

## Supplementary Information

### **A chromenone analog as an ATP-competitive, DNA non-intercalative topoisomerase II catalytic inhibitor with preferences toward alpha isoform**

Seojeong Park,<sup>‡,a</sup> Soo-Yeon Hwang,<sup>‡,a</sup> Jaeho Shin,<sup>b</sup> Hyunji Jo,<sup>a</sup>  
Younghwa Na,<sup>b,\*</sup> and Youngjoo Kwon,<sup>a,\*</sup>

<sup>a</sup>College of Pharmacy & Graduate School of Pharmaceutical Sciences, Ewha Womans  
University, Seoul, 03760, Korea.

<sup>b</sup>College of Pharmacy, CHA University, Pocheon, 11160, Korea.

<sup>‡</sup> *S.P. and S.-Y.H contributed equally.*

<sup>\*</sup> *Y.K. and Y.N. are co-corresponding authors.*

<sup>\*</sup>Corresponding Authors:

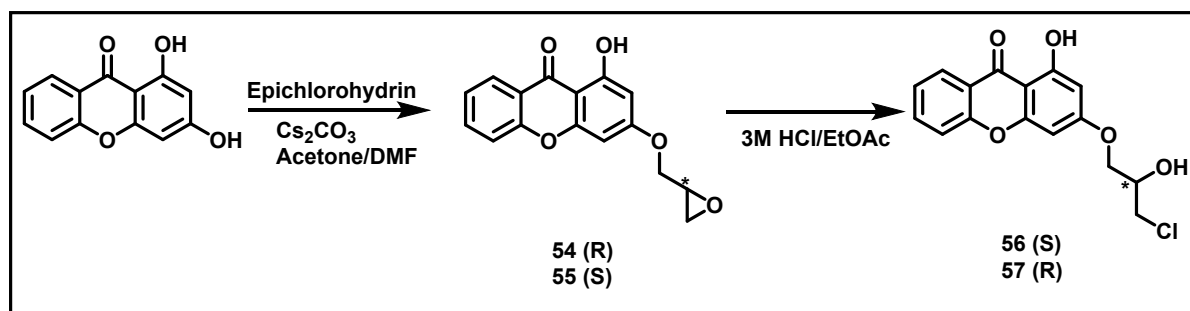
Tel: +82-31-8017-9896. Fax: 82-31-8017-9420. E-mail: yna7315@cha.ac.kr (Y.N.)

Tel: +82-2-3277-4653. Fax: 82-2-3277-3051. E-mail: ykwon@ewha.ac.kr (Y.K.)

## 1. Experimental

### 1.1 Chemistry general

The solvents and reagents used were of the highest commercial grade available and used as received. The TLC plates were Kieselgel 60 F254 (art A715, Merck). Silica gel 60 (0.040–0.063 mm ASTM, Merck) was used for column chromatography. The purity was assessed by HPLC (Shimadzu LC-20AD) analysis under the following conditions; column, SunFire C18 (4.6 mm × 150 mm, 5 μm); mobile phase, isocratic 60% acetonitrile in water over 15 min, flow rate; 1.0 mL/min; detection, UV detector (Shimadzu Spd-M20A diode array detector). The purity of compound is described as percent (%). The <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were recorded on a Varian NMR AS 400 MHz instrument. Chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane as an internal standard and coupling constants (*J* values) are in Hertz. Mass spectral investigations were performed on a AB SCIEX API 4000 equipped with an electrospray ionization (ESI) source. The melting points were measured on Gallenkamp Melting Point Apparatus without correction. Synthesis and structure elucidation of compounds **50–53** were reported previously [i].



Scheme S1. Synthetic method for compounds 54-57

#### 1.1.1 (*R*)-1-Hydroxy-3-(oxiran-2-ylmethoxy)-9*H*-xanthen-9-one (54)

The reaction mixture of 1,3-dihydroxyxanthone (0.50 g, 2.17 mmol), (*R*)-epichlorohydrin (0.51 mL, 6.51 mmol) and Cs<sub>2</sub>CO<sub>3</sub> (0.71 g, 4.34 mmol) in DMF/acetone (15 mL/20 mL) was stirred at ~80°C for 14 h and then cooled to room temperature. The reaction mixture was poured into

water and extracted with dichloromethane. Organic layer was washed with water and brine and then dried over anhydrous  $\text{MgSO}_4$ . Solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (eluent: dichloromethane) to give compound **54** (0.21 g, 34.5%) as a weak green solid. m.p. 188-192°C;  $R_f$  0.57 ( $\text{CH}_2\text{Cl}_2$ ); HPLC:  $R_T$  6.59 min (purity; 99.8%);  $^1\text{H-NMR}$  ( $\text{DMSO-d}_6$ , 400 MHz)  $\delta$  2.74 (dd,  $J = 4.8, 2.4$  Hz, 1H), 2.88 (dd,  $J = 4.8, 4.0$  Hz, 1H), 3.36-3.39 (m, 1H), 3.99 (dd,  $J = 15.2, 6.4$  Hz, 1H), 4.53 (dd,  $J = 15.2, 2.4$  Hz, 1H), 6.45 (d,  $J = 2.0$  Hz, 1H), 6.68 (d,  $J = 2.0$  Hz, 1H), 7.50 (ddd,  $J = 8.0, 8.0, 0.8$  Hz, 1H), 7.62 (dd,  $J = 8.0, 0.8$  Hz, 1H), 7.89 (ddd,  $J = 8.0, 8.0, 1.6$  Hz, 1H), 8.16 (dd,  $J = 8.0, 1.6$  Hz, 1H), 12.79 (s, 1H);  $^{13}\text{C-NMR}$  ( $\text{DMSO-d}_6$ , 100 MHz)  $\delta$  43.7, 49.4, 69.9, 93.4, 97.5, 103.3, 117.8, 119.8, 124.6, 125.3, 135.9, 155.5, 157.3, 162.6, 165.5, 180.2 ppm; HRMS-ESI ( $m/z$ )  $[\text{M}+\text{H}]^+$   $\text{C}_{16}\text{H}_{13}\text{O}_5$  calcd 285.0757, found 285.0761.

### 1.1.2 (*S*)-1-Hydroxy-3-(oxiran-2-ylmethoxy)-9*H*-xanthen-9-one (**55**)

The reaction mixture of 1,3-dihydroxyxanthone (0.50 g, 2.17 mmol), (*S*)-epichlorohydrin (0.51 mL, 6.51 mmol) and  $\text{Cs}_2\text{CO}_3$  (0.71 g, 4.34 mmol) in DMF/acetone (15 mL/20 mL) was stirred at ~80°C for 14 h and then cooled to room temperature. The reaction mixture was poured into water and extracted with dichloromethane. Organic layer was washed with water and brine and then dried over anhydrous  $\text{MgSO}_4$ . Solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (eluent: dichloromethane) to give compound **55** (0.22 g, 35.8%) as a weak green solid. m.p. 188-192°C;  $R_f$  0.57 ( $\text{CH}_2\text{Cl}_2$ ); HPLC:  $R_T$  6.63 min (purity; 99.8%);  $^1\text{H-NMR}$  ( $\text{DMSO-d}_6$ , 400 MHz)  $\delta$  2.74 (dd,  $J = 4.8, 2.4$  Hz, 1H), 2.88 (dd,  $J = 4.8, 4.0$  Hz, 1H), 3.36-3.40 (m, 1H), 3.99 (dd,  $J = 15.6, 7.2$  Hz, 1H), 4.53 (dd,  $J = 15.2, 2.4$  Hz, 1H), 6.45 (d,  $J = 2.4$  Hz, 1H), 6.69 (d,  $J = 2.4$  Hz, 1H), 7.49 (ddd,  $J = 8.0, 8.0, 0.8$  Hz, 1H), 7.62 (dd,  $J = 8.0, 0.8$  Hz, 1H), 7.88 (ddd,  $J = 8.0, 8.0, 1.6$  Hz, 1H), 8.15 (dd,  $J = 8.0, 1.6$  Hz, 1H), 12.79 (s, 1H);  $^{13}\text{C-NMR}$  ( $\text{DMSO-d}_6$ , 100 MHz)  $\delta$  43.7, 49.3, 69.9, 93.4, 97.5,

103.3, 117.8, 119.8, 124.6, 125.3, 135.9, 155.5, 157.2, 162.6, 165.5, 180.2 ppm; HRMS-ESI (m/z) [M+H]<sup>+</sup> C<sub>16</sub>H<sub>13</sub>O<sub>5</sub> calcd 285.0757, found 285.0766.

### 1.1.3 (*S*)-3-(3-Chloro-2-hydroxypropoxy)-1-hydroxy-9*H*-xanthen-9-one (**56**)

A reaction mixture of compound **54** (0.05 g, 0.18 mmol) in aqueous 3M-HCl in ethyl acetate (6.7 mL) was stirred at room temperature for 30 min and the solvent was removed under reduced pressure. The residue was treated with ethyl ether and then solvent was removed. Finally, the residue was treated with ethylacetate and hexane and then solid was filtered and collected. After dry under vacuum compound **56** (0.05 g, 86.9%) was obtained as a weak brown solid. m.p. 148-152°C; R<sub>f</sub> 0.33 (ethyl acetate:*n*-hexane = 1:3); HPLC: R<sub>T</sub> 5.71 min (purity; 97.7%); <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 400 MHz) δ 3.68 (dd, *J* = 10.8, 5.6 Hz, 1H), 3.76 (dd, *J* = 11.2, 4.8 Hz, 1H), 4.06-4.09 (m, 1H), 4.11-4.18 (m, 1H), 5.66 (brs, 1H), 6.41 (d, *J* = 2.4 Hz, 1H), 6.65 (d, *J* = 2.0 Hz, 1H), 7.48 (ddd, *J* = 8.0, 8.0, 1.2 Hz, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.87 (ddd, *J* = 8.0, 8.0, 1.6 Hz, 1H), 8.14 (dd, *J* = 8.0, 1.6 Hz, 1H), 12.78 (s, 1H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 100 MHz) δ 46.3, 68.4, 69.9, 93.3, 97.5, 103.2, 117.7, 119.8, 124.6, 125.3, 135.9, 155.5, 157.3, 162.6, 165.7, 180.1 ppm; HRMS-ESI (m/z) [M+H]<sup>+</sup> C<sub>16</sub>H<sub>14</sub>ClO<sub>5</sub> calcd 321.0524, found 321.0523.

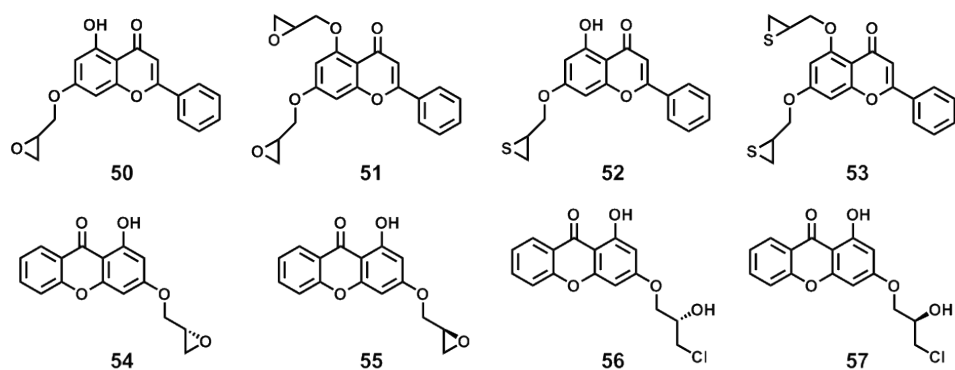
### 1.1.4 (*R*)-3-(3-Chloro-2-hydroxypropoxy)-1-hydroxy-9*H*-xanthen-9-one (**57**)

A reaction mixture of compound **55** (0.03 g, 0.11 mmol) in aqueous 3M-HCl in ethyl acetate (5.0 mL) was stirred at room temperature for 30 min and the solvent was removed under reduced pressure. The residue was treated with ethyl ether and then solvent was removed. Finally, the residue was treated with ethylacetate and hexane and then solid was filtered and collected. After dry under vacuum compound **57** (0.03 g, 88.8%) was obtained as a weak orange solid. m.p. 148-152°C; R<sub>f</sub> 0.33 (ethyl acetate:*n*-hexane = 1:3); HPLC: R<sub>T</sub> 5.75 min

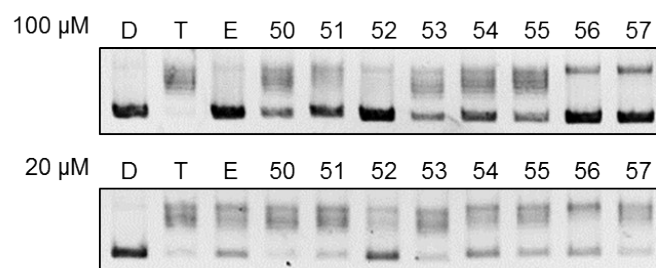
(purity; 99.1%);  $^1\text{H}$ -NMR (DMSO- $\text{d}_6$ , 400 MHz)  $\delta$  3.68 (dd,  $J$  = 11.2, 5.6 Hz, 1H), 3.76 (dd,  $J$  = 11.2, 4.8 Hz, 1H), 4.05-4.10 (m, 1H), 4.11-4.19 (m, 1H), 5.65 (d,  $J$  = 5.2 Hz, 1H), 6.43 (d,  $J$  = 2.0 Hz, 1H), 6.67 (d,  $J$  = 2.0 Hz, 1H), 7.49 (ddd,  $J$  = 8.0, 8.0, 1.2 Hz, 1H), 7.61 (dd,  $J$  = 8.0, 0.8 Hz, 1H), 7.88 (ddd,  $J$  = 8.0, 8.0, 1.6 Hz, 1H), 8.15 (dd,  $J$  = 8.0, 1.6 Hz, 1H), 12.79 (s, 1H);  $^{13}\text{C}$ -NMR (DMSO- $\text{d}_6$ , 100 MHz)  $\delta$  46.3, 68.4, 69.9, 93.4, 97.6, 103.3, 117.8, 119.9, 124.6, 125.3, 135.9, 155.5, 157.3, 162.6, 165.8, 180.2 ppm; HRMS-ESI ( $m/z$ )  $[\text{M}+\text{H}]^+$   $\text{C}_{16}\text{H}_{14}\text{ClO}_5$  calcd 321.0524, found 321.0526.

## 2. Supplementary Figures

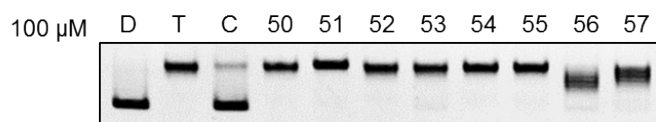
(A)



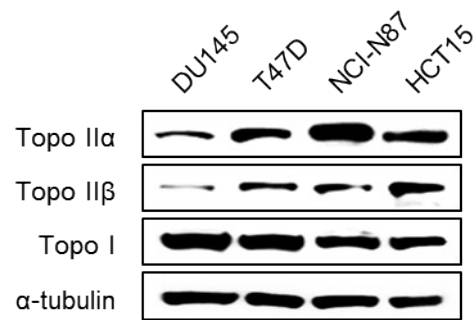
(B) Topo II $\alpha$  inhibitory activity



(C) Topo I inhibitory activity



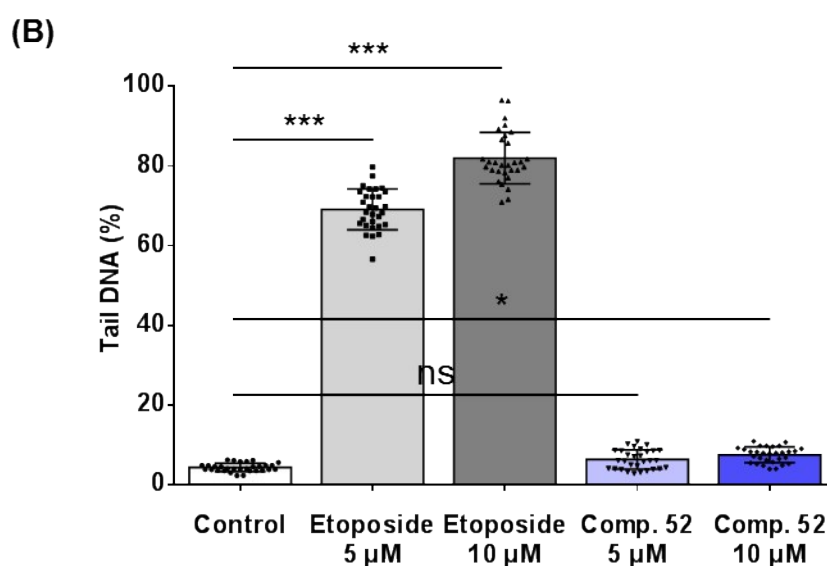
**Figure S1. (A)** Structures of synthesized and evaluated compounds (**50–57**). **(B)** Topo II $\alpha$  relaxation assay. Lane D: pBR322 DNA only; Lane T: pBR322 DNA + topo II $\alpha$ ; Lane E: pBR322 DNA + topo II $\alpha$  + Etoposide; Lane 50–57: pBR322 DNA + topo II $\alpha$  + compound **50–57**. **(C)** Topo I relaxation assay. Lane D: pBR322 DNA only; Lane T: pBR322 DNA + topo I; Lane C: pBR322 DNA + topo I + camptothecin; Lane 50–57: pBR322 DNA + topo I + compound **50–57**.



**Figure S2.** All of the cell lines commonly expressed topoisomerase I, IIα and IIβ. Notably, topo IIα was most highly expressed in NCI-N87 among the four cell lines.

**(A)**

Compound/ Treated concentration	Etoposide 250 $\mu$ M	ICRF-187 250 $\mu$ M	Compound <b>52</b>	
			250 $\mu$ M	500 $\mu$ M
% Inhibition of decatenation (mean $\pm$ STD)	87.9 $\pm$ 4.6	86.4 $\pm$ 3.4	41.1 $\pm$ 4.1	95.2 $\pm$ 1.1

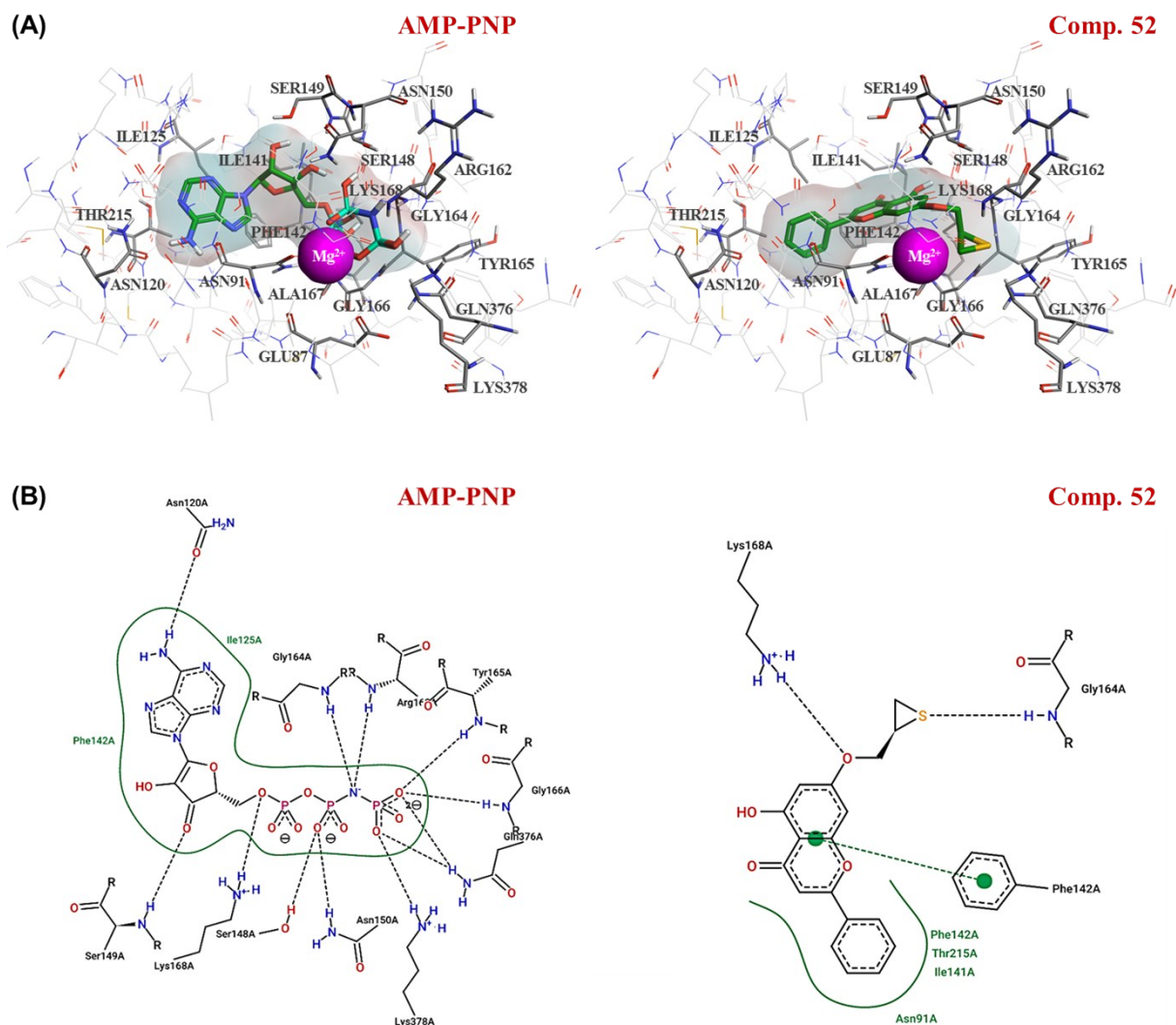


**(C)**

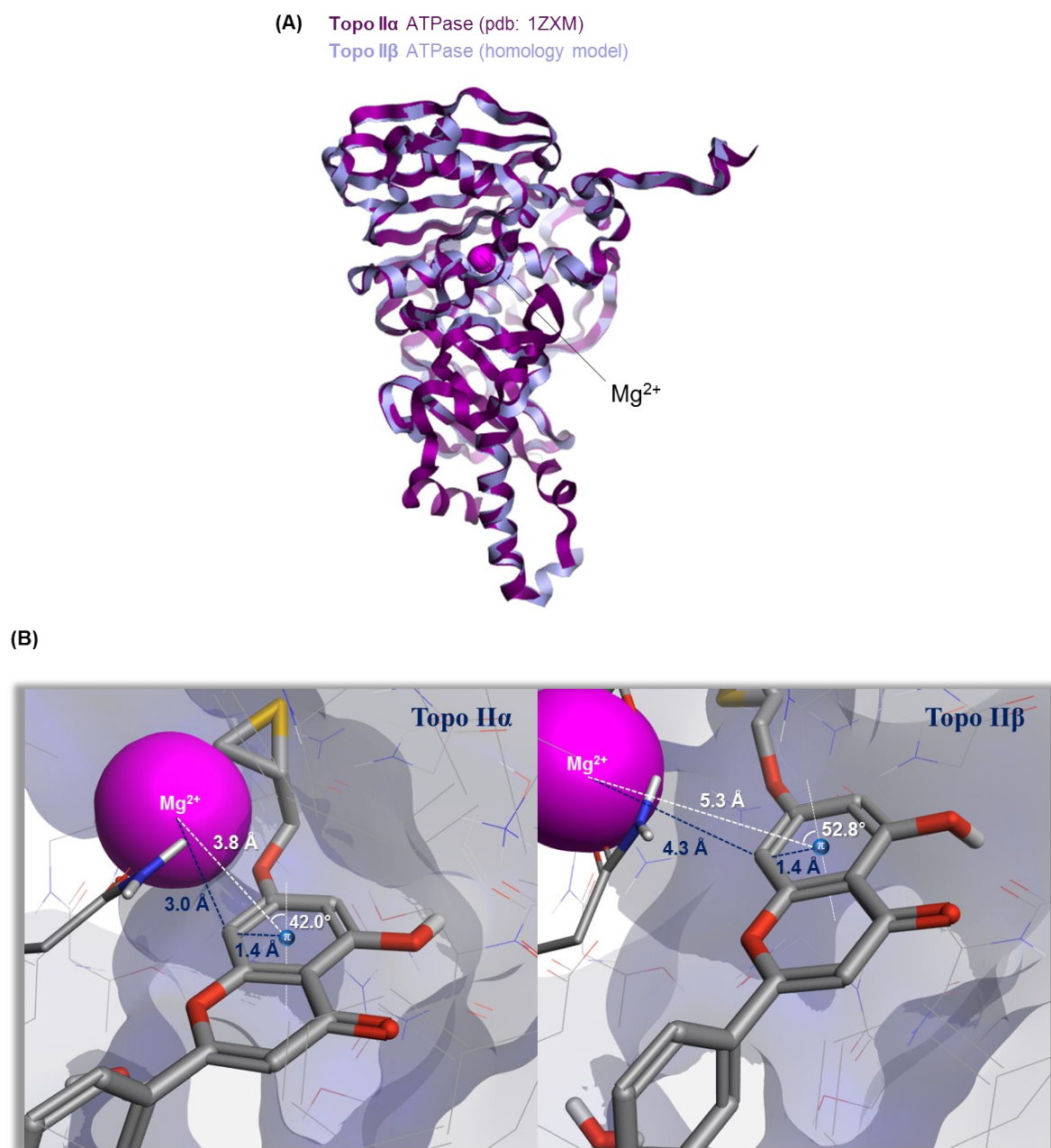
Human topo II $\alpha$ inhibition (%)				
ATP (mM)	Etoposide	Compound <b>52</b>		
	100 $\mu$ M	100 $\mu$ M	50 $\mu$ M	10 $\mu$ M
1.0	72.8	69.8	63.3	20.5
2.0	75.1	32.4	31.6	0

**Figure S3. (A)** Decatenation inhibitory activity (%) calculated from three different experiments of kDNA decatenation assay in **Figure 2A**. **(B)** Quantification of comet assay, included in **Figure 2C**. Extent of the comet tail generation was quantified by randomly selecting comet lengths of NCI-N87 cells (n=30) and displayed in vertical bar graph. **(C)** Quantification of ATP-concentration dependent topo II $\alpha$  relaxation assay, included in **Figure 3A**. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and ns; non-significant.

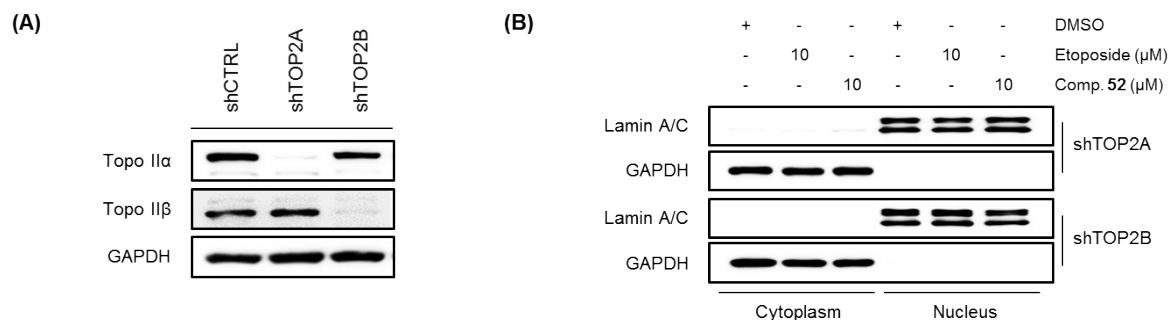




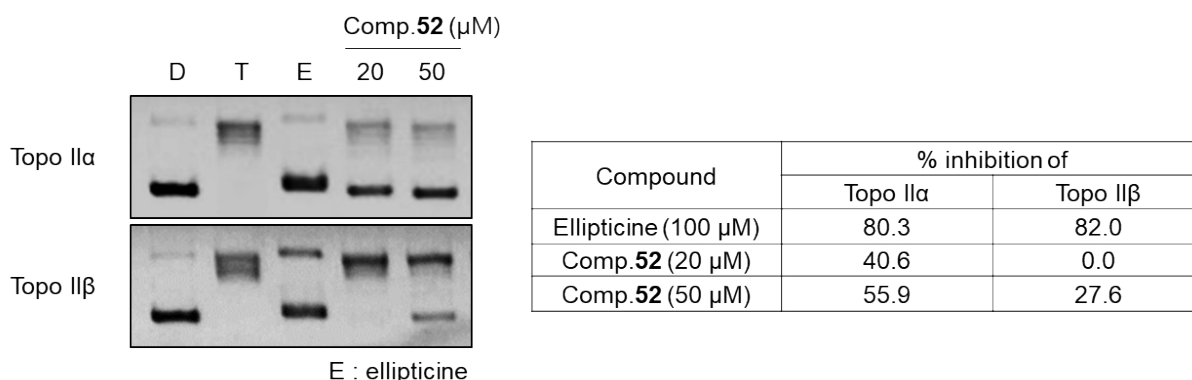
**Figure S4. (A)** Binding module of AMP-PNP and compound **52** to the ATP-binding site. Important residues involved in the interaction are represented in sticks. Magnesium is represented as magenta-colored sphere. **(B)** 2D interaction diagrams of the docking model from **Figure S4A** generated through PoseView (<https://proteins.plus>). Compound **52** also formed hydrogen bonds with Gly164 and Lys168 residues in the topo II $\alpha$  ATPase domain.



**Figure S5.** (A) Generated homology model of topo II $\beta$  overlapped with topo II $\alpha$ . Structure of the topo II $\alpha$  ATPase domain (pdb: 1ZXN) was used as the template. RMSD value between the two structures was calculated to be 1.023 Å. (B) Distance and angle formed between the Mg<sup>2+</sup> cation and the aromatic ring of compound **52** in topo II $\alpha$  (left) and II $\beta$  (right), respectively. Distance  $\leq 5.0$  Å and angle  $\leq 50^\circ$  were criteria for qualifying cation- $\pi$  interaction. According to the criteria, Mg<sup>2+</sup>-compound **52**  $\pi$  contact was only found in topo II $\alpha$ .



**Figure S6. (A)** shTOP2A and shTOP2B cells exhibited clear reduction of topo II $\alpha$  and II $\beta$  expression, respectively. To generate stable knockdown cell lines, shRNA transfected NCI-N87 cells were cultured with 1~3  $\mu$ g/mL of puromycin. **(B)** shTOP2A and shTOP2B NCI-N87 cells were treated with Etoposide or compound **52** for 24 h. Cytoplasmic and nuclear were then fractionated using NE-PER<sup>TM</sup> nuclear cytoplasmic extraction reagent. Fractionation efficiency was analysed by western blot analysis. GAPDH and Lamin A/C were used for the cytoplasmic and nuclear fraction markers, respectively.



**Figure S7.** Topo II $\alpha$  and II $\beta$  relaxation assay. Lane D: pBR322 DNA only; Lane T: pBR322 DNA + topo II $\alpha$  or topo II $\beta$ ; Lane E: pBR322 DNA + topo II $\alpha$  + Ellipticine 100  $\mu$ M; Lane 20 and 30: pBR322 DNA + topo II $\alpha$  + compound **52** 20 and 50  $\mu$ M, respectively. To assess the preference of compound **52** toward topo II $\alpha$ , DNA relaxation assay was performed using recombinant human topo II $\alpha$  and II $\beta$  enzymes (Inspiralis Limited) under the condition in which 100  $\mu$ M ellipticine (known as a DNA intercalating topo II inhibitor) inhibited both topo II $\alpha$  and II $\beta$  with similar intensity (% inhibition: 80.3% for topo II $\alpha$  and 82.0% for topo II $\beta$ ). Compound **52** at 20  $\mu$ M inhibited topo II $\alpha$  (40.6%) but not topo II $\beta$ , while the percent inhibition of compound **52** at 50  $\mu$ M was 55.9% for topo II $\alpha$  but 27.6% for topo II $\beta$ . This result is consistent with results presented in figures 1, 2A, 4A and 4B and corroborates compound **52** as a topo II $\alpha$ -specific inhibitor.

### 3. Supplementary table

Compound	%Inhibition of			$^{*}IC_{50}$ ( $\mu$ M)			
	Topo I	Topo II $\alpha$					
	100 $\mu$ M	100 $\mu$ M	20 $\mu$ M	DU145	T47D	NCI-N87	HCT15
Camptothecin	80.6	NT	NT	$0.34 \pm 0.01$	$0.15 \pm 0.04$	$0.13 \pm 0.05$	$0.10 \pm 0.06$
Etoposide	NT	76.1	27.5	$2.83 \pm 0.12$	$1.65 \pm 0.44$	$1.41 \pm 0.06$	$6.06 \pm 0.95$
Adriamycin	NT	NT	NT	$1.48 \pm 0.02$	$0.83 \pm 0.04$	$1.54 \pm 0.43$	$2.12 \pm 0.40$
50	0.0	31.9	9.0	$12.83 \pm 0.26$	$2.20 \pm 0.03$	$1.91 \pm 0.03$	$3.43 \pm 0.13$
51	1.0	52.6	6.9	$0.97 \pm 0.01$	$1.45 \pm 0.02$	$1.23 \pm 0.01$	$1.36 \pm 0.01$
<b>52</b>	<b>0.0</b>	<b>62.5</b>	<b>33.8</b>	<b>&gt;50</b>	<b>&gt;50</b>	<b><math>3.75 \pm 0.1</math></b>	<b>&gt;50</b>
53	0.0	34.8	7.5	$1.05 \pm 0.03$	$1.55 \pm 0.03$	$1.55 \pm 0.04$	$2.55 \pm 0.02$
54	0.0	36.5	19.7	$38.79 \pm 0.40$	$7.12 \pm 0.04$	$3.78 \pm 0.09$	$9.94 \pm 0.11$
55	0.0	33.7	13.3	$37.55 \pm 0.20$	$23.09 \pm 0.72$	$3.78 \pm 0.09$	$4.86 \pm 0.01$
56	0.0	75.1	21.5	$5.98 \pm 0.18$	$1.83 \pm 0.04$	$6.16 \pm 0.04$	$37.22 \pm 0.60$
57	0.0	72.2	7.8	$9.69 \pm 0.11$	$8.31 \pm 0.23$	$6.53 \pm 0.06$	>50

**Table S1.** Topo I and II $\alpha$  inhibitory and antiproliferative activities of compounds (**50-57**). NT: Not Tested; HCT-15: human colorectal adenocarcinoma; T47D: Human breast ductal carcinoma; DU145 (human prostate tumor cell line; NCI-N87: human gastric carcinoma cell line; Adriamycin: positive control for antiproliferative activity; Etoposide: positive control for topo II $\alpha$  and antiproliferative activity; Camptothecin: positive control for topo I and antiproliferative activity. \*Results are presented as the means  $\pm$  SDs of three independent experiments performed in triplicate.

## 4. Experimental Procedures

### Cell culture and cell viability assay

Cytotoxicity were evaluated using diverse cancer cell lines following the previous method <sup>1</sup>. HCT15 (human colon cancer cell line), T47D (human breast cancer cell line) DU145 (human prostate cancer cell line) and NCI-N87 (human gastric cancer cell line) were grown in RPMI1640 medium (Welgene, Korea) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, USA) and 1% penicillin-streptomycin (Thermo, USA) in a 5% CO<sub>2</sub> incubator at 37 °C. Diverse cancer cells were seeded with 10<sup>4</sup> cell/well in a 96-well cell culture plate and incubated for 24 h. On day 2, the existing medium was suctioned from each well and replaced with serum-free RPMI1640 medium. After 4 h incubation, each compound was diluted in serum-free medium and treated with designated concentrations for 72 hours. After 3 days-incubation, 5 µL of Ez-cytoX (Dogen, Korea) was added to each well for 1~4 hours and cell viability was measured by absorbance at 450 nm using Tecan Infinite M200 PRO Multi-Detection Microplate Reader (Tecan Group Ltd., Switzerland).

### *In vitro* DNA topoisomerase I and II $\alpha$ relaxation assay.

All the test compounds were dissolved in DMSO and the DNA topoisomerase I and II inhibitory activity of each compound was measured as follows according to previously reported methods <sup>2</sup>. Mixture comprising of 100 ng supercoiled pBR322 plasmid DNA (Thermo Scientific, USA) and 0.2~1 unit of recombinant human DNA topo I (TopoGEN INC., USA) or topo II $\alpha$  (USB Corp., USA) was incubated with or without the prepared compounds in the assay buffer (For topo I, 10 mM Tris-HCl (pH 7.9), 150 mM NaCl and 0.1% BSA, 0.1 mM spermidine and 5% glycerol; for topo II, 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM ATP and 15 mg/mL BSA) for 30 min at 37 °C. The reaction

with final volume of 10  $\mu$ L was stopped by adding the topo stop buffer (For topo I, 10% SDS solution containing 0.2% bromophenol blue, 0.2% xylene cyanol and 30% glycerol; for topo II, 7 mM EDTA). The reaction products were electrophoresed on 0.8% agarose gel at 50 V for 1 h with TAE electrophoresis buffer. The gels were stained in an EtBr solution (0.5 mg/mL) and visualized by transillumination with UV light and were quantitated using Alpha Tech Imager<sup>TM</sup> (Alpha Innotech Corp., USA).

### **kDNA decatenation assay**

The kinetoplast DNA (kDNA) decatenation assay was performed following the manufacturer's protocol and previously reported method <sup>3</sup>. kDNA (TopoGEN INC., USA) 75~100 ng was added in the topo II reaction buffer followed by addition of compound as designated concentration in figure legend. Reactions were initiated by the addition of 2~3 units of topo II $\alpha$ , kDNA and compound mixtures were incubated for 30 min at 37 °C. The reaction was terminated by the addition of topo II stop buffer followed by treatment with 2  $\mu$ L of proteinase K (Sigma, USA) at 55 °C for 30 min to eliminate the protein. Samples were resolved by electrophoresis on a 1.2% agarose gel containing 0.5  $\mu$ g/ml EtBr in TAE buffer. DNA bands were visualized as followed *in vitro* topoisomerase relaxation assay.

### **Alkaline comet assay**

To evaluate DNA damage, comet assay was performed using single-cell gel electrophoresis with a Comet Assay<sup>®</sup> Kit (Trevigen Inc., USA) according to the manufacturer's protocol and previously reported method <sup>2</sup>. NCI-N87 cells, seeded in a density of  $1 \times 10^5$  cells per well in 60 mm dishes, were treated with compound **52** or Etoposide for 24 hours and harvested by trypsinization followed by resuspending cells in 1 mL of ice-cold PBS. Then, 80  $\mu$ L of cells were mixed with LM agarose, solidified on the slides, lysed, electrophoresis under alkaline

conditions and stained with SYBR Gold (Thermo, USA). The comet images were obtained using an inverted fluorescence microscope (Zeiss, Axiovert 200) at 10× magnification and percent tail DNA was analyzed by Komet™ 5.0 software (kinetic imaging Ltd, UK). The proportion of the comet tails were quantified by randomly selecting comet lengths of NCI-N87 cells and representative images were displayed.

#### **DNA topo II $\alpha$ cleavable complex assay.**

According to the previously reported method <sup>4</sup> with minor modification, 100 ng supercoiled DNA pBR322 (Thermo, USA) was incubated with 2~3 units of topo I $\alpha$  enzyme (USB Corp., USA) at 37 °C for 30 min and the reaction was stopped by topo II stop buffer followed by digestion with proteinase K (Sigma-Aldrich, USA) at 45 °C for 30 min. After addition of 6X gel loading dye (New England Biolabs, UK), the samples were electrophoresed with a 1.5% agarose gel containing 0.5  $\mu$ g/mL EtBr in TAE buffer. The DNA band was visualized and measured following the same method of *in vitro* topoisomerase relaxation assay.

#### **Band depletion assay and Western blot analysis**

The cleavage complex of topoisomerase II $\alpha$  with DNA in cells were evaluated with Etoposide and compound **52** according to the previously reported method <sup>5</sup>. NCI-N87 cells were seeded in 6-well plate in a density of  $2 \times 10^5$  cells. On Day 2, cells were treated with 50  $\mu$ M compound for 2 hr. Then cells were harvested by trypsinization and suspended in 1 mL of phosphate-buffered saline (PBS) spiked with 50  $\mu$ M each compound to prevent dissociation of the DNA-Topo II cleavable complex. The suspended cells were centrifuged and lysed using denaturing buffer (62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA and 2% SDS) and sonicated with 20% amplification for 2 sec. Total protein amounts were normalized using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA). The samples were mixed with 2X SDS loading



buffer (1 M Tris-HCl (pH 6.8), 10% SDS, 0.04% bromophenol blue, 20% glycerol, 10%  $\beta$ -mercaptoethanol) and separated by SDS-PAGE. The protein bands were detected using ECL solution reagent (GE Healthcare, USA) and LAS-3000 (Fuji Photo Film Co., Ltd., Japan). Band images were analyzed with Multi-Gauge Software (Fuji Photo Film Co. Ltd.). All indicated primary antibodies were purchased from Cell Signaling Technology.

#### **Competitive EtBr displacement assay.**

Competitive EtBr displacement assay was carried out according to previously reported methods<sup>2, 6</sup>. ctDNA (30 mM) was pre-mixed with EtBr (20 mM) in the 10 mM tris buffer solution (pH 7.2), followed by shaking incubation for 30 min at RT. And then, 10 to 40  $\mu$ M concentration of compounds (m-AMSA and compound **52**) were added to the 96 well white plate (Nunc<sup>TM</sup>, Denmark) containing pre-incubated EtBr, ctDNA and tris buffer mixture. After addition of the compounds, plate was continuously incubated for 30 min at room temperature with shaking. The fluorescence intensity of EtBr-ctDNA was measured by Tecan Infinite M200 PRO Multi-Detection Microplate Reader (Tecan Group Ltd., Switzerland) with excitation at 471 nm and the emission spectrum was collected at 500 to 700 nm.

#### **Cell topoisomerase inhibition assay.**

To assess the ability of compounds to inhibit the function of topo activity in topo II $\alpha$  and II $\beta$  knockdown NCI-N87 cells were treated at designated concentrations in Figures 6 for 24 h and harvested. The topo II knockdown NCI-87 cells, shTOP2A and shTOP2B, were prepared using topo II $\alpha$ -targeting lentivirus-mediated small hairpin RNA (shRNA) interference (SHCLING-NM\_001067, Sigma, USA) and topo II $\beta$ -targeting shRNA lentiviral Particles (Santa Cruz, USA) with puromycin selection for at least 2 weeks. The extent of protein levels of topo II $\alpha$  and II $\beta$  was monitored with western blot analysis. After a 24 h treatment of compounds with

cells, nuclear lysate of each cell line was prepared according to the protocol provided by the manufacturer using the NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo scientific, USA). The protein

concentration in each nuclear extract was measured by Bradford assay (Thermo, USA). The topoisomerase reaction was performed with 10-20 ng of nuclear extract, 100 ng of pBR322 plasmid as a substrate, and topo II reaction buffer. Cell topoisomerase inhibitory activity was determined following the same method of *in vitro* topoisomerase relaxation assay.

### ***In silico docking study.***

The ATP-binding domain of human topo II $\alpha$  coordinates were retrieved from the Protein Data Bank (PDB: 1ZXN) and prepared through Sybyl-X 2.1.1. software (Tripos Associates Inc.). Waters were removed and the hydrogen atoms were added to the original crystal structure. The magnesium ion was assigned a charge of +0.9<sup>7</sup>. 3D structure file for compound **52** was also generated through Sybyl-X 2.1.1. and further minimized and optimized via Cresset Flare™ software V2.0<sup>8</sup> before the docking process. To perform docking study, the energy grid was defined based on the clustered internal AMP-PNP ligand from the topo II $\alpha$  ATPase domain. Docking was further carried out in accurate mode within default settings. Results were demonstrated in optimized docking poses and in four distinct scoring functions: Rank score, dG score, virtual screening (VS) score and electrostatic complementarity score. PoseView (<https://proteins.plus>) was further utilized to visualize the binding module in 2D.

### **Homology modeling of topo II $\beta$ ATPase domain.**

Homology model of topo II $\beta$  ATPase domain was generated by ORCHESTRAR in the Sybyl-X 2.1.1 software (Tripos Associates Inc.) using crystal structure of human topo II $\alpha$  ATP-binding domain (PDB: 1ZXN) as template. Region of amino acid residues 35-367 of topo II $\beta$

which showed maximum identity in sequence alignment was used to build the 3D protein model. Two essential water molecules and the magnesium ion were defined as conserved region in the modeling process. Generated final model was minimized energetically using a Tripos force field with Gasteiger-Huckel charges.

### **Statistical Analysis.**

All data are expressed as the mean  $\pm$  standard deviation, with each experiment performed in triplicate. Comparison of the differences was conducted with analysis of variance (ANOVA) or Student's t-test using the Prism statistical software package (GraphPad<sup>TM</sup> Software, USA). The differences were considered statistically significant when the p value was  $* < 0.05$ ,  $** < 0.01$  and  $*** < 0.001$ .

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