay was used to avoid false inhibition arising from a decrease in enzymatic activity. The final volume of the reaction mixture was 10 μL and the reaction was terminated by adding 2.5 μL of stop solution (topoisomerase I: 0.5% sarcosyl, 0.00025% bromophenol blue, and 2.5% glycerol; topoisomerase IIα: 0.7 mM EDTA). The reaction samples were then electrophoresed on a 0.8% agarose gel at 60 V for 1 hour with TAE running buffer. Gels were stained for 15 minutes in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by trans-illumination with UV light and quantitated using AlphaImager™ (Alpha Innotech Corporation). Camptothecin and etoposide were used as positive controls for topoisomerase I and topoisomerase II, respectively.

Cell viability assay. Cell viability was evaluated with the Cyto X cell viability assay kit (LPS Solution Corporation, Korea) to measure the antiproliferative activity of compounds in various cancer cell lines. HCT15, T47D, DU145, and MCF 10A cells were seeded in 96-well plates at a density of 10^4 cells per well and grown for 1 day at 37 °C in a 5% CO_2 atmosphere. After cells were incubated for 4 hours in serum-free medium (RPMI1640 or DMEM/F-12 only), they were treated with serum free medium containing diverse concentrations of compounds for 72 hours. 5 μL of Cyto X was added to each well and cells were incubated at 37 °C for an additional 2 hours. The absorbance of each well was determined using an ELISA Microplate reader (VERSAmax, Molecular Devices) at a wavelength of 450 nm. The IC_{50} values were evaluated using Table Curve 2D (SPSS Inc., Chicago).

kDNA decatenation assay. The decatenation assay was performed in a total reaction volume of 10 μL containing 50 ng of kinetoplast DNA (kDNA) (Topogen, USA) in reaction buffer solution (10 mM tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL bovine serum albumin, 1 mM ATP). Compounds were added at final concentrations of 10, 50, and 100 μM. Reactions were initiated by adding 3 units of human topoisomerase IIα. The reaction mixtures were incubated for 30 minutes at 37 °C. The reaction was terminated by the addition of 2.5 μL of stop solution (0.7 mM EDTA) followed by treatment with 2 μL of 0.25 mg/mL proteinase K (Roche) and incubation at 55 °C for 30 minutes to eliminate proteins. The reaction mixtures were then resolved by electrophoresis on a 1.2% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide at 35 V for 5 hours with TAE running buffer. DNA bands were visualized by UV and quantitated using AlphaImager™.

Stabilization of the cleavage complex assay. The cleavage complex assay was performed in a total reaction volume of 10 μL containing 125 ng of supercoiled pBR322 DNA in reaction buffer solution (10mM tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, and 1 mM ATP). Compounds were added at final concentration of 10 or 50 μM. Reactions were initiated

by adding 3 units of human topoisomerase IIα, and mixtures were incubated for 30 minutes at 37 °C. The reaction was terminated by the addition of 2.5 μL of stop solution (0.7 mM EDTA) followed by treatment with 2 μL of 0.25 mg/mL proteinase K (Roche) at 45 °C for 30 minutes to eliminate proteins. Samples were resolved by electrophoresis on a 1% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide at 35 V for 5 hours with TAE running buffer. DNA bands were visualized with UV light and quantitated using AlphaImager™.

Alkaline comet assay. The alkaline single cell gel electrophoresis (comet assay) was performed using the Alkaline Comet Assay® kit (Trevigen, USA). MCF7 cells were seeded in 6-well cell culture plates (SPL, Korea) at a density of 10^5 cells per well and grown for 1 day at 37 °C. Cells were incubated either with or without etoposide and compounds for 24 hours in serum-free media. Before harvesting cells, LMAgarose was melted in a beaker of boiling water with the cap loosened and then cooled in a 37 °C water bath. Trypsinized cells were combined with molten LMAgarose at a ratio of 1:10 and 80 μL was immediately pipetted onto a CometSlide™. If necessary, the side of a pipette tip was used to spread agarose/cells over the sample area to ensure complete coverage of the sample area and the slides were placed flat at 4 °C in the dark for 40 minutes. Then slides were immersed in lysis solution which was cooled at 4 °C before use. After 30 minutes, slides were incubated in alkaline unwinding solution (0.2 M NaOH, 1 mM EDTA) for 30 minutes at room temperature and electrophoresed at 15 V for 20 minutes in electrophoresis solution (0.2 M NaOH, 1 mM EDTA, pH > 13). Then, slides were washed twice with double distilled water and once with 70% ethanol for 5 minutes. Next, samples were dried at 37 °C for 2 hours and stained with diluted SYBR® Green Staining Solution for 15 minutes in the dark. Then, they were mounted with coverslips and sealed with clear nail polish. Images were captured with a Zeiss HBO100 microscope illumination system (Carl Zeiss, Germany) equipped with an epq100-isolated epifluorescence condenser. About 50 spots of MCF7 cells were randomly analyzed with an image analysis system (Komet 5.5, Konetic Imaging Ltd, UK). Komet 5.5 software was used to calculate the lengths of the comet tails. The mean values were taken to represent the extent of DNA damage.

DNA unwinding assay. The unwinding assay was performed to determine if a compound induced DNA unwinding, which is characteristic of a DNA intercalator. A total of 10 μL of reaction mixture consisting of 1 μL supercoiled pHOT1 (100 ng/μL), 1 μL 10 × Reaction Buffer (100 mM Tris-Cl, pH 7.9, 1.5 M NaCl, 1% BSA, 1 mM spermidine, 50% glycerol), 1 μL topoisomerase I (4 units) and 6 μL DDW was incubated at 37 °C for 30 minutes. After incubation, 1 μL of each compound solution was added at a final concentration of 100, 200, 500, or 1000 μM, and mixtures were incubated at 37 °C for 30 minutes. Reactions were terminated by the addition of 1 μL topoisomerase I stop buffer. The reaction mixtures were resolved on 1% agarose gel at 15 V for 14 hours. After electrophoresis, the gels was stained in TAE buffer with ethidium bromide for 10 minutes and destained with water for 15 minutes. The gels

were visualized using AlphaImager™. In DNA unwinding assay, supercoiled DNA is fully relaxed at the first step, and then compounds are added and intercalate into relaxed DNA. During electrophoresis step, the intercalated compounds are released from the DNA because of their small size and the DNA intercalated by compounds is re-coiled.

ATPase assay. ATPase assays were performed with an ATP dehydrogenase reaction using a malachite green phosphate assay kit (BioAssay Systems, USA). The reaction was performed in 100 μL of a mixture containing reaction buffer (10 mM tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA), 100 ng supercoiled pBR322 DNA, 250 μM ATP, and 2 units of human topoisomerase IIα per well in 96-well plates and incubated at 37 °C for 30 minutes. Then, the reaction mixture was incubated for 30 min at room temperature with 20 μL per well of working reagent (reagent A:B = 100:1). The absorbance of each well was determined using an ELISA microplate reader at a wavelength of 620 nm.

UV-vis absorption measurements. UV-vis spectra were measured on a UV-vis Spectrophotometer, U-3000 (HITACHI, Japan) in 0.01 M Tris buffer (pH 7.4). The concentration of ctDNA (Sigma Chemical Co.) ranged from 0 to 500 μM. TI-1-190 was dissolved in DMSO, from which working solutions were prepared by dilution in 0.01 M Tris buffer to a concentration of 50 μM. All measurements were performed at room temperature.

Fluorescence measurements. Fluorescence measurements were taken on a multi-functional microplate reader, Infinite M200 PRO (TECAN, Switzerland). Emission spectra were recorded in the range of 390 - 460 nm using an excitation wavelength of 350 nm. Fluorescence titrations were conducted by maintaining a constant concentration of TI-1-190 (50 μM) and varying the concentration of ctDNA. The concentration range of ctDNA was 0 - 50 μM. In the case of the EB displacement assay, the concentration of EB solution was 20 μM and 30 μM of ctDNA was used for titration with varying concentration of m-AMSA or TI-1-190. The concentration range of m-AMSA or TI-1-190 was 0 - 40 μM. EB-ctDNA complex emission spectra were recorded from 500 - 700 nm with a fixed excitation at 471 nm. All measurements were performed at 25 °C.

Potassium iodide (KI) quenching assay. Iodide quenching experiments were carried out in the presence and absence of ctDNA. Fluorescence intensity of TI-1-190 (30 μM) was taken with or without ctDNA (30 μM) and with a varying concentration of KI (0 - 9 mM). Excitation was done at 350 nm and emission was recorded at 420 nm.

Circular dichroism measurement. CD spectra of DNA and DNA- TI-1-190 complexes were recorded on a CD-ORD Spectropolarimeter (Jasco Co.) with a temperature controller. All CD spectra were recorded in the range of 230 - 300 nm. DNA- TI-1-190 molar ratios of 3:1, 1:1, and 1:2 were used. The results are expressed as CD (mdeg).

Western blotting analysis. MCF7 cells were washed with PBS and trypsinized. The cells were lysed in lysis buffer (300 mM NaCl, 1% triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM tris-HCl (pH 7.4), 1 mM phenylmethanesulfonylfluoride (PMSF), 1% protease inhibitor cocktail) and incubated on ice for 30 minutes. The cells were centrifuged at 12,000 rpm for 20 minutes at 4 °C. The supernatant was collected and stored at -20 °C until further use. The amount of soluble proteins in the lysate was determined with a BCA Protein Assay Kit (Pierce, USA). The lysates were mixed with 2 × loading buffer (1 mM Tris-HCl (pH 6.8), glycerol, 10% SDS, bromophenol blue, beta mercaptoethanol), heated at 95 °C for 3 minutes, and incubated on ice for 20 minutes. About 40 - 60 μg of proteins were loaded to detect cyclin D, $p27^{kip1}$, topoisomerase IIα, caspase-3, bcl-2, bax, PARP, γH2AX, and α-tubulin. Samples were run on 12% sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a 0.2 μm polyvinylidenedifluoride transfer membrane (PALL, USA) at 200 mA for 2 hours. Then, the membranes were blocked with 5% skim milk in Tris buffered saline containing 0.1% tween 20 (TBST) for 1 hour at room temperature. After the membranes were washed with TBST every 20 minutes three times, they were incubated with primary antibodies diluted at a ratio of 1:1000 in TBST with 5% BSA at 4 °C overnight. The next day, the membranes were washed with TBST every 20 minutes three times and incubated with diluted secondary antibodies, anti-rabbit IgG horseradish peroxidase at a ratio of 1:10000, for 2 hours at room temperature. The membranes were washed again with TBST every 20 minutes three times and detected with ECL western blotting detection reagent (Abclon, Korea and GE Healthcare, United Kingdom). Western blot images were taken with an LAS-3000 (Fuji Photo Film Co., Ltd., Japan) and analyzed using Multi-Gauge Software (Fuji Photo Film Co., Ltd., Japan).

Cell cycle analysis with PI staining. MCF7 cells were seeded at a density of 5×10^4 cells per 60 mm cell culture plate. After 1 day, the cells were treated with compounds in serum-free media. The cells were washed with PBS (pH 7.4) and trypsinized and harvested by centrifugation at 2,000 rpm for 3 minutes. The cell pellet was fixed with 70% ethanol, then incubated at -20 °C for at least 3 hours. Fixed cells were centrifuged at 3000 rpm for 3 minutes and mixed with 500 μL PBS staining solution (propidium iodide 50 mg/mL, RNase 0.1 mg/mL, triton X-100 0.05% in PBS) and incubated at 37 °C for 40 minutes. The cells were centrifuged at 3,000 rpm for 3 minutes and the supernatant was removed and mixed with 500 μL PBS. Fluorescence was measured on Fluorescence Activated Cell Sorting (FACS)-Caliber flow cytometer (BD Biosciences, USA). At least 10,000 cells were measured for each

sample.

Apoptosis detection with Annexin V and PI. Apoptotic cell death induced by **TI-1-190** and etoposide were quantified by flow cytometry using the annexin V-fluorescein isothiocyanate (FITC) kit (BD Pharmingen, U.S.A.) in accordance with the manufacturer's protocol. Briefly, cells were plated on a six-well plate at a density of 1×10^4 cells per well and incubated with or without the indicated concentration of the tested compound for 24 hours. Floating cells as well as residual attached cells were collected and washed twice with PBS. The cell pellets were resuspended in 100 μL of 1 × binding buffer at a concentration of 1×10^5 cells/mL. Five milliliters of annexin V-FITC and 10 μL of PI were added to the cell suspension. After incubation for 15 minutes at room temperature, stained samples were examined using a FACS-Calibur flow cytometer (USA). Analysis was performed using the BD CellQuest Pro software supplied with the instrument.

Statistical analysis. All experiments were performed at least three times and all data are expressed as mean ± standard deviation (SD). Statistics were calculated by one-way analysis of variance (ANOVA) with GraphPad Instat version 3.10 (GraphPad Software, USA) and the differences between two values were considered statistically significant when the p values were < 0.05, < 0.01, or < 0.001, which are labeled with single (*), double (**), and triple asterisks (***), respectively.