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Induction of apoptosis in colon cancer cells by a novel topoisomerase I inhibitor TopIn

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ABSTRACT

The tumor suppressor p53 plays an important role in cellular emergency mechanisms through regulating the genes involved in cell cycle arrest and apoptosis. To identify small molecules that can activate p53-responsive transcription, we performed chemical screening using genetically engineered HCT116 reporter cells. We found that TopIn (7-phenyl-6H-[1,2,5]oxadiazolo[3,4-e]indole 3-oxide) efficiently activated p53-mediated transcriptional activity and induced phosphorylation of p53 at Ser15, thereby stabilizing the p53 protein. Furthermore, TopIn upregulated the expression of $p21^{WAF1/CIP1}$, a downstream target of p53, and suppressed cellular proliferation in various colon cancer cells. Additionally, TopIn induced DNA fragmentation, caspase-3/7 activation and poly ADP ribose polymerase cleavage, typical biochemical markers of apoptosis, in p53 wild-type and mutated colon cancer cells. Finally, we found that TopIn inhibited topoisomerase I activity, but not topoisomerase II, *in vitro* and induced the formation of the topoisomerase I-DNA complex in HCT116 colon cancer cells. Unlike camptothecin (CPT) and its derivative SN38, TopIn did not affect the activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP) or multidrug-resistant protein-1 (MDR-1). These results suggest that TopIn may present a promising new topoisomerase I-targeting anti-tumor therapeutics.

1. Introduction

The tumor suppressor p53 plays a key role in the cellular protection to genotoxic stress and is the most frequently mutated protein in cancer cells [1,2]. A lack of p53 function is accompanied by a high rate of genomic instability, rapid tumor progression, resistance to anti-cancer therapy, and increased angiogenesis [3,4]. In response to DNA damage, p53 rapidly accumulates through attenuated proteolysis, leading to increased p53-mediated transcriptional activity [5,6]. Activated p53 up-regulates target genes involved in cell cycle arrest and/or apoptosis, leading to suppression of malignant transformation and the maintenance of genomic integrity [7,8].

Human topoisomerase I is a monomeric nuclear protein of 91 kDa, encoded by a gene located on chromosome 20 [9], that is involved in numerous cellular processes, including DNA replication, transcription, recombination, DNA repair, and chromatin assembly [10]. Topoisomerase I catalyzes the unwinding of supercoiled DNA by creating transient single strand-breaks. During this catalytic process, topoisomerase I forms a phosphotyrosine covalent bond at the 3′ end of the nicked DNA (topoisomerase I-DNA cleavable complex), followed by passage of the intact strand through the break, and re-ligating the nicked DNA, thereby relieving, by one turn, the torsional constraint [11,12]. Expression of topoisomerase I is associated with tumor growth, tumor differentiation, and a poor prognosis for survival in colon cancers [13,14]. As levels of topoisomerase I are frequently elevated in colorectal cancer compared with healthy colonic tissue [15,16], this may confer tumor specificity to TopoI inhibitor-mediated anti-cancer strategies.

In the present study, using cell-based synthetic chemical screening, we identified TopIn as an activator of the p53 pathway. We further demonstrate that TopIn suppressed the proliferation of colon cancer cells and induced apoptosis through the inhibition of topoisomerase I activity.

2. Materials and methods

2.1. Chemicals

Doxorubicin was purchased from Sigma (St. Louis, MO) and dissolved in Me_2SO. TopIn was synthesized by ChemBridge and dissolved in Me_2SO.

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2.2. Cell culture

Cell lines were purchased from the American Type Culture Collection. RKO, IMR90, and WI-38 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 120 μg/mL penicillin, and 200 μg/mL streptomycin. SW480 cells were cultured in Leibovitz's L-15 supplemented with 10% FBS, 120 μg/mL penicillin, and 200 μg/mL streptomycin. HCT116 and HT29 cells were maintained in Macoy's 5a supplemented with 10% FBS, 120 μg/mL penicillin, and 200 μg/mL streptomycin. HCT15 and DLD-1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 120 μg/mL penicillin, and 200 μg/mL streptomycin.

2.3. Transfection of plasmid DNA and luciferase assays

Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase assays were performed using the Dual luciferase assay kit (Promega).

2.4. Caspase assays

The activity of caspases-3/7 was determined in cell lysates using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega).

2.5. Screening for small molecule activators of the p53 pathway

HCT116-p53-FL reporter cells were established by selecting HCT116 cells transfected with a synthetic p53-dependent firefly luciferase reporter with 1 mg/mL G418. The cells were then inoculated into 96-well plates at a density of 15,000 cells per well, and grown for 24 h. The cells were incubated with synthetic compounds from ChemBridge at a final concentration of 20 μM for 15 h and the plates assayed for firefly luciferase activity and cell viability.

2.6. Western blotting

To analyze protein expression, lysates were resolved by SDS–PAGE (Invitrogen), transferred to a PVDF membrane (Amersham Bioscience), using a wet-blotting transfer apparatus, and blocked in 5% nonfat milk in TBS-T (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Membranes were then probed with anti-p53 (Santa Cruz Biotechnology), anti-phospho-p53 (Ser15) (Santa Cruz Biotechnology), anti-$p21^{WAF1/CIP1}$ (Santa Cruz Biotechnology), anti-PARP (Cell Signaling Technology), anti-topoisomerase 1 (Topogen Inc.), and anti-actin (Cell Signaling Technology) antibodies. Membranes were then labeled with HRP-conjugated anti-mouse IgG (Amersham Biosciences). Western blot signals were visualized using the ECL system (Amersham Biosciences).

2.7. Cell viability assays

Cells were seeded into 96-well plates and treated with TopIn for 2 days. Cell viability was determined using an MTT-based cell viability assay kit (Roche) according to manufacturer's protocol.

2.8. DNA fragmentation assays

HCT116 and HCT15 cells were exposed to TopIn for 24 h. The cells were then suspended in 250 μL TE buffer (pH 7.5) containing 1 mg/mL RNase A (Qiagen, Hilden, Germany). Cells were lysed by the addition of an equal volume of 1.2% SDS and gentle mixed by inversion. Following a 5-min incubation, 350 μL of a CsCl-containing precipitation solution (3 M CsCl, 1 M potassium acetate, 0.67 M acetic acid) was added, the samples were incubated on ice for 15 min, and spun (14,000*g*) at room temperature. The supernatant (700 μL) was then transferred to a miniprep spin column (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany). The spin columns were spun (1 min, 14,000*g*), washed with wash buffer, respun, and the DNA was then eluted with 50 μL TE (pH 8.0). DNA fragmentation was assessed by 1.5% agarose gel electrophoresis.

2.9. Topoisomerase assays

Human topoisomerase I and II were obtained from Sigma (St. Louis, MO). Topo I and Topo II assay kits were purchased from Topogen Inc. (Columbus, Ohio). Assays were performed according to the manufacturer's protocol.

2.10. Band depletion assays

Treated cells were lysed in 100 μL of alkaline lysis solution (200 mM NaOH, 2 mM EDTA) for 30 min on ice. The lysate was neutralized by the addition of 8 μL of 1.2 M Tris (pH 8.0) and 8 μL of 2 M HCl. The neutralized lysate was mixed with 13.2 μL of 10× S7 nuclease reaction buffer (50 mM $MgCl_2$, 50 mM $CaCl_2$, 5 mM DTT, 1 mM EDTA, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 50 μg/mL pepstatin A, 1 mM PMSF) and 60 units of staphylococcal S7 nuclease. After 20 min of nuclease digestion on ice, 4× LDS sample buffer was added to each sample. The lysates were resolved by SDS–PAGE and probed with anti-topoisomerase I antibody.

2.11. Intracellular calcein and estrone-3-sulfate accumulation assays

Mardin-Darby canine kidney II (MDCKII)-MDR1 and BCRP cells [17,18] were seeded in 96-well plates (10^4 cells/well). Once the cells had reached 95% confluency, the growth medium was discarded and the cells were washed and pre-incubated with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 20 mM glucose, 9 mM sodium bicarbonate, and 25 mM Hepes for 30 min at 37 °C. Uptake of calcein-AM (0.5 μM) and [^{3}H] estrone-3-sulfate (0.1 μM) was initiated through the addition of irinotecan, SN38, and TopIn (1, 10, and 100 μM), followed by incubation at 37 °C for 30 min. The cells were then placed on ice and washed with ice-cold DPBS. The cells were then lysed in 250 μL of 1% Triton X-100 (Sigma Aldrich) in PBS for 1 h. Cell lysates (200 μL) were transferred to 96-well plates, and the fluorescence due to intracellular calcein was measured using a Victor3 plate reader (Perkin-Elmer LAS, Waltham, MA) with excitation and emission wavelengths of 485 and 535 nm, respectively. The radioactivity of [^{3}H] estrone-3-sulfate in the cells was measured using a liquid scintillation counter (MicroBeta TriLux, PerkinElmer). A Bradford protein assay kit (Bio-Rad) was used to determine the cellular protein content of each lysate. Cyclosporin A (CsA) and fumitremorgin C (FTC), inhibitors of P-gp and BCRP [19,20], respectively, were used as positive controls.

3. Results and discussion

3.1. Identification of TopIn as an activator of the p53 pathway

To identify cell-permeable synthetic compound activators of the p53 signaling pathway, we used a cell-based screening system. HCT116-p53-FL reporter cells that stably harbored a synthetic p53-dependent luciferase reporter plasmid were grown in 96-well plates. Following the addition of each compound, p53-dependent reporter activity was assessed (Fig. 1A). One of the compounds from this screen was 7-phenyl-6H-[1,2,5]oxadiazolo[3,4-e]indole 3-oxide (TopIn; Fig. 1B). Treatment of HCT116-p53 reporter cells with different concentrations of TopIn consistently produced a dose-dependent increase in p53 responsive transcription

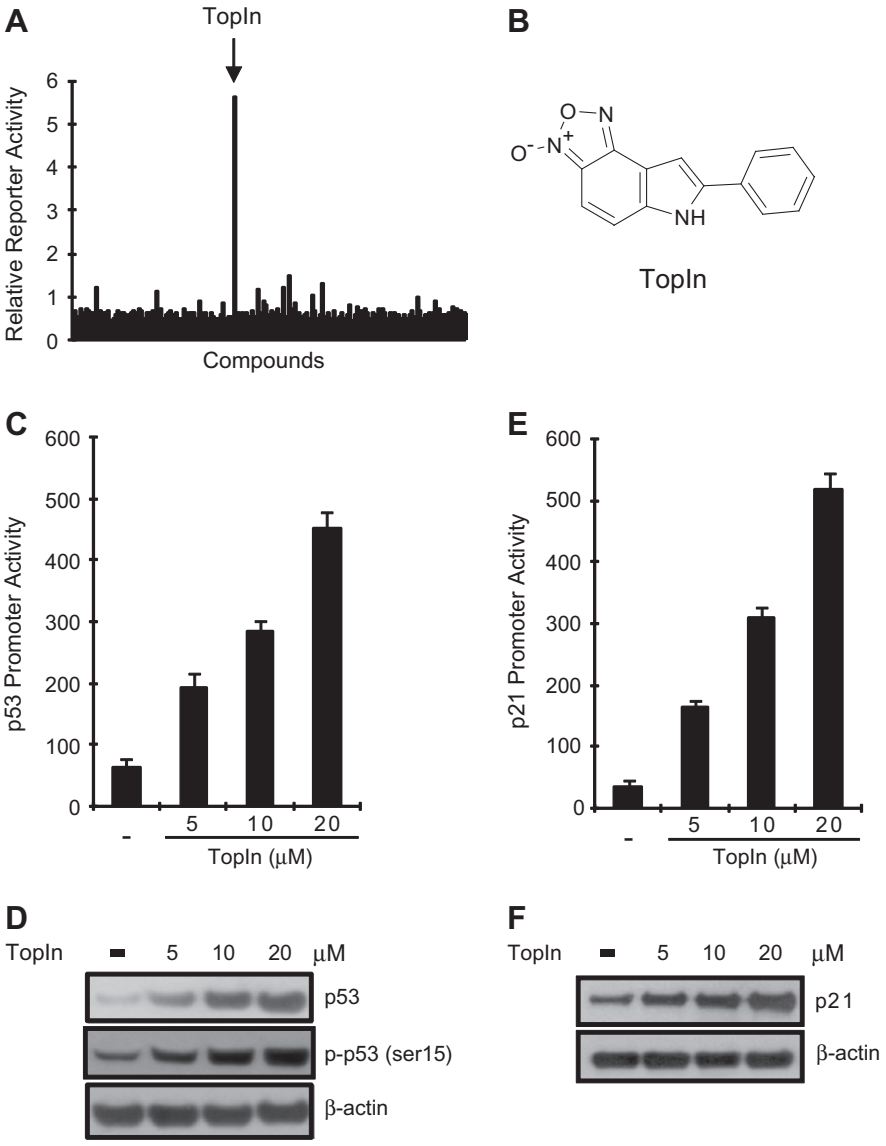

Fig. 1. Identification of TopIn as an activator of the p53 pathway. (A) Screening of small molecules that activate the p53 pathway. Compounds modulating p53-dependent reporter activity were screened using HCT116-p53-FL reporter cells. (B) Chemical structure of TopIn. (C) Dose-dependent increases in p53-dependent reporter activity by TopIn. HCT116-p53-FL reporter cells were incubated with the indicated concentrations of TopIn and luciferase activity was determined following a 15-h incubation. (D) Cell extracts from HCT116 cells treated with either vehicle (DMSO) or the indicated concentration of TopIn were subjected to Western blot analysis with anti-p53 and anti-phospho-p53 (Ser15) antibodies. (E) HCT116 cells were co-transfected with $p21^{WAF1/CIP1}$-FL and pCMV-RL, and incubated with the indicated concentrations of TopIn for 15 h. Luciferase activity was measured 39 h following transfection. The $p21^{WAF1/CIP1}$ promoter activity was assessed in relative light units (RLU), normalized to the constitutive *Renilla* luciferase activity. (F) Cell extracts from HCT116 cells treated with either vehicle (DMSO) or TopIn where analyzed by Western blotting with $p21^{WAF1/CIP1}$ antibodies. In (C) and (E), the results are the average of three experiments ± SD. In (D) and (F), the blots were re-probed with anti-actin antibodies as a loading control.

(Fig. 1C). Because p53 transcription is dependent on the levels of intracellular p53, a process regulated by ubiquitin-mediated proteasome degradation, we sought to examine the effects of TopIn on p53 stabilization. As shown in Fig. 1D, p53 accumulated in cells following treatment of HCT116 colon cancer cells with TopIn, consistent with the p53-dependent reporter activity. We also observed TopIn-mediated induction of p53 in RKO and MCF-7 cells (data not shown).

p53 is phosphorylated at Ser15, which leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2 [21]. MDM2 inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation. We thus sought to determine the effects of TopIn on Ser15 phosphorylation. Western blot analysis using phospho-specific p53 antibodies demonstrated that the phosphorylation of p53 at Ser15 was induced by incubation of HCT116 cells with TopIn (Fig. 1D) suggesting this to be the mechanism of TopIn-induced p53 accumulation and activation.

We further examined the effects of TopIn on the expression of p53 downstream genes in HCT116 cells. Cells transfected with a reporter construct containing the $p21^{WAF1/CIP1}$ promoter, which harbors a p53 responsive region were treated with TopIn and promoter activity was assessed using the luciferase assay. As shown in Fig. 1E, $p21^{WAF1/CIP1}$ promoter activity was upregulated following treatment with TopIn. Consistent with the reporter assays, we observed increases in the protein levels of $p21^{WAF1/CIP1}$ in response to TopIn (Fig. 1F). Taken together, these results suggest that TopIn is an activator of the p53 pathway.

3.2. TopIn inhibits cellular proliferation and induces apoptosis in colon cancer cells

Previous studies have demonstrated that p53, which is accumulated as a result of anticancer drugs, such as adriamycin and camptothecin (CPT), inhibits the growth of cancer cells [22]. As TopIn

induced the accumulation of p53, we hypothesized that TopIn may also suppress cancer cell proliferation. We thus evaluated the effects of TopIn on the growth of various cancer cell lines. As shown in Fig. 2A, TopIn efficiently inhibited the growth of human cancer cells with IC_{50} values ranging from 0.5 to 4.39 μM. Interestingly, compared with cancer cells, TopIn displayed lower cytotoxicity in WI-38 (IC_{50} = 20 μM) and IMR-90 (IC_{50} = 15 μM) cells, which are non-transformed cells (Fig. 2A). These results suggest that TopIn preferentially inhibited the growth of cancer cell lines.

We next investigated the ability of TopIn to induce apoptosis in HCT116 and HCT15 cells, which contain wild type and mutant p53, respectively. Treatment of these cells with TopIn led to significant inter-nucleosomal DNA fragmentation, in a dose-dependent manner (Fig. 2B). Additionally, TopIn increased the proteolytic cleavage of poly ADP ribose polymerase (PARP), a biochemical marker of apoptosis (Fig. 2C). TopIn also increased the activity of caspases-3/7 in HCT116 and HCT15 cells, consistent with an induction of apoptosis (Fig. 2D).

3.3. Identification of topoisomerase I as a molecular target of TopIn

Previous studies have demonstrated that p53 is accumulated by DNA-damaging agents, including DNA intercalation, inhibition of topoisomerase I and II, and DNA cross-linking [23]. Because DNA intercalating agents inhibit topoisomerase II activity, we performed *in vitro* topoisomerase II assays using catenated DNA as a substrate. While etoposide, a known topoisomerase II inhibitor, inhibited the formation of decatenated DNA at a concentration of 100 μM, TopIn had no effect on topoisomerase II activity at the same concentration (Fig. 3A). We next investigated whether TopIn

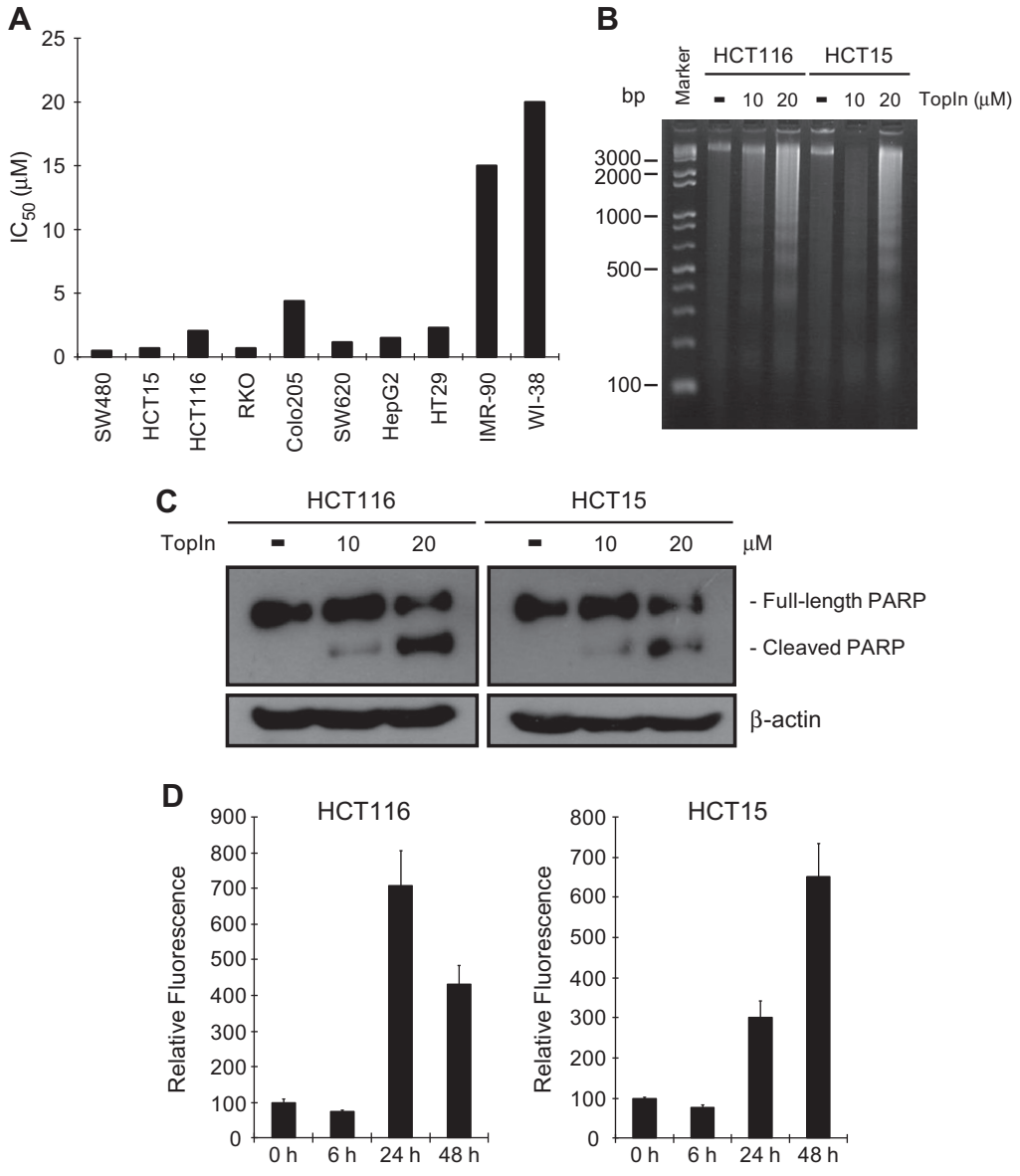

Fig. 2. TopIn induces apoptosis in colon cancer cells. (A) Effects of TopIn on cell viability. Cells were incubated with the indicated concentrations of TopIn for 48 h. Cell viability was examined using the MTT assay. The IC_{50} values were calculated with the *WinNonlin* Professional software (Pharsight Corporation). (B) Cells were treated with the indicated concentrations of TopIn for 24 h. Fragmented DNA was extracted and separated by 1.5% agarose gel electrophoresis. (C) Cell extracts from HCT116 and HCT15 cells treated with either vehicle (DMSO) or the indicated concentration of TopIn for 24 h, were analyzed by Western blotting with anti-PARP antibodies. The blots were re-probed with anti-actin antibodies as a loading control. (D) Cells were treated with 10 μM of TopIn and the activity of caspases 3/7 determined with the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega).

inhibited topoisomerase I activity. Supercoiled DNA was incubated with TopIn in the presence or absence of topoisomerase I and DNA topology was analyzed by agarose gel electrophoresis. As shown in Fig. 3B, supercoiled DNA was converted into nicked circular DNA in the presence of increasing concentrations of TopIn (Fig. 3B).

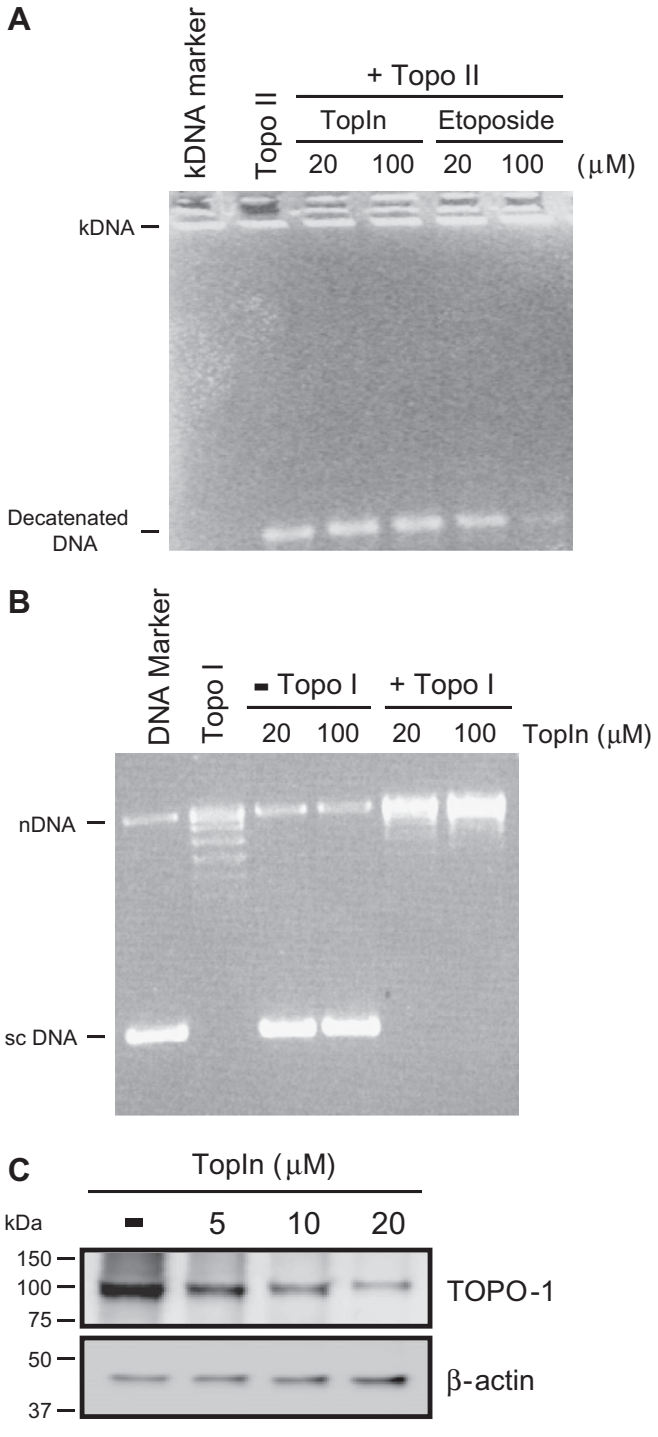

Fig. 3. TopIn inhibits the activity of topoisomerase I. (A) The effects of TopIn on topoisomerase II. Kinetoplast network DNA was incubated with the indicated concentrations of TopIn and etoposide in the presence of topoisomerase II. kDNA, catenated kinetoplase DNA. (B) Effect of TopIn on topoisomerase I activity *in vitro*. Supercoiled plasmid DNA (SC) was incubated with the indicated concentrations of TopIn in the presence or absence of topoisomerase I. nDNA, nicked DNA; scDNA, supercoiled DNA. (C) HCT116 cells were treated with TopIn for 24 h and then cell extracts were prepared for Western blotting with anti-TOPO-1 antibodies. To confirm equal loading, the blots were re-probed with anti-actin antibody.

Topoisomerase I forms transient covalent complexes with DNA, and treatment with topoisomerase I inhibitors stabilizes this complex by preventing the re-ligation step of the topoisomerase I catalytic cycle without affecting the DNA cleavage reaction [24]. When topoisomerase I is covalently bound to DNA, it migrates more slowly than free topoisomerase I on SDS–PAGE, resulting in a decrease in free topoisomerase I. We thus examined whether TopIn reduced the levels of free topoisomerase I. As shown in Fig. 3C, the levels of free topoisomerase I were markedly decreased in the presence of TopIn in HCT116 cells. These results indicate that TopIn inhibits topoisomerase I activity apparently via stabilizing the cleavage complex between the DNA and topoisomerase I.

3.4. TopIn does not modulate the activity of BCRP or MDR1

Previous studies have demonstrated that CPT and its derivatives modulate the activity of the ABC transporter proteins BCRP and MDR-1 [25,26]. To evaluate the functionality of BCRP, the inhibitory effect of fumitremorgin (FTC), a known inhibitor of BCRP, on the BCRP-mediated efflux of estrone-3-sulfate was measured. FTC significantly inhibited estrone-3-sulfate efflux and increased the cellular accumulation of estrone-3-sulfate, by 4.5-fold, in MDCKII-BCRP cells. Additionally, consistent with previous results

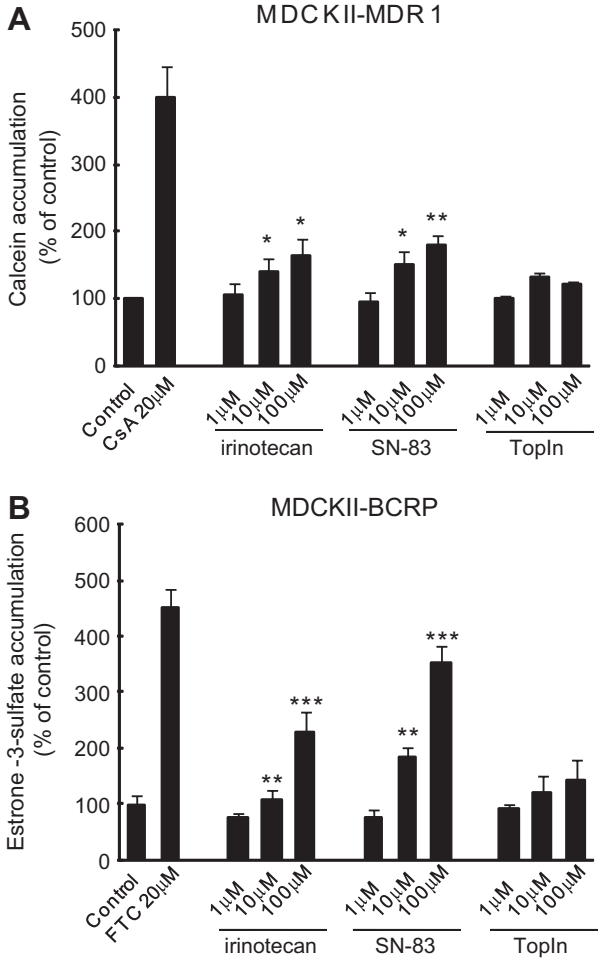

Fig. 4. TopIn does not affect the activity of ABC transporters. (A) Cellular accumulation of 0.1 μM estrone-3-sulfate was assessed following incubation for 30 min in the presence of FTC (20 μM each), irinotecan, SN-38, and TopIn (1, 10, 100 μM) in MDCKII- BCRP cells. (B) Cellular accumulation of 0.5 μM calcein-AM was assessed following incubation for 30 min in the presence of CsA (20 μM), irinotecan, SN-38, and TopIn (1, 10, 100 μM) in MDCKII-MDR1 cells. Each data point represents the mean ± S.D. of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Statistical analyses were performed using an unpaired *t*-test.

[25,26], irinotecan, a derivative of CPT, and its active metabolite, SN38, significantly inhibited BCRP-mediated estrone-3-sulfate efflux, by 2.3- and 3.5-fold, at 100 μM, respectively. However, when the effects of TopIn were assessed, no modulation of the cellular accumulation of estrone-3-sulfate was evident (Fig. 4A). We also examined the effects of TopIn on the activity of the ABC transporter MDR-1. As shown in Fig. 4B, moderate inhibition of MDR1 activity was evident in the presence of irinotecan and SN38, by 1.6- and 1.9-fold, at 100 μM respectively, while the intracellular accumulation of calcein, a substrate of MDR-1, remained unchanged in the presence of TopIn. These results suggest that Topin, unlike CPT, does not affect BCRP or MDR-1 activity.

Topoisomerase I inhibitors, such as CPT and its derivatives, have been widely used as cancer chemotherapeutics. Irinotecan exhibits a broad spectrum of activity against advanced lung, ovarian, and colorectal cancers [27,28], while SN38 displays a 100- to 1000-fold greater cytotoxicity than the parent compound [29]. Mechanistic studies have demonstrated that CPT and its derivatives inhibit topoisomerase I activity by stabilizing a covalent topoisomerase I-DNA complex [24]. During DNA replication, an advancing replication fork is arrested through its collision with a covalent topoisomerase I-DNA complex, leading to formation of a toxic DNA double-strand break, conferring S phase-specific cytotoxicity [30]. Additionally, this collision has been suggested to be responsible for G2-M cell cycle arrest [31] and the generation of many DNA damage signals, including the activation of "ataxia telangiectasia-mutated (ATM)/ataxia telangiectasia and Rad3-related" (ATR), DNA protein kinase activation, p53 phosphorylation and stabilization, H2AX phosphorylation, and nuclear factor κB activation [32]. In this study, we present several lines of evidence suggesting that topoisomerase I is a molecular target of TopIn. First, similar to other topoisomerase I inhibitors, TopIn induced the accumulation of p53 and activated the expression of its target gene, $p21^{WAF1/CIP1}$, thereby inhibiting cellular proliferation and promoting apoptosis in colon cancer cells. Second, TopIn, like CPT, induced topoisomerase I-mediated DNA breaks *in vitro*. Finally, band depletion assays demonstrated that TopIn reduced the levels of free topoisomerase I in HCT116 colon cancer cells, indicating that TopIn is able to trap reversible topoisomerase complexes.

CPT and its derivatives have limitations in clinical use because of toxic side effects, such as myelosuppression and diarrhea [33,34]. Thus, non-CPT topoisomerase I inhibitors are of great interest in overcoming the limitations of CPT and its derivatives. In this study, we identified a novel topoisomerase I inhibitor (TopIn) that displayed different chemical structure to CPT and its derivatives. CPT is a known substrate for BCRP and MDR-1 [35,36]. Interestingly, TopIn inhibited the cell growth of MDR-1-positive HCT15 cells (IC_{50} = 0.69 μM) in comparison with MDR-1-negative HCT116 cells (IC_{50} = 2.06 μM), suggesting that the potency of TopIn is independent of the MDR-1 levels, a serious problem in anti-cancer therapeutics.

In conclusion, through cell-based small molecule screening, we identified TopIn as an inhibitor of topoisomerase I. TopIn induced the stabilization of p53 and activated the expression of its target gene, $p21^{WAF1/CIP1}$, thereby suppressing the proliferation of colon cancer cells. In view of the different chemical scaffold from CPT and its derivatives, TopIn may potentially be developed into a therapeutic agent for the treatment of colon cancer.

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