

# Induction of Radiosensitization by Indolocarbazole Derivatives: The Role of DNA Topoisomerase I

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## ABSTRACT

DNA topoisomerase I (TOP1) mediates the induction of radiosensitization (RS) by camptothecin derivatives in mammalian cells. Many indolocarbazole (INDO) derivatives have been shown to induce TOP1-mediated DNA damage (T1DD). In the current study, we characterized the cytotoxic and radiosensitizing activities of six INDO derivatives in relation to their efficiencies to induce T1DD. Evaluated by clonogenic survival assay, the INDO derivatives F1, F5, and F7, but not F43, F44, or F71, were shown to induce significant levels of RS in the human breast cancer MCF-7 cells at noncytotoxic concentrations. Analyzed by the single-hit multitarget (SHMT) model, F1, F5, and F7, like camptothecin, induce RS by obliterating the "shoulder" of radiation survival curve. In contrast to the Chinese hamster

DC3F cells, the TOP1 mutant DC3F/C10 cells demonstrated cross-resistance to the cytotoxicity of F7 and the induction of RS by F7 and F1. The efficiencies to induce T1DD were determined by 1) drug-stimulated TOP1 cleavage assay in vitro and 2) K<sup>+</sup>-SDS coprecipitation assay in vivo. These compounds exhibited varying efficiencies in inducing T1DD with the following order: F71, F7 > F44, F1 > F5 > F43. It is surprising that the individual efficiency of these compounds to induce T1DD correlates well with their individual cytotoxicity but not RS activity. Taken together, our data demonstrate that certain, but not all, INDO derivatives capable of inducing T1DD can induce RS in mammalian cells. The INDO derivatives F1, F5, and F7 have the potential to be developed as a new class of radiation sensitizers.

Combining chemotherapy with radiotherapy has become the mainstay of therapy for a wide variety of human cancers (Hellman, 1997; Rotman et al., 1998). In addition to having their own cytotoxic effects, certain chemotherapeutic drugs can enhance radiation cytotoxicity by inducing radiosensitization (RS) (Cook and Mitchell, 1995; McGinn et al., 1996). Numerous clinical trials have shown a superior treatment outcome with combination chemoradiotherapy versus either modality alone in various settings (Hellman, 1997; Rotman et al., 1998). However, the efficacy of most chemoradiation regimens remains largely limited by the cumulative normal tissue toxicities from combining two modalities. A better understanding of the cytotoxic interactions between chemotherapeutic compounds and radiation and the development of

new radiation sensitizers that can enhance radiotherapy selectively toward cancer cells remain major challenges for cancer researchers.

The catalytic activity of TOP1 is essential for many aspects of nucleic acid metabolism, including DNA replication, RNA transcription and regulation of DNA supercoiling (Wang, 2002). TOP1 is also a primary target for many anticancer drugs (Li and Liu, 2001), including camptothecin derivatives (Hsiang et al., 1985) and DNA minor groove-binding drugs (Chen et al., 1993; Sim et al., 1997). Instead of inhibiting the DNA topology modifying activity, TOP1-targeted drugs stabilize a key covalent TOP1-DNA intermediate (named the TOP1 cleavable complex) that is formed between the tyrosine-723 residue of TOP1 and a 3'-phosphate at the break site during the transient DNA cleavage stage (Hsiang et al., 1985). The drug-trapped TOP1 cleavable complexes can serve as DNA breaking poisons and damage DNA through interactions with cellular processes such as replication of DNA (Hsiang et al., 1989; D'Arpa et al., 1990). The presence of

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**ABBREVIATIONS:** RS, radiosensitization; TOP1, DNA topoisomerase I; INDO, indolocarbazole; T1DD, TOP1-mediated DNA damage; DMSO, dimethyl sulfoxide; CHO, Chinese hamster ovary; SER, sensitization enhancement ratio; SHMT, single-hit multitarget.

elevated levels of TOP1 in tumor cells rather than normal tissue cells has provided a molecular basis for targeting TOP1 in cancer therapy (Giovanella et al., 1989; Pantazis et al., 1993).

Many INDO derivatives have been shown to be capable of inducing TOP1-mediated DNA damage (T1DD) and represent a different class of TOP1 drugs (Yamashita et al., 1992; Yoshinari et al., 1993; Bailly et al., 1998; Labourier et al., 1999; Prudhomme, 2000; Moreau et al., 2003). Based on its cross-resistance toward a camptothecin-resistant mutant TOP1, the INDO derivative R-3 in particular has been proposed to share common steric and electronic features with camptothecin (Labourier et al., 1999). However, some INDO derivatives interact with DNA with a higher affinity than do camptothecin derivatives (Yoshinari et al., 1993; Bailly et al., 1998). In addition, certain structural analogs of INDO exhibit inhibitory activities toward protein kinase C (Pereira et al., 1996; Moreau et al., 1998), protein kinase A (Pereira et al., 1996), and TOP1 kinase (Pereira et al., 1996; Anizon et al., 1997; Moreau et al., 1998, 1999, 2003; Labourier et al., 1999). It is notable that the TOP1 kinase inhibitory activity of the INDO derivative R-3 (identical to F7 in the present study) seems to be distinguished by its ability to induce TOP1-mediated DNA cleavage (Labourier et al., 1999).

Camptothecin derivatives have been shown to induce RS in mammalian cells (Mattern et al., 1991; Boothman et al., 1994; Chen et al., 1997). Our previous work demonstrated TOP1 mediates camptothecin-induced RS in a stereospecific manner (Chen et al., 1997). One hypothesis derived from this observation is that compounds similar to camptothecin that are capable of targeting TOP1, such as the INDO derivatives, may induce RS in mammalian cells. We show in the present study that only three of the six tested INDO derivatives induce significant levels of RS in mammalian cells. In addition, we also characterize the role of TOP1 in mediating the cytotoxic and RS activities of these INDO derivatives.

## Materials and Methods

**Drugs and Materials.** INDO derivatives were synthesized as published previously (Pereira et al., 1996; Anizon et al., 1997; Moreau et al., 1998; Moreau et al., 1999). Camptothecin lactone (NSC 94600) and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). All drugs were dissolved in DMSO at a concentration of either 10  $\mu$ M or 10  $\mu$ g/ml and kept frozen in aliquots at  $-20^{\circ}\text{C}$ . Except for fetal bovine serum, which was obtained from Inovar Biologicals (Gaithersburg, MD), media and other reagents for tissue culture work were purchased from Invitrogen (Carlsbad, CA). Purified human TOP1 and the pHOT1 plasmid were purchased from TopoGEN, Inc. (Columbus, OH).

**Cell Cultures.** The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The Chinese hamster lung fibroblast DC3F and its camptothecin-resistant DC3F/C10 sublines were kindly provided by Dr. Yves Pommier of the National Cancer Institute (Bethesda, MD) and grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2  $\mu$ M glutamine, and 0.1  $\mu$ M nonessential amino acids (Taniyawa et al., 1993). The Chinese hamster ovary (CHO) cells were obtained from Dr. Terence Dermody of the Vanderbilt University Medical Center (Nashville, TN) and grown in Ham's medium supplemented with 10% heat-inactivated fetal bovine serum. All cell lines were grown as stock cultures maintained at  $37^{\circ}\text{C}$  in a humid-

ified atmosphere of 5%  $\text{CO}_2$  and 95% air at pH 7.3. Under these conditions, the doubling times and plating efficiencies for DC3F, DC3F/C10, and MCF-7 cells were 10 h, 40 to 50%; 15 h, 45 to 55%; and 22 h, 50 to 60%, respectively.

**Clonogenic Survival Assay.** Clonogenic survival assays were conducted as described previously (Chen et al., 1997). In brief, stock cultures of exponentially growing cells were trypsinized, rinsed, plated into cultured dishes, and incubated at  $37^{\circ}\text{C}$  18 to 24 h before experimental studies. After drug incubation and irradiation, cells were trypsinized, rinsed, counted, and plated for colony formation. An equivalent amount of DMSO was added in the control dishes (final concentration,  $\leq 0.1\%$ ). After 7 to 14 days of incubation, colonies were fixed with methanol/acetic acid (3:1) and stained with crystal violet. Colonies consisting of  $>50$  cells were counted. All survival points were determined in triplicate, and experiments were conducted a minimum of two times. Error bars shown in the figures represent standard deviations (S.D.) and are shown when larger than the symbol.

**Irradiation of Cells.** Drug-treated and control cells in medium were irradiated using a cobalt-60 source (Eldorado 8; Theratronics, Ottawa, ON, Canada) at a dose rate of 105 cGy/min.

**Analysis of Survival Curves.** Chemoradiation survival curves were corrected for cytotoxicity induced by drug treatment alone. Each sensitization enhancement ratio (SER) was calculated by dividing the radiation dose to induce 10% cell survival in the absence of radiosensitizer with the radiation dose to induce 10% cell survival in the presence of radiosensitizer. The updated programs developed by Dr. N. Albright (San Rafael, CA) (Albright, 1987) were used for curve fitting for radiation survival curves with the linear quadratic and the single-hit multitarget (SHMT) models in MCF-7 cells.

**In Vitro Drug-Stimulated TOP1 Cleavage Assay.** Drug-stimulated DNA cleavage assay using purified human TOP1 was performed according to the protocol provided by TopoGEN Inc. (Columbus, OH), with modifications. In brief, supercoiled pHOT1 plasmid DNA (0.2  $\mu$ g) was incubated with 1 unit of human TOP1 in cleavage buffer (50 mM Tris, pH 7.8, 50 mM KCl, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, and 1 mM EDTA) in the presence of varying concentrations of the drug under study. After a 30-min incubation at  $37^{\circ}\text{C}$ , the reactions were terminated by adding SDS to 1.0% and proteinase K to 200  $\mu$ g/ml. The proteinase K treatment continued at  $37^{\circ}\text{C}$  for 1 h. The sample was then mixed with loading dye (containing sucrose and bromophenol blue) and separated in 1% agarose gel in 0.08 M Tris-phosphate, 0.008 M EDTA, and pH 8.0 buffer, at room temperature for 16 h. The gel was stained with ethidium bromide (1 mg/ml), destained, and photographed under UV light. The photograph was scanned and the intensity of the nicked DNA of each lane was measured using the ImageJ program (<http://rsb.info.nih.gov/ij/>). The intensity of the nicked band of each lane was plotted after the background intensity (pHOT1 + TOP1 lane) was subtracted.

**In Vivo  $\text{K}^+$ -SDS Coprecipitation Assay for Protein-DNA Cross-Links.** The  $\text{K}^+$ -SDS coprecipitation assay, as described previously (Chen et al., 1990), was used to quantitate the formation of covalent topoisomerase-DNA cross-links in drug-treated cells. In brief, the DNA in logarithmic growing CHO cells was labeled overnight in medium containing 1  $\mu\text{Ci}/\text{ml}$  of [*methyl*- $^3\text{H}$ ]thymidine. These cells were trypsinized and diluted in fresh medium before being aliquoted into a 24-well plate. After incubation overnight, the labeled cells were treated with various concentrations of drugs for 30 min, washed with phosphate-buffered saline once, and lysed by adding 1 ml of prewarmed lysis solution (1.25% SDS, 5 mM EDTA, and 0.4 mg/ml salmon sperm DNA, pH 8.0;  $65^{\circ}\text{C}$ ). After shearing chromosomal DNA by passing the lysate through a 19-gauge needle, each sample was transferred to a tube containing 0.25 ml of 325 mM KCl. The sample was vortexed vigorously for 10 s, cooled on ice for 10 min, and centrifuged at 3000 rpm for 10 min at  $4^{\circ}\text{C}$ . The pellet was then resuspended in 1 ml of a wash solution (100 mM KCl, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, and 10 mM Tris-HCl, pH 8.0) and placed at  $65^{\circ}\text{C}$  for 10 min with occasional mixing. The sample was

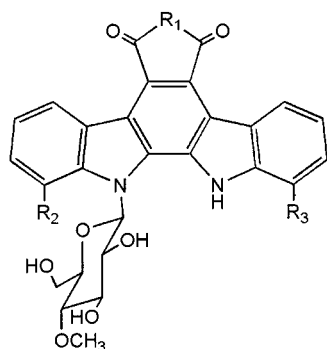
again cooled on ice for 10 min and centrifuged at 3000 rpm for 10 min at 4°C. After the pellet from each sample was washed again, the pellet was resuspended in 0.2 ml of 65°C water and added to 5 ml of scintillation fluid to determine radioactive counts. The background precipitable counts were obtained by treating the lysate with proteinase K (400 µg/ml) at 65°C for 2 h. Data are arithmetic means (± S.D.) of three determinations, and the background counts have been subtracted.

## Results

**INDO Derivatives F1, F5, and F7, but Not F43, F44, or F71, Induced RS in Human Breast Cancer MCF-7 Cells.** Six INDO derivatives (see Fig. 1 for their chemical structures) were tested for their RS activities by clonogenic survival assay in the human breast cancer MCF-7 cells. As shown in Fig. 2A, significant levels of RS were induced by 1-h preincubation with 2 µg/ml of F1, F5, and F7. On the contrary, no RS was induced by F43, F44, and F71 at 2 µg/ml (Fig. 2; Table 1) or at 10 µg/ml (data not shown). As measured by the SER at 10% cell survival, the magnitudes of RS induced by F1, F5, and F7 (SER = 1.9, 1.7, and 1.7, respectively) compare favorably to camptothecin (SER = 1.4) (Table 1). In notable contrast to camptothecin in inducing RS at cytotoxic concentrations (Table 1), F1, F5, and F7 exhibited potent RS activities at a noncytotoxic concentration of 2 µg/ml in MCF-7 cells (Table 1). F1, F5, and F7 also consistently induced RS at concentrations that caused little cytotoxic effect in the Chinese hamster lung fibroblast DC3F cells (Table 3 and 4).

**F1, F5, and F7 Induce RS by Obliterating the “Shoulder” of the Radiation Survival Curve.** The programs developed by Albright (1987) were used to analyze the chemoradiation survival curves in the MCF-7 cells (Fig. 2A). The SHMT model gave a qualitatively good fit (Fig. 2B). Figure 2C shows the respective radiation survival curve parameters. The D<sub>0</sub>, defined as the radiation dose required for reducing the survival fraction to 37% of its previous value, usually denotes the radiation sensitivity of the cells (Hall, 1994). On

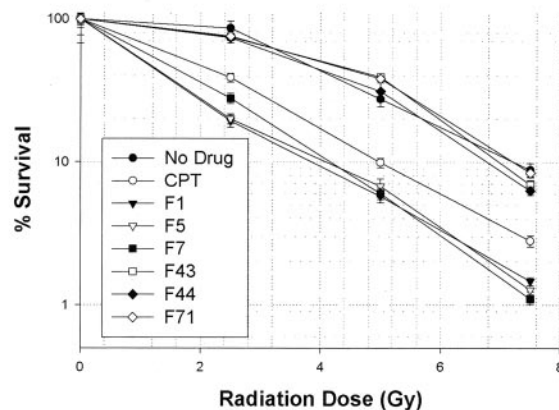
the other hand, the D<sub>q</sub>, defined as the straight portion of the survival curve extrapolated backward and cutting the dose axis drawn through a survival fraction of unity, represents the width of the shoulder of the survival curve (Hall, 1994).



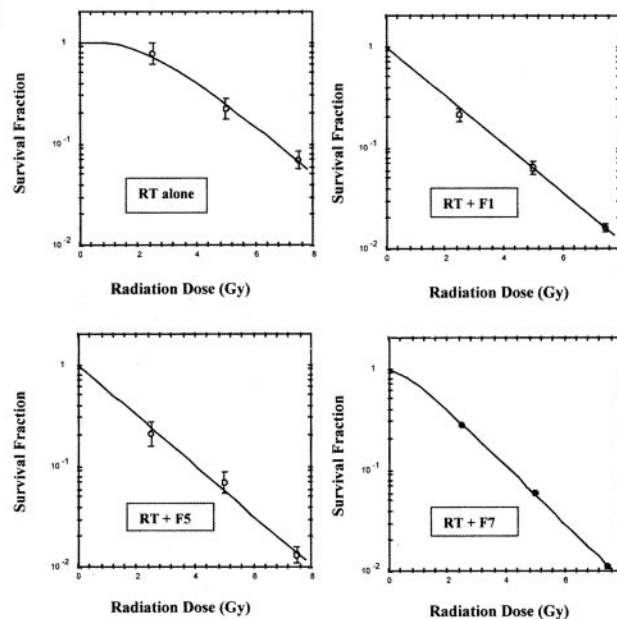
INDO derivative	R <sub>1</sub>	R <sub>2</sub> = R <sub>3</sub>
F1 (rebeccamycin)	NH	Cl
F5	O	H
F7 (also known as R-3)	NOH	H
F43	NCH <sub>3</sub>	Cl
F44	NCH <sub>3</sub>	H
F71	NH	H

Fig. 1. Chemical structures of INDO derivatives used in this study.

A



B



C

Parameters	RT alone	F1	F5	F7	CPT
D <sub>0</sub>	1.8	1.8	1.8	1.5	1.8
D <sub>q</sub>	2.7	0.0	0.0	0.7	0.8

**Fig. 2.** Induction of radiosensitization by INDO derivatives and camptothecin (CPT) in the human breast cancer MCF-7 cells. Clonogenic survival assays of exponentially growing MCF-7 cells were conducted as described under *Materials and Methods*. Cells were preincubated for 1 h with 2 µg/ml concentrations of various INDO derivatives or 1 µM CPT, followed by treatment with 0, 2.5, 5, or 7.5 Gy of radiation. Programs developed by Dr. N. Albright (Albright, 1987) were used for curve-fitting with the SHMT model. A, chemoradiation survival curves. SHMT survival curves (B) and SHMT survival curve parameters (C) for F1, F5, F7, or CPT. Points, mean of triplicates; bars, SD. D<sub>0</sub> and D<sub>q</sub> are measurements of the radiation sensitivity of the cells and the width of the shoulder of the survival curve, respectively (Hall, 1994). Please see the text for the definition of D<sub>0</sub> and D<sub>q</sub>.



Similar to camptothecin, the INDO derivatives F1, F5, and F7 induced significant reductions in Dq, but caused no apparent alteration in D<sub>0</sub> (Fig. 2, B and C). The analysis indicates that obliteration of the “shoulder” of the radiation survival curve, but not alteration of its terminal slope (1/D<sub>0</sub>), accounts in large part for the RS induction of F1, F5, and F7.

**INDO Derivatives Exhibit Varying Efficiencies in Inducing T1DD.** The activities to induce T1DD of the six INDO derivatives were characterized by the drug-stimulated TOP1 cleavage assay in vitro and the K<sup>+</sup>-SDS coprecipitation assays in vivo. In the drug-stimulated TOP1 cleavage assay, these compounds were tested for their abilities to convert the “supercoiled” form of the pHOT1 DNA substrate into the “nicked” form by stimulating TOP1-mediated DNA breaks. As shown in Fig. 3A, the six INDO derivatives exhibit varying efficiencies in stimulating TOP1-mediated nicking of the pHOT1 plasmid DNA in a dose-dependent manner similar to that of camptothecin. As a negative control, the DNA topoisomerase II-targeted etoposide shows no activity in inducing TOP1-mediated nicking of DNA. To better separate the “nicked” from the “relaxed” DNA population, the gel in Fig. 3A was further run for 4 h in the presence of ethidium bromide before being photographed (Fig. 3B). The relative intensity of the nicked bands induced by INDO derivatives and camptothecin from Fig. 3B was quantified as described under *Materials and Methods* and is shown in Fig. 3C. Therefore, the efficiencies of the six INDO derivatives in inducing TOP1-mediated DNA cleavage can be divided into two groups: 1) the potent group consists of F7 and F71 that exhibit similar activities as camptothecin and 2) the less potent group consists of F44, F5, F1, and F43.

As shown in Fig. 3A, unlike camptothecin, different INDO derivatives cause varying degrees of “shifting” of the supercoiled DNA, as well as alterations in the distribution of various DNA topoisomers. This finding indicates stronger interactions between DNA and drug molecules of the INDO derivatives than with camptothecin. Based on the amount of drug-associated “supercoiled DNA” (Fig. 3A), F71 and F44 have the strongest interactions with DNA, followed by F7.

The ability to stabilize the reversible protein-DNA cross-links, termed the “cleavable complexes”, is a unique feature of TOP1-targeted drugs (Hsiang et al., 1985). Quantification of the protein-DNA cross-links in cells can be used as a measure of the

drug potency in poisoning TOP1 intracellularly (Hsiang et al., 1985; Chen et al., 1993). As determined by the K<sup>+</sup>-SDS coprecipitation assay (Fig. 4), the potency of INDO derivatives in poisoning TOP1 in the Chinese hamster CHO cells was found to adhere to the following order: F71 > F7 = F1 > F44 > F5 > F43. These drug-induced protein-DNA cross-links were reversible by dilution or a brief 65°C heat treatment (data not shown) and most likely represent the TOP1-DNA complexes (Hsiang et al., 1985; Chen et al., 1993). The potencies of the six INDO derivatives to induce protein-DNA complexes in vivo seem to correlate well with their efficiencies to induce TOP1-mediated DNA cleavage in vitro (Table 2). This finding further indicates that the protein-DNA cross-links induced by INDO derivatives in the CHO cells primarily represent the TOP1-DNA cleavable complexes.

**The Abilities of the Six INDO Derivatives to Induce T1DD Correlate with their Cytotoxicities, but Not RS Activities.** The relationship between the ability to induce T1DD, the cytotoxicity, and the RS activity among the six INDO derivatives was investigated. The cytotoxicities of 1-h drug treatment of the six INDO derivatives in the CHO cells were determined by clonogenic survival assay. Measured by the drug concentration leading to 50% cell death (LD<sub>50</sub>), the cytotoxic potencies of the INDO derivatives adhere to the following order: F71, F44 > F1, F7 > F5, F43 (Table 2). A good correlation seems to exist between the cytotoxicity and the efficiency to induce T1DD among the six INDO derivatives (Table 2). Our data support the notion that TOP1 is the major cytotoxic target of these INDO derivatives (Pereira et al., 1996; Anizon et al., 1997; Moreau et al., 1998; Bailly et al., 1999). It is interesting, however, that the cytotoxicity and the efficiency to induce T1DD do not correlate well with the RS activity in these INDO derivatives (Table 2). For example, although they demonstrated no RS activity, F71 and F44 are potent inducers for T1DD and cytotoxicity (Table 2). On the other hand, F5 exhibits high potency in inducing RS but little activity in inducing T1DD and cytotoxicity.

**TOP1 Mutant DC3F/C10 Cells Exhibited Cross-Resistance to the Cytotoxicity of F7, and Less Sensitivity to the Induction of RS by F7 and F1.** To further elucidate the role of TOP1 in mediating the cytotoxic effects of the six INDO derivatives, clonogenic survival assays were performed in the Chinese hamster lung fibroblast DC3F and its camptothecin-resistant TOP1 mutant DC3F/C10 cells (Tanizawa et al., 1993). The amino acid mutation from Gly<sup>505</sup> to Ser<sup>505</sup> of the mutant TOP1 has been shown to be responsible for the camptothecin-resistance phenotype of DC3F/C10 cells (Tanizawa et al., 1993). As shown in Table 3, the DC3F/C10 cells demonstrated resistance of more than 12-fold to the cytotoxic effects of a 30-min camptothecin treatment compared with their parental DC3F cells. In comparison, the camptothecin-resistant DC3F/C10 cells demonstrated more than 5-, 2.5- and 2.7-fold cross-resistance to F7, F44, and F71, respectively (Table 3). The fact that DC3F/C10 cells exhibited cross-resistance to F7, F44, and F71, although only at moderate levels, indicates that TOP1 is a cytotoxic target of these compounds. Because of the lack of cytotoxic effect, the relative resistance of F1, F5, or F43 could not be determined in DC3F/C10 cells (Table 3).

The RS activities of camptothecin and the INDO derivatives F1 and F7 were also examined in the DC3F and DC3F/C10 cells by clonogenic survival assays. Consistent with the

TABLE 1

SER values in the human breast cancer MCF-7 cells treated with INDO derivatives or camptothecin (CPT) in combination with radiation (RT)

Clonogenic survival assays as described under *Materials and Methods* were performed in the MCF-7 cells. Cells were pretreated with 2 µg/ml of various INDO derivatives or 1 µM CPT for 1 h, rinsed off the drug, before receiving radiation treatment. The percentage of cell survival without RT and RT dose at 10% survival were determined graphically from Fig. 2. SER indicates the radiation dose to induce 10% cell survival in the absence of drug divided by the radiation dose to induce 10% cell survival in the presence of drug.

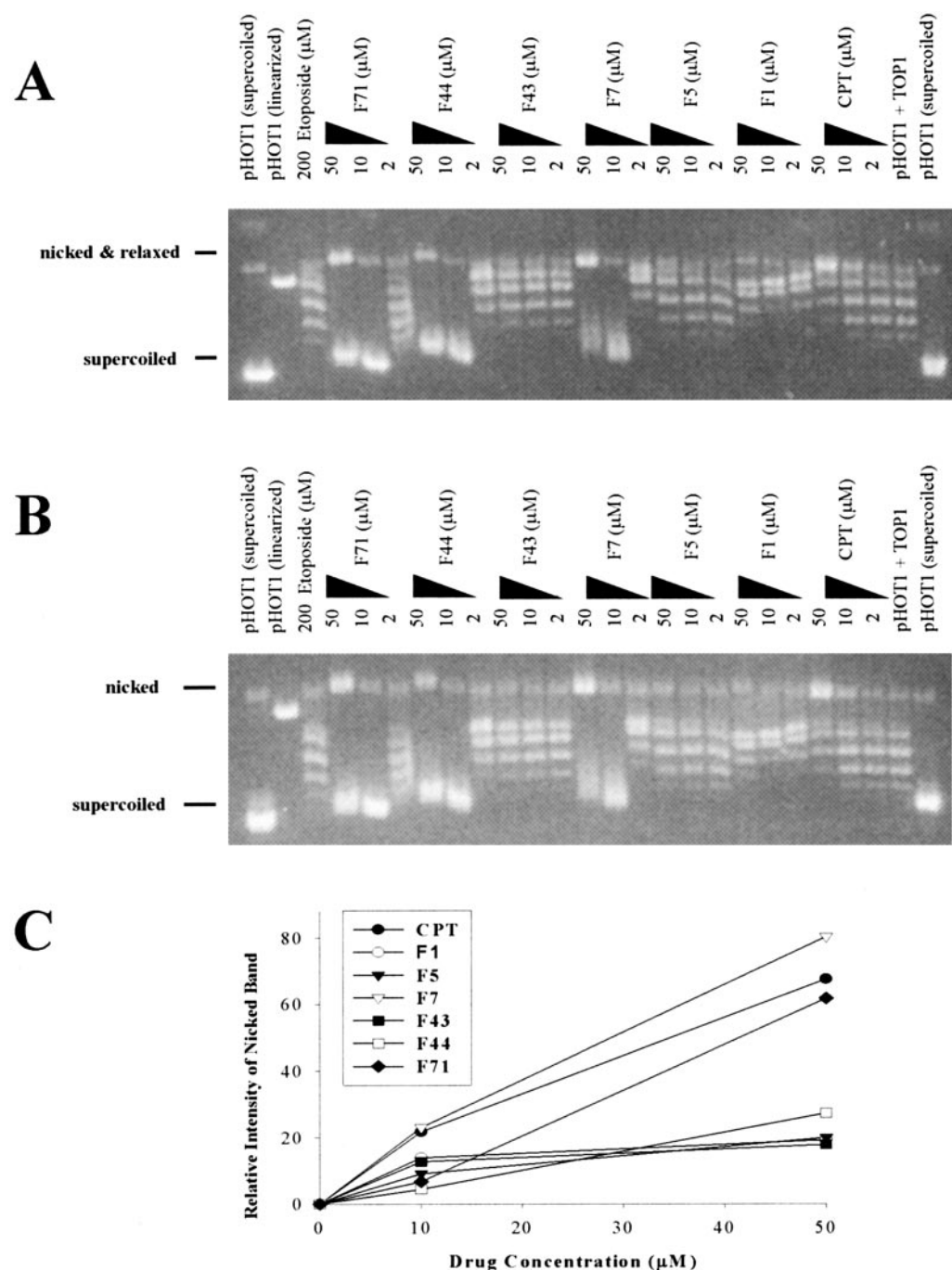
Drug Treatment	Cell Survival without RT	RT Dose at 10% Survival	SER
	%/TD	Gy	
No drug	100 ± 11	7.2	1.0
F1	94 ± 8	3.8	1.9
F5	98 ± 2	4.2	1.7
F7	98 ± 2	4.2	1.7
F43	95 ± 5	7.0	1.0
F44	100 ± 2	6.9	1.0
F71	85 ± 10	7.2	1.0
CPT	72 ± 5	5.0	1.4

findings of our previous study (Chen et al., 1997), as shown in Table 4, the TOP1 mutant DC3F/C10 cells exhibit significantly lower sensitivity to the induction of RS by 1-h camptothecin pretreatment at various drug concentrations than their parental DC3F cells: in comparison with the SER values of 1.4 and 1.5 induced by 0.1 and 1  $\mu$ M camptothecin, respectively, in the DC3F cells, significantly lower SER values of 1.0 and 1.1 were induced by 1 and 10  $\mu$ M camptothecin, respectively, in the DC3F/C10 cells. This finding fits the theory that TOP1 mediates the RS activity of camptothecin (Chen et al., 1997). Likewise, the TOP1 mutant DC3F/C10 cells also exhibited lower sensitivity than the DC3F cells to the induction of RS by F7 and F1 (Table 4). In contrast to the SER of 1.3 in the DC3F cells, a lower SER value of 1.1 was

induced by 5  $\mu$ g/ml F1 in the DC3F/C10 cells. Likewise, in contrast to the SER of 1.5 in the DC3F cells, little RS (SER = 1.0) was induced by 5  $\mu$ g/ml F7 in the DC3F/C10 cells. These findings indicate that TOP1 is involved in mediating the RS activities of F1 and F7. Consistent with the results in the MCF-7 cells (Fig. 2), F71, F44, and F43 induced no RS activity in either the DC3F or the DC3F/C10 cells at concentrations as high as 10  $\mu$ g/ml (data not shown).

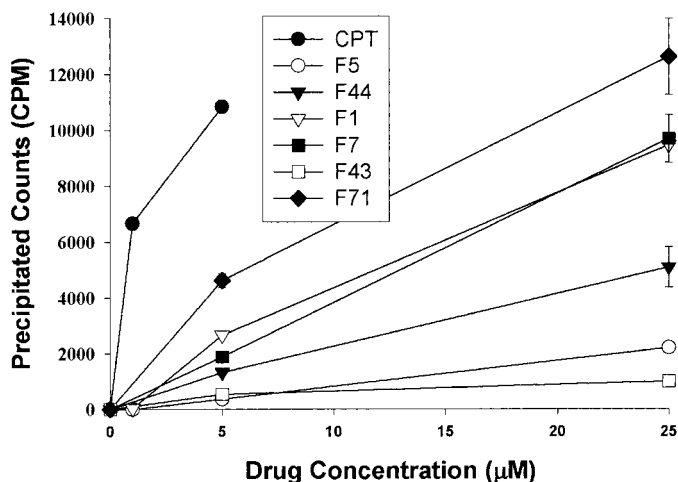
## Discussion

Different INDO derivatives are known to exert diverse biochemical activities, including induction of T1DD in mammalian cells (Yamashita et al., 1992; Pantazis et al., 1993;



**Fig. 3.** Induction of TOP1-mediated DNA cleavage by INDO derivatives and camptothecin (CPT). The in vitro drug-stimulated DNA cleavage assay using purified human TOP1 was carried out as described under *Materials and Methods*. The concentrations of various drugs are marked in the figure. After reactions, the samples were analyzed by 1% agarose gel electrophoresis in buffer containing 0.08 M Tris-phosphate and 0.008 M EDTA, pH 8.0, at room temperature for 16 h. The positions of the linear, nicked, relaxed, and supercoiled pHOT1 DNA are labeled. The initial gel was stained with ethidium bromide (1  $\mu$ g/ml), washed, and photographed under UV light (A). To better separate the "nicked" from the "relaxed" DNA population, the gel was further run for 4 h in the presence of ethidium bromide before being photographed (B). The relative intensity of the nicked DNA of each lane from (B) was measured using the ImageJ program as described under *Materials and Methods* (C). The intensity of the nicked band of each lane was plotted after the background intensity (pHOT1 + TOP1 lane) was subtracted.

Yoshinari et al., 1993; Pereira et al., 1996; Anizon et al., 1997; Bailly et al., 1998; Moreau et al., 1998, 2003; Laborier et al., 1999; Moreau et al., 1999; Prudhomme, 2000). However, before this study, the RS activity of INDO derivatives was mostly unknown. Among the six INDO derivatives tested in the current study, F1, F5, and F7, but not F43, F44, or F71, were found to exhibit RS activity in mammalian cells



**Fig. 4.** Induction of protein-DNA cross-links by various INDO derivatives and camptothecin (CPT). The formation of covalent TOP1-DNA cross-links in CHO cells induced by a 30-min drug treatment was measured by the in vivo  $K^+$ -SDS coprecipitation assay as described under *Materials and Methods*. The background precipitable counts were obtained by treating the lysate with proteinase K (400  $\mu$ g/ml) at 65°C for 2 h. Data are arithmetic means ( $\pm$  S.D.) of three determinations, and the background counts have been subtracted. Points, mean of triplicates; bars, S.D.

**TABLE 2**

Activities in inducing TOP1-mediated DNA damage, cytotoxicity, and RS of the six INDO derivatives

Activity of each INDO derivative in inducing TOP1-mediated DNA cleavage in vitro (second column) or covalent protein-DNA cross-links in CHO cells (third column) was denoted as high (+++), modest (++), or low (+), based on Fig. 4. Cytotoxic activity of each INDO derivative is based on the  $LD_{50}$  (drug concentration leading to 50% cell death) determined graphically from clonogenic survival assays in the CHO cells as described under *Materials and Methods*. The drug treatment time was 1 h. RS activity of each INDO derivative was based on experiments conducted in the MCF-7 (as shown in Fig. 2) and DC3F cells (as shown in TABLE 4). + indicates RS activity; - indicates no RS activity.

INDO derivatives	TOP1-Mediated DNA Cleavage <sup>a</sup>	Protein-DNA Cross-Links <sup>b</sup>	Cytotoxicity <sup>c</sup>	RS <sup>d</sup>
			$\mu$ g/ml	
F1	+	++	$5.0 \pm 0.3$	+
F5	+	+	$>10$	+
F7	+++	++	$7.3 \pm 0.2$	+
F43	+	+	$>10$	-
F44	++	++	$2.4 \pm 0.2$	-
F71	+++	+++	$2.0 \pm 0.4$	-

**TABLE 3**

Cross-resistance pattern of the DC3F/C10 cells to INDO derivatives  
 $LD_{50}$  was determined graphically from clonogenic survival assay of each drug.

Drugs	$LD_{50}$		RR
	DC3F	DC3F/C-10	
CPT ( $\mu$ M)	0.8	$>10$	$>12.0$
F1 ( $\mu$ g/ml)	$>25$	$>25$	N.D.
F7 ( $\mu$ g/ml)	4.0	$>20$	$>5.0$
F44 ( $\mu$ g/ml)	3.2	8.0	2.5
F71 ( $\mu$ g/ml)	1.5	4.1	2.7

$LD_{50}$ , drug concentration that leads to 50% cell death; RR (relative resistance) =  $LD_{50}$  of DC3F/C10 cells divided by  $LD_{50}$  of DC3F cells. N.D., not determined.

(Fig. 2). Two features of these INDO derivatives are considered "ideal" for radiation sensitizers (Chen et al., 1999; Herscher et al., 1999). First, these compounds exhibit excellent RS activities that are more potent than that of camptothecin (Table 1). Second, these compounds can induce RS in mammalian cells at concentrations with little or no cytotoxicity (Table 1 and 2). Therefore, these INDO derivatives can potentially enhance tumor control selectively at the irradiated site(s) and cause no significant systemic untoward effects for cancer patients. Further preclinical investigations in animal model systems are needed to confirm these special features of INDO derivatives, which may make them "interesting radiation sensitizers" (Herscher et al., 1999).

Similar to camptothecin (Chen et al., 1997), obliteration of the "shoulder" of the survival curve but not its terminal slope ( $1/D_0$ ) seems to account for the observed RS effect induced by INDO derivatives F1, F5, and F7 (Fig. 2). The "shoulder" of the radiation survival curve is considered a measure of the repair capacity for "sublethal damage" of the irradiated cells (Hall, 1994; Hellman, 1997; Rotman et al., 1998). At least two possible mechanisms exist by which these INDO derivatives can suppress the repair capacity for "sublethal damage" of the irradiated cells. First, these INDO derivatives may exert their RS activities by "saturating" cellular machinery that is responsible for repairing sublethal DNA damage caused by ionizing radiation (Chen et al., 1999). TOP1 poisons are S-phase-specific agents that are known to trap the TOP1-DNA cleavable complexes and induce various types of DNA damage in actively proliferating cells (Hsiang et al., 1989; Li and Liu, 2001). Double-strand DNA breaks, TOP1-linked DNA breaks, and arrest of DNA replication forks are three major camptothecin-induced T1DDs that have been observed in a cell-free simian virus 40-based in vitro replication system (Tsao et al., 1993). It is possible that any or a combination of the three T1DDs induced by INDO derivatives in S-phase may serve as the substrate(s) and "saturate" cellular repair machinery for radiation-induced sublethal DNA damage. Consistent with this notion, we have recently demonstrated that TOP1-mediated RS is significantly enhanced in mammalian cells deficient in the nonhomologous end joining pathway (Shih et al., 2003), on major double-strand break repair pathways known to be involved in repairing radiation-induced DNA damage (Jeggo, 1998). On the other hand, INDO derivatives may exert their RS activities partly by efficiently eliminating the S-phase population of the cell cycle, which is relatively more resistant to radiation and may contribute to the "shoulder" of the radiation survival curve (Hall, 1994).

Loss of clonogenic survival generally may reflect antiproliferative effects, including induction of apoptotic and non-apoptotic forms of cell death. The induction of apoptosis as a potential RS mechanism for the radiosensitizing F5 compound with camptothecin as the control was examined in the human colon cancer HCT-116 cells. Apoptotic nuclei were observed in less than 5% of cells treated with radiation alone, radiation plus 1-h drug pretreatment, or 1-h treatment with drug alone during a time course of 24 h (A. Y. Chen and S.-J. Shih, unpublished results). There was no discernible difference between F5 and camptothecin in inducing apoptosis. Therefore, our preliminary results seem to indicate that triggering an apoptotic cell death may play little role in the induction of RS by INDO derivatives. It has been shown that genomic instability can manifest several generations after



cellular exposure to physical or chemical DNA-damaging agents, including ionizing radiation (Marder and Morgan, 1993). For example, a delayed, nonapoptotic form of reproductive cell death caused by genomic instability has been observed in cells surviving ionizing radiation (Chang and Little, 1992). The nature of the primary lesion(s) and the mechanism of this delayed chromosomal instability induced by radiation remain largely unknown. However, it has been speculated that a subset of a variety of radiation-induced DNA lesions, including DNA base alterations, DNA-DNA and DNA-protein cross-links, and single- and double-strand DNA breaks may trigger cellular processes, including gene deletions, and eventually lead to delayed chromosomal instability (Marder and Morgan, 1993). It will be interesting to test whether INDO derivatives may induce RS by enhancing such delayed chromosomal instability induced by radiation.

The potency of the six INDO derivatives to induce protein-DNA complexes *in vivo* was found to correlate well with their efficiency to induce TOP1-mediated DNA cleavage *in vitro* (Table 2). This observation implies that there is no significant difference in the permeability of the six INDO compounds to cross the lipid bilayer of the cell membrane to intracellularly affect their targeting TOP1. The efficiency of these INDO derivatives to induce T1DD was consistently well correlated with their cytotoxicities (Table 2). In the past, albeit with some controversies, TOP1 has been shown to mediate the cytotoxic effects of different INDO derivatives (Bailly et al., 1998; Labourier et al., 1999; Prudhomme, 2000; Urasaki et al., 2001; Woo et al., 2002; Moreau et al., 2003). Our data indicate that TOP1 is the major intracellular cytotoxic target for the six INDO derivatives tested in the present study. In addition, we also demonstrated that the TOP1 mutant DC3F/C10 cells exhibited cross-resistance to the INDO derivatives F7, F44, and F71 (Table 3). This finding may suggest a "functional analogy" between these INDO derivatives and camptothecin in "trapping" the TOP1-DNA cleavable complexes and inducing T1DD. Our observation agrees with the published work from Bailly et al. (1999) showing that the camptothecin-resistant TOP1 mutant F361S is cross-resistant to the INDO derivative *R*-3 (which is

the same as F7 in the current study). Further studies are needed to determine the structural basis of this analogy between camptothecin and INDO derivatives in trapping the TOP1-DNA cleavable complexes and its contribution to the compound's biological effects, including cytotoxicity and RS.

The cross-resistance of the TOP1 mutant DC3F/C10 cells to the RS induction of F7 and F1 indicates that TOP1 may be involved in mediating their RS activities (Table 4). However, it is surprising that not all the INDO derivatives capable of inducing T1DD exhibited RS activities in our cultured cell systems (Table 3). In fact, we demonstrated no clear correlation between the efficiency to induce T1DD and the potency to induce RS activity among these INDO derivatives (Table 3). For example, the F71 and F44, although demonstrating high potencies in inducing T1DD, exhibited no RS activity in either the human MCF-7 cells or the Chinese hamster DC3F cells (Fig. 2, Table 2). Conversely, F5 exhibits little potency in inducing T1DD, but is potent in inducing RS (Fig. 2, Table 2). The dissociation between the ability to induce T1DD and the RS activity in these INDO derivatives remains unclear. As has been demonstrated by different researchers, some structural derivatives of INDO possess other biological activities, including inhibitory effects toward protein kinase C (Pereira et al., 1996; Moreau et al., 1998), protein kinase A (Pereira et al., 1996), and TOP1 kinase (Pereira et al., 1996; Anizon et al., 1997; Moreau et al., 1998, 1999, 2003; Labourier et al., 1999). Therefore, it is plausible that certain TOP1-unrelated biological activities of F71 and F44 may interfere with and negate their TOP1-mediated RS activities. On the other hand, based on the observation that F71 and F44 contain higher affinities with DNA than do F7, F5, and F1 (Fig. 3), it is also conceivable that the strong interactions with DNA of F71 and F44 may "interfere" with their TOP1-mediated RS activities. Another less likely possibility is that the RS activities of these INDO derivatives may be governed by mechanisms unrelated to TOP1. Further investigation will elucidate the dissociation between the cytotoxicity and the RS activity of these INDO derivatives and may provide important information regarding the mechanism of TOP1-mediated

TABLE 4

Induction of radiosensitization in the DC3F and DC3F/C10 cells by camptothecin (CPT) and the INDO derivatives F1 and F7

Radiation dose at 10% Survival is determined graphically from clonogenic survival assays conducted with cells pre-incubated with drug for 1 h followed by treatment with 0-, 2.5-, 5-, or 7.5-Gy doses of radiation. SER indicates the radiation dose at 10% survival without drug pretreatment divided by radiation dose at 10% survival with 30-min drug pretreatment.

Drugs	DC3F Cells		DC3F/C10 Cells	
	Radiation Dose at 10% Survival <sup>a</sup>	SER	Radiation Dose at 10% Survival	SER
	Gy		Gy	
No Drug Control	6.0	1.0	5.2	1.0
Camptothecin				
0.1 $\mu$ M	4.3	1.4	N.D.	N.D.
1.0 $\mu$ M	3.9	1.5	5.2	1.0
10.0 $\mu$ M	N.D.	N.D.	4.6	1.1
F1				
1.0 $\mu$ g/ml	4.9	1.2	N.D.	N.D.
5.0 $\mu$ g/ml	4.6	1.3	4.8	1.1
25.0 $\mu$ g/ml	N.D.	N.D.	4.5	1.2
F7				
2.5 $\mu$ g/ml	4.6	1.3	N.D.	N.D.
5.0 $\mu$ g/ml	3.9	1.5	5.2	1.0
10.0 $\mu$ g/ml	3.6	1.7	4.9	1.1
20.0 $\mu$ g/ml	N.D.	N.D.	4.1	1.3

N.D., not determined.

ated RS, as well as the development of novel radiation sensitizers.

In summary, our results indicate that certain, but not all, INDO derivatives capable of trapping TOP1 cleavable complexes can induce RS in mammalian cells. With the ability to induce RS at relatively noncytotoxic concentrations, the INDO derivatives F1, F5, and F7 have the potential to be developed as a new class of radiation sensitizers. Further studies are needed to establish the critical structural and biochemical determinants for the induction of RS by these INDO derivatives.

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