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ORIGINAL ARTICLE

Therapeutic potential and molecular mechanism of a novel sulfonamide anticancer drug, indisulam (E7070) in combination with CPT-11 for cancer treatment

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Abstract
Purpose Indisulam (*N*-(-3-chloro-7-indolyl)-1,4-benzenedisulfonamide; E7070) is an experimental anticancer agent. Microarray analysis indicates that indisulam downregulates several genes involved in drug resistance, and this finding led us to test the effect of combining indisulam with other anticancer drugs. We investigated the antitumor effect and mechanism of synergism when indisulam was administered in combination with CPT-11.
Methods In vitro cytotoxic activity was examined using a cell counter kit, and the combination effect was determined by isobologram analysis. The level of topoisomerase IIα was measured by Western blotting. The in vivo antitumor effect was assessed in mice inoculated with human colorectal cancer SW620 cells.
Results Isobologram analysis indicated that a 24-h exposure to indisulam and SN-38, an active metabolite of CPT-11, had a synergistic effect in HCT116 and SW620 cells and an additive effect in HCT15 and WiDr cells. Prolongation of exposure to 48 h resulted in a synergistic effect in HCT15 and WiDr cells. Treatment with SN-38 alone increased the amount of intracellular topoisomerase IIα in all cell lines tested. Co-treatment with indisulam suppressed the SN-38-induced upregulation of topoisomerase IIα after 24 h of exposure in HCT116 and SW620 cells and after 48 h of exposure in HCT15 and WiDr cells. This apparent association between a synergistic effect and suppression of SN-38-mediated upregulation of topoisomerase IIα suggests that indisulam enhances SN-38 cytotoxicity by suppressing topoisomerase IIα upregulation to compensate for topoisomerase I inhibition by SN-38. Synergy was also observed in xenografted tumors and was accompanied by complete suppression of topoisomerase IIα upregulation induced by CPT-11 treatment.
Conclusion These observations prompted the clinical evaluation of indisulam and CPT-11 combination therapy.

Keywords Indisulam · SN-38 · Topoisomerase IIα · Isobologram analysis

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Introduction

Indisulam (*N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide; E7070) (Fig. 1a) is a novel anticancer drug that we selected from our sulfonamide compound collections by antitumor screening and flow cytometric analysis [1, 2]. Phase II studies of indisulam are presently being completed in solid tumors, including colorectal, breast, and renal cancers [3–6]. The results in an in vitro tumor panel consisting of 42 human cancer cell lines suggest that indisulam has a novel antitumor mechanism [7]. Indisulam affects energy metabolism and cell cycle regulation, but its mechanism of action remains unclear. Recently, microarray analysis has

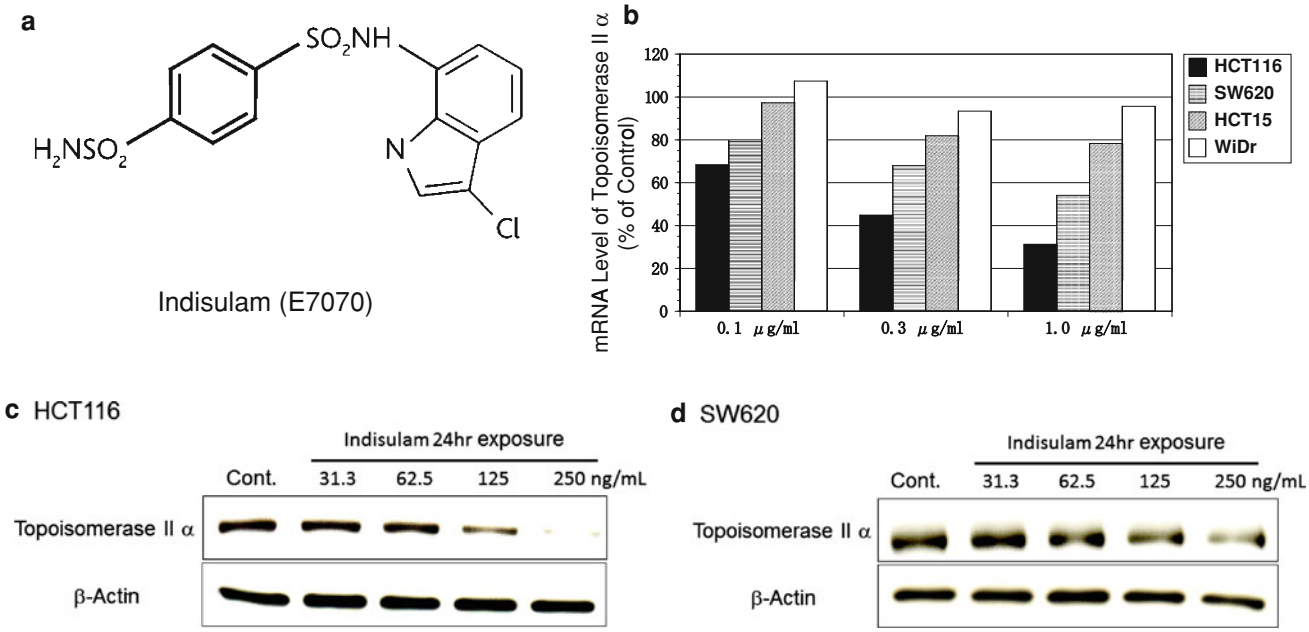

Fig. 1 Effect of indisulam on mRNA and protein levels of topoisomerase IIα. **a** structure of indisulam. **b** after 24 h of exposure to indisulam at the indicated concentrations, samples of total RNA were prepared. Expression of topoisomerase IIα was determined by real-time quantitative reverse transcription-PCR. Analysis of each gene was conducted in duplicate and normalized against their expression in HCT116, SW620, HCT15, and WiDr cells using 18S ribosomal RNA as the internal control. **c** after 24 h of exposure, whole lysates were prepared. The protein level of topoisomerase IIα was determined by Western blotting

indicated that indisulam downregulates several genes involved in cytotoxic drug resistance and cell proliferation, including those encoding cyclin H (related to nucleotide excision repair [NER]), glutathione synthase (GS; involved in detoxification of platinum), thymidylate synthase (TS; a target molecule of 5-fluorouracil [5-FU]), and topoisomerase IIα [8].

The topoisomerase I inhibitor CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyl-oxy-camptothecin) is a hydrophilic analog of camptothecin that has greater cytotoxic activity and less overall toxicity than camptothecin [9]. It is a key chemotherapeutic agent against colorectal cancer [10] and has definite anti-tumor activity in patients with refractory lymphoma, ovarian cancer, or small-cell or non-small-cell lung cancer.

Treatment with topoisomerase I inhibitors upregulates topoisomerase IIα expression [11, 12], and in cell lines resistant to topoisomerase I inhibitors topoisomerase IIα activity and/or expression is increased [13–15]. These findings suggest that upregulation of topoisomerase IIα is a survival characteristic to prevent topoisomerase I inhibitor-induced toxicity. We hypothesize that indisulam enhances the antitumor effect of CPT-11 by suppressing the upregulation of topoisomerase IIα induced by CPT-11. In this study, we test this possibility and consider the relevance of indisulam and CPT-11 combination therapy in the clinical setting.

Materials and methods

Chemicals and reagents

Indisulam was synthesized at the Kashima plant of Eisai Co., Ltd.; CPT-11 was obtained from Daiichi Sankyo Co., Ltd.; SN-38 (an active metabolite of CPT-11) was donated by Yakult Honsha Co., Ltd.

Tumor cell lines and cell cultures

Human colorectal cancer HCT116, SW620, HCT15, and WiDr cells were purchased from the American Type Culture Collection. All cell lines were maintained in RPMI-1640 (Sigma Chemical) supplemented with 10% FBS (Cansera International Inc.), penicillin-G (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2 in air.

Real-time quantitative reverse transcription-PCR

Real-time quantitative reverse transcription-PCR was used to measure the expression of topoisomerase IIα. RNA was isolated from untreated cells and indisulam-treated cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with a High-Capacity cDNA Archive Kit (Applied

Biosystems) according to the manufacturer's instructions. The PCR primers were Taqman probes (Applied Biosystems) for topoisomerase IIα (Hs00172214m1) and for 18S ribosomal RNA (Hs99999901s1), which was the internal control. Amplifications were conducted in duplicate with an ABI Prism 7700 sequencing detector.

Western blot analysis

Cultured cells (1.6×10^5 cells) were inoculated into 6-well plates. After a 24-h pre-incubation, the drugs were added. After a 24- or 48-h exposure, cells were washed twice in phosphate-buffered saline (PBS), and whole-cell extracts were prepared by adding cell lysis buffer (1% SDS, 10 mM Tris (pH 6.8), 2 mM EDTA, 0.1 mM sodium orthovanadate, 2 μg/mL leupeptin, and 1 mM A-PMSF). For in vivo tumors, tumor-bearing mice were divided into three dosing groups, and each group was given either indisulam 25 mg/kg alone, CPT-11 62.5 mg/kg alone, or both. Three mice were used for each treatment. Indisulam was administered intravenously once a day for 5 days, and CPT-11 was administered intravenously on days 1 and 5. Twenty-four hours after the final administration, the tumors were removed and frozen with liquid N_2. Approximately 100 mg tumor was homogenized in ice-cold tris buffer (1 mM Tris–HCl (pH 7.5), 10% glycerol, 1 mM P-APMSF, 1 mM orthovanadate, 2 mM EDTA, and 50 μg/mL leupeptin).

After determination of protein concentration by using Bradford protein assays (Bio-rad Laboratories), an equal volume of sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris (pH 6.8), and 10% glycerol) was added, and the mixture was boiled for 5 min. Ten micrograms of protein was analyzed by 4–20% SDS-PAGE gels and transferred to a PVDF membrane (Hybond P, Amersham). The membranes were probed with 0.1 μg/mL monoclonal mouse IgG Topoisomerase II (Ab-1) (Oncogene Research Products) in 5% (w/v) skim milk powder and 0.05% (v/v) Tween 20 in PBS. Detection was carried out by the addition of horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences UK Ltd.), followed by SuperSignal West Pico Chemiluminescent Substrate (PIERCE). Images were captured with an ImageMaster VDS-CL multi-imaging system (Amersham Biosciences).

Subcutaneous xenograft model in athymic mice

SW620 cells were harvested from cell culture flasks by using 0.05% trypsin–EDTA solution and collected by centrifugation at $450 \times g$ for 5 min. Cell pellets were resuspended in Hanks's balanced solution to 5×10^7 cells/mL. A 0.1-mL/mouse suspension of SW620 cells was subcutaneously implanted into the right flank of female athymic Balb/c *nu/nu* mice (Japan SLC, Inc.). All mice were maintained under SPF conditions and received sterile rodent chow and water ad libitum. In all studies, tumors were allowed to reach 150–300 mm^3 before drug treatment was initiated.

Drug treatment and evaluation of combination effect in vivo

Animals with established tumors were randomized into treatment groups of 6 mice each. The day on which treatment commenced was designated day 1. Indisulam was administered daily for 5 days (qdx5 regimen). CPT-11 was administered every 4 days, for three rounds (q4dx3 regimen). Indisulam and CPT-11 combination was administered in accordance with three dosing schedules as follows: simultaneous administration, indisulam on days 1–5 (qdx5) and CPT-11 on days 1, 5, and 9 (q4dx3); indisulam pre-treatment, indisulam on days 1 to 5 (qdx5) and CPT-11 on days 6, 10, and 14 (q4dx3); and CPT-11 pre-treatment, CPT-11 on days 1, 5, and 9 (q4dx3), and indisulam on days 10–14 (qdx5). Tumors were measured every 3 or 4 days with calipers, and tumor weights were determined by calculating the volume of an ellipsoid by using the formula $(\text{length} \times \text{width}^2)/2$. Relative tumor volumes (RTVs) were determined by using the following formula: tumor volume on day *n*/tumor volume on day 1. All procedures were performed in an animal facility accredited by the Center for Accreditation of Laboratory Animal Care, which is overseen by the Japan Health Sciences Foundation.

Antitumor effects were quantified as relative tumor volume (RTV). In accordance with the method reported by Slinker [16], combination effects were evaluated using two-way analysis of variance (ANOVA). The RTVs of the non-treated control group, indisulam 25 mg/kg group, CPT-11 62.5 mg/kg group, and indisulam 25 mg/kg and CPT-11 62.5 mg/kg combination group were statistically analyzed. When the main effects of each drug were statistically significant and the *P* value of interaction was <0.05, the combination effect was considered to be synergistic. Statistical analysis was conducted with the software package, SAS6.12 (SAS institute Japan Ltd.). Doses that resulted in mortality or a body weight loss greater than 20% were considered toxic.

Cytotoxicity assay and evaluation of combination effect in vitro

Drug sensitivity was determined by using a Cell Counting Kit (Dojindo). Cells (2,000–4,000) were plated in triplicate into 96-well, flat-bottomed microplates and incubated for 24 h. Indisulam and SN-38 were then added at various doses (indisulam: 0.0762–500 μg/mL; SN-38: 0.0762–500 ng/mL). After incubation of the plates for 24 h or 48 h,

Table 1 IC_{50} values of indisulam and SN-38 against 4 human colorectal cancer cell lines

Cell line	Indisulam (μg/mL)		SN-38 (ng/mL)	
	24-h exposure	48-h exposure	24-h exposure	48-h exposure
HCT116	0.198 ± 0.009[a]	0.095 ± 0.002	1.52 ± 0.10	1.11 ± 0.07
SW620	13.3 ± 0.4	0.322 ± 0.008	7.93 ± 0.447	7.68 ± 0.71
HCT15	67.9 ± 4.9	1.95 ± 0.58	11.4 ± 1.77	10.9 ± 0.9
WiDr	48.4 ± 0.6	20.8 ± 1.0	53.7 ± 8.83	40.8 ± 8.9

[a] Mean ± SD

drugs were washed out with fresh medium replacement. Seventy-two hours after the start of drug exposure, 10 μL of WST-8 solution was added to each well and the plates were incubated at 37°C for a further 2 h. Absorbance at a wavelength of 450 nm was measured with a Microplate Reader (TECAN).

The combination effect of indisulam and SN-38 was analyzed by using a modified isobologram method [17, 18]. Briefly, three isoeffect curves (models I, IIA, and IIB), which were based on the growth inhibition curves of indisulam alone and SN-38 alone, were drawn. The total area enclosed by the three lines represented as an "envelope of additivity." When the experimentally observed IC_{50} of a combination was plotted on the left side of this envelope, the combination was considered to show a supra-additive (synergistic) interaction. When the observed IC_{50} was plotted within the envelope, the combination was regarded as additive, and when it was on the right side of the envelope and within the dotted-line square, the combination was considered to be sub-additive. When the observed IC_{50} was plotted outside the square, this combination was considered to be protective.

Results

Indisulam suppresses expression of topoisomerase IIα

Previously, we reported that the topoisomerase IIα gene is a response marker for indisulam [8]. Treatment with indisulam for 24 h partially down-modulated the expression of topoisomerase IIα mRNA in a dose-dependent manner, parallel to sensitivity, in the colorectal cancer cell lines HCT116, SW620, HCT15, and WiDr (Fig. 1b). (The sensitivities to indisulam and SN-38 are summarized in Table 1). Suppression of topoisomerase IIα mRNA was reflected at the protein level. In the relatively sensitive cell lines HCT116 and SW620, topoisomerase IIα suppression was observed with indisulam doses of 62.5 ng/mL or more (Fig. 1c).

Combination effect of indisulam and SN-38 on cultured cell lines

Isobolograms were drawn by using three isoeffect curves (mode I, mode IIA, and mode IIB) based on 24-h growth inhibition curves with indisulam or SN-38 alone (Fig. 2). In the sensitive cell lines HCT116 and SW620, a 24-h drug exposure resulted in a supra-additive effect (Fig. 2a, b), but the same treatment resulted in only an additive effect in the low-sensitivity cell lines HCT15 and WiDr (Fig. 2c, d). These results indicated that there was some interaction between the direct anti-proliferative or cytotoxic effects of indisulam and SN-38, at least in HCT116 and SW620 cells.

Effect of indisulam and SN-38 combination on topoisomerase IIα levels

To address the possibility that the mechanism of synergy of the indisulam and SN-38 combination was related to the retrodirective effect on topoisomerase IIα of indisulam and SN-38, we examined whether indisulam suppressed SN-38–induced topoisomerase IIα. After 24 h of drug exposure, SN-38-induced topoisomerase IIα was observed in all cell lines (Fig. 3a–d). Indisulam co-treatment completely suppressed the upregulation of topoisomerase IIα in HCT116 and SW620 cells. Interestingly and unexpectedly, suppression was observed even at the lowest dose of indisulam (31.3 ng/mL)—a dose at which indisulam alone did not suppress the expression of topoisomerase IIα. In contrast, indisulam in combination with SN-38 did not suppress topoisomerase IIα in HCT15 and WiDr cells (Fig. 3c, d).

Combination effect and effect of prolongation of exposure time on topoisomerase IIα levels

As we previously reported, the antitumor effect of indisulam is exposure time dependent [3, 7]. Prolongation of exposure strengthened the antitumor effect of indisulam (Table 1). Therefore, we examined the effect of a 48-h drug exposure on the level of topoisomerase IIα in HCT15 and WiDr cells. From 24 to 48 h, SN-38 further upregulated topoisomerase IIα. When indisulam and SN-38 were co-administered, indisulam completely canceled out the upregulation from 24 to 48 h (Fig. 4c, d). Furthermore, prolongation of exposure resulted in the combination effect switching from additive to synergistic (Fig. 4a, b). Therefore, suppression of topoisomerase IIα upregulation by SN-38 treatment was coincident with the synergism of the indisulam and SN-38 combination.

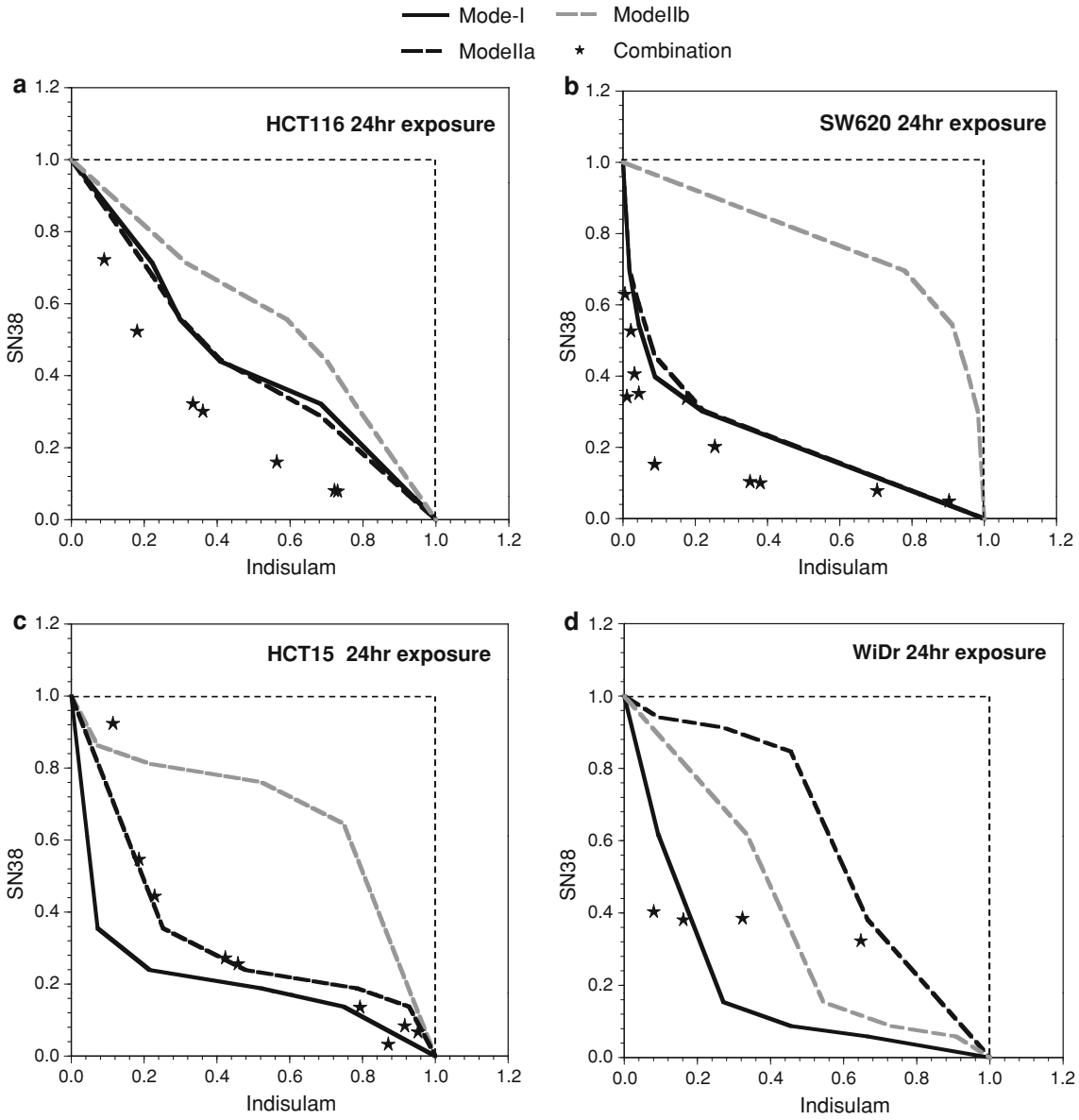

Fig. 2 Isobolograms of indisulam treatment in combination with SN-38 to HCT116, SW620, HCT15, and WiDr cells. **a**, **b**, **c**, and **d** are isobolograms based on the IC_{50} values for 24-h drug exposure in HCT116, SW620, HCT15, and WiDr cells, respectively

Antitumor effect of the indisulam and CPT-11 combination in a human colorectal cancer SW620 xenograft model

The in vivo antitumor effect of the indisulam and CPT-11 combination was evaluated in a human colorectal cancer SW620 xenograft model using three administration schedules: simultaneous administration (indisulam: days 1–5; CPT-11: days 1, 5, and 9); indisulam pre-treatment (indisulam: days 1–5; CPT-11: days 6, 10, and 14); and CPT-11 pre-treatment (CPT-11: days 1, 5, and 9; indisulam: days 10–14). Indisulam alone or CPT-11 alone was administered using a simultaneous dosing schedule (indisulam: days 1–5; CPT-11: days 1, 5, and 9). The simultaneous dosing of tumor regression was recorded on day 21. The RTV values on day 21 were 6.28, 1.83, 2.11, and 0.15 in the control, indisulam 25 mg/kg, CPT-11 62.5 mg/kg, and combination groups, respectively. Two-way ANOVA produced a P value of interaction <0.05. Additionally, this combination effect was significantly superior to monotherapies at their MTDs (indisulam 40 mg/kg; CPT-11 100 mg/kg) (Fig. 5a). These data suggest that this combination effect was synergistic. The combination effects of the other two schedules were comparable to that of the simultaneous schedule (mRTV was 0.15, 0.17, and 0.26 for the simultaneous, indisulam pre-treatment, and CPT-11 pre-treatment schedules, respectively) (Fig. 5a). This suggests that the indisulam and

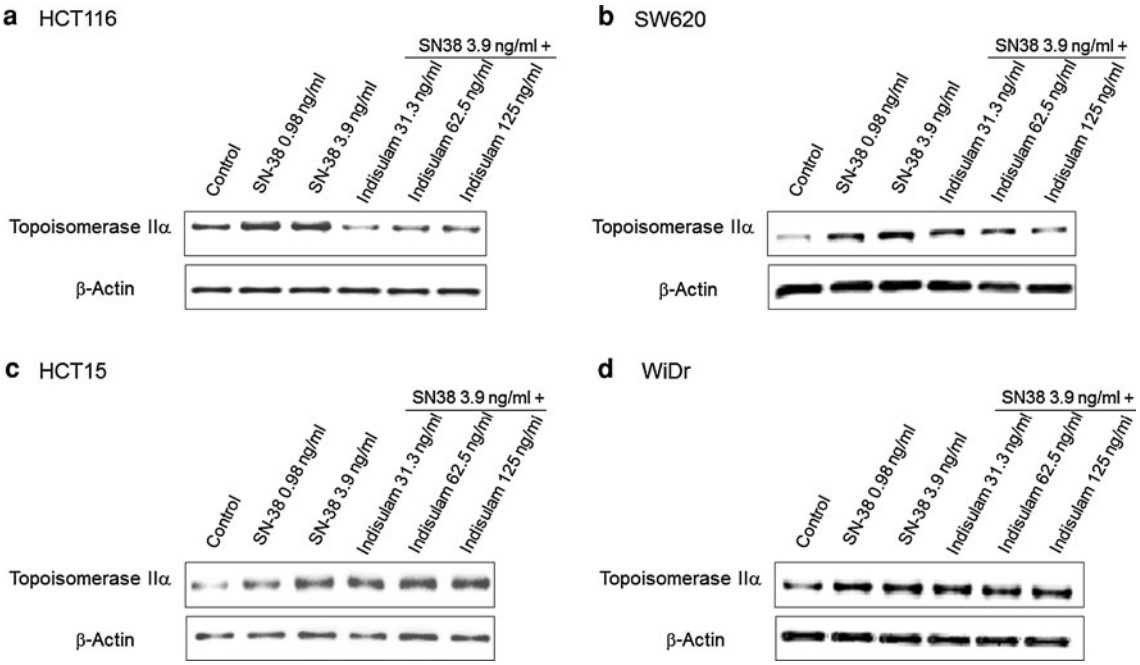

Fig. 3 Changes in intracellular topoisomerase IIα levels induced by treatment of SN-38, indisulam, or their combination. Cells were treated with indisulam, SN-38, or both at the indicated concentrations for 24 h and then cell lysates were prepared. Western blotting was performed using antibodies against topoisomerase IIα and *β*-actin

CPT-11 combination is not dose-schedule dependent. In all treatments, body weight loss was within 10% of initial body weight, and there were no significant differences in body weight loss among the treatments.

We performed a pharmacokinetic study to check whether there were any differences in drug concentrations in blood and tumors between the monotherapy and the combination therapy. There were no significant differences in the levels of indisulam, CPT-11, or SN-38 in blood or tumor between the monotherapy and combination therapy (Online Resource Supplementary Fig. 1a–c). This observation suggests that indisulam and CPT-11 synergy is not caused by a metabolic interaction.

We then compared the levels of topoisomerase IIα in tumors from an SW620 xenograft model administered indisulam and CPT-11 combination therapy, indisulam alone, or CPT-11 alone. Indisulam 25 mg/kg was administered on days 1–5, and CPT-11 62.5 mg/kg was administered on days 1 and 5 in both the combination treatment and the monotherapies. The tumors were removed, and whole tumor extract was prepared 24 h after the final administration (day 6). Indisulam alone did not affect the level of topoisomerase IIα at 25 mg/kg, although indisulam decreased the level of topoisomerase IIα at the MTD (40 mg/kg; data not shown). CPT-11 alone approximately doubled the level of topoisomerase IIα. In combination, indisulam completely suppressed the increase induced by CPT-11 (Fig. 5b). These results show that the indisulam and CPT-11 combination has a synergistic antitumor effect and that the synergy is accompanied by suppression of the topoisomerase IIα upregulation induced by CPT-11 at 25 mg/kg indisulam and 62.5 mg/kg CPT-11 (62.5% of MTD).

Finally, we examined the synergy between indisulam and CPT-11 at two different dose ratios: (1) 20 mg/kg indisulam and 75 mg/kg CPT-11 (50% MTD indisulam and 75% MTD CPT-11) and (2) 30 mg/kg of indisulam and 50 mg/kg of CPT-11 (75% MTD indisulam and 50% MTD CPT-11). In both combination ratios, synergy was observed (Fig. 5c).

Discussion

Various studies have examined the relationship between topoisomerase expression and topoisomerase inhibitors. Some of these reports have concluded that, in cell lines resistant to topoisomerase I inhibitors or IIα inhibitors, the targeted topoisomerase is often downregulated or mutated, with a reciprocal increase in the activity and/or level of the non-targeted topoisomerase [13–15, 19–21]. Furthermore, longer lasting upregulation of topoisomerase IIα has been observed in cell lines that are more resistant to SN-38 [11]. DNA topoisomerase I and II are functionally related nuclear enzymes that, in concert, catalyze the relaxation of supercoiled chromosomal DNA during DNA replication.

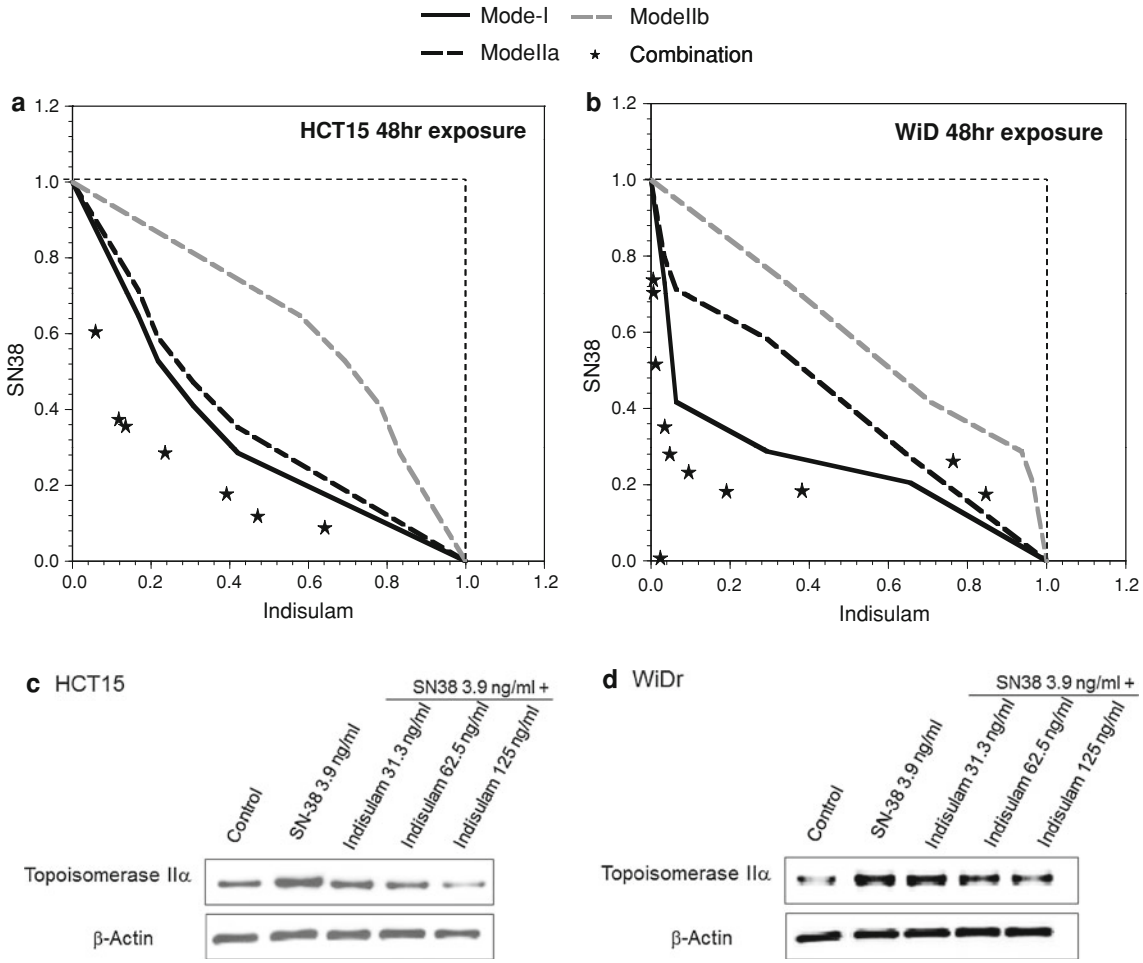

Fig. 4 Change in intracellular topoisomerase IIα levels and isobolograms with 48 h of drug exposure. **a** and **b** are isobolograms based on the IC_{50} values for 48 h of drug exposure in HCT15 and WiDr cells, respectively. **c** and **d**, HCT15 and WiDr cells were treated with indisulam, SN-38, or both at the indicated concentrations for 48 h, and cell lysates were prepared. Western blotting was performed using antibodies against topoisomerase IIα and *β*-actin

The relaxation of DNA by either topoisomerase I or II involves the transient single- or double-strand breakage of DNA, followed by strand passage and relegation of the DNA strand. The topoisomerases are extensively involved in DNA replication, transcription, and recombination, and in sister chromatin segregation, and as such are essential in maintaining cell viability [22]. That is, topoisomerase I and topoisomerase IIα share the same essential function—the regulation of DNA structure.

Previously, we reported that the expression of 13 genes downregulated by indisulam treatment was closely associated with the antitumor action of indisulam because of significant correlations between rank orders of their transcriptional repression and growth suppression in 36 drug-treated human cancer cell lines [8]. From these results, we hypothesized that upregulation of topoisomerase IIα is a compensatory cellular response to escape from the toxicity of topoisomerase I inhibitors and that indisulam enhances the antitumor effect of topoisomerase I inhibitors by suppressing the topoisomerase IIα upregulation induced by topoisomerase I inhibitors.

Indisulam and SN-38 combination showed a supra-additive effect in vitro, and this synergistic effect was coincident with indisulam's suppression of SN-38-induced topoisomerase IIα. Therefore, our hypothesis appears valid.

There is no molecular-based explanation for the upregulation of topoisomerase IIα by topoisomerase I inhibitors. Despite some reports that this upregulation of topoisomerase IIα occurs at the mRNA level [11, 13], SN-38 did not increase the levels of topoisomerase IIα mRNA in our study using SW620 cells. By contrast, SN-38 temporally decreased the level of topoisomerase IIα mRNA to about 50% for 6 h after the start of treatment, after which the level of topoisomerase IIα mRNA returned to the basal level within 24 h. The level of topoisomerase IIα was increased 24 h after administration. In the authors' opinion, the

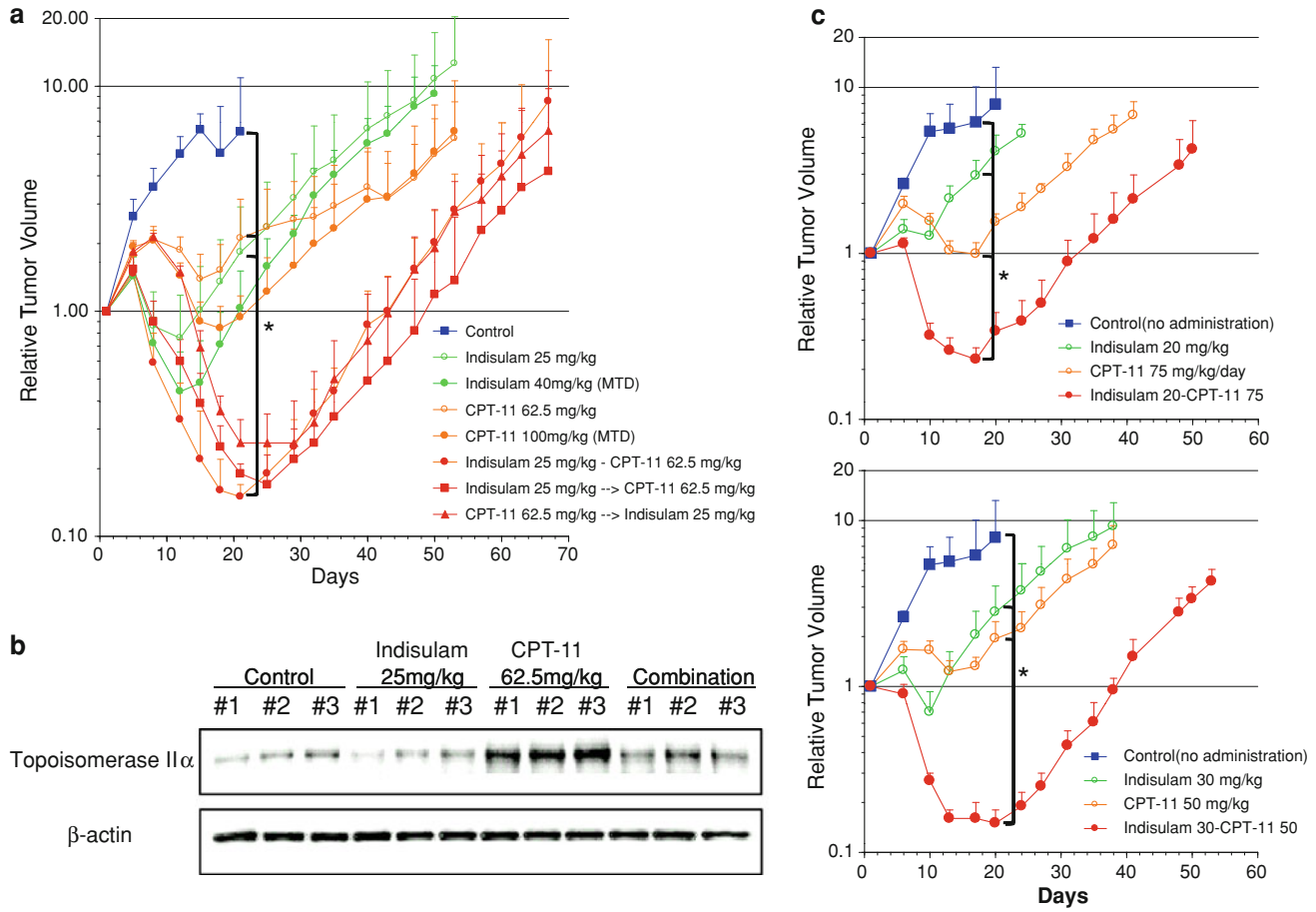

Fig. 5 Antitumor effects and changes in topoisomerase IIα level after treatment with indisulam and CPT-11 combination in a human colorectal cancer SW620 xenograft model in mice (**a**), SW620 cells (5×10^6) were subcutaneously injected into athymic nude mice. Treatment was started 15 days after implant, when the mean tumor size was 226 mm^3 (day 1). There were 6 mice per group. Indisulam alone was administered on days 1–5 at 25 mg/kg (*green open circles*) and 40 mg/kg (*green solid circles*). CPT-11 alone was administered on days 1, 5, and 9 at 62.5 mg/kg (*orange open circles*) or 100 mg/kg (*orange solid circles*). For the combination treatments, 25 mg/kg indisulam and 62.5 mg/kg CPT-11 were administered in accordance with three dosing schedules: simultaneous (indisulam 25 mg/kg and CPT-11 62.5 mg/kg; *red solid circles*); indisulam pre-treatment (indisulam 25 mg/kg → CPT-11 62.5 mg/kg; *red solid squares*); and CPT-11 pre-treatment (CPT-11 62.5 mg/kg → indisulam 25 mg/kg; *red solid triangles*). Control animals (*blue squares*) were not treated. The *graph* indicates the changes in relative tumor volume (RTV, mean ± SD). **b** SW620 cells (5×10^6) were subcutaneously injected into athymic nude mice. Treatment was started when the mean tumor size was more than 150 mm^3 (day 1). In the indisulam monotherapy group and combination group, 25 mg/kg of indisulam was administered on days 1–5. In the CPT-11 monotherapy group and combination group, 62.5 mg/kg of CPT-11 was administered on days 1 and 5. Twenty-four hours after the final administration, tumors were removed and lysates were prepared. Topoisomerase IIα and *β*-actin were detected by Western blotting. **c** SW620 cells (5×10^6) were subcutaneously injected into athymic nude mice. Treatment was started 15 days after implant, when the mean of tumor size was 226 mm^3 (day 1). All administrations followed the simultaneous schedule described in (**a**). Combinations of indisulam 20 mg/kg and CPT-11 75 mg/kg (**c** upper), and indisulam 30 mg/kg and CPT-11 50 mg/kg (**c** lower) were tested (indisulam alone, *green open circles*; CPT-11 alone, *orange open circles*; combination, *red solid circles*). Control animals (*blue solid squares*) were not treated. The *graph* indicates change in RTV (mean ± SD). *$P < 0.05$ (interaction with two-way ANOVA)

upregulation of topoisomerase IIα by topoisomerase I inhibitors is caused not only by the increase in mRNA level, but also by protein stability. Indisulam suppressed the recovery of topoisomerase IIα mRNA after temporary downregulation (Online Resource, Supplementary Fig. 2). This suppression of the recovery of the mRNA level may lead to suppression of protein production. It is possible that indisulam also decreases the stability of topoisomerase IIα. Although the exact mode of action of the antitumor effect of indisulam is not yet understood, screening of the indisulam-binding protein has revealed that indisulam binds to NADH-dependent cytosolic malate dehydrogenase (cMDH) and lactate dehydrogenase (LDH) and inhibits their intrinsic dehydrogenase activities by competing with NADH [23]. cMDH and LDH are important enzymes in energy metabolism, especially in the glycolysis pathway. Topoisomerase IIα is sensitive to the energy status of cells, and glucose deprivation induces the quick degradation of

topoisomerase IIα [24, 25]. Therefore, it is likely that indisulam induces degradation of topoisomerase IIα by disrupting energy metabolism through the inhibition of these dehydrogenases.

Interestingly, indisulam suppressed SN-38–induced topoisomerase IIα expression, even at low doses (31.3 ng/mL), whereas indisulam alone did not suppress the steady-state level of topoisomerase IIα expression at either the mRNA or the protein level. Western blotting using tumor xenografts gave similar data. Furthermore, even at 20 mg/kg indisulam, which is 50% of the MTD, indisulam enhances the antitumor effect of CPT-11 in vivo. These observations suggest that the induction pathway of topoisomerase IIα is more sensitive to indisulam than the regulation pathway of steady-state topoisomerase IIα. Similarly, synergism has been reported with combinations of topoisomerase I inhibitors and topoisomerase II inhibitors [26]. Mammalian DNA topoisomerase II is the primary target of a number of antitumor agents, such as doxorubicin, daunorubicin, VP-16, and amsacrine [27]. These agents interfere with the breakage–reunion reaction of DNA topoisomerase II by trapping a covalent enzyme–DNA complex, called the *cleavable complex*, in which DNA strands are broken and their 5′ termini are covalently linked to the protein. The amount of cleavable complex is associated with the cytotoxicity of topoisomerase II inhibitors. Topoisomerase I inhibitors upregulate topoisomerase IIα and treatment with topoisomerase II inhibitors results in increased formation of the cleavable complex. Consequently, the scheduling of therapy with a combination of CPT-11 and a topoisomerase II inhibitor is critical for success [28]. Sequential administration of CPT-11 followed by a topoisomerase II inhibitor has led to synergistic cytotoxicity, whereas concurrent administration has led to antagonism [29]. On the other hand, schedule dependency was not observed with the CPT-11 and indisulam combination. CPT-11 and indisulam may each enhance the antitumor effect of the other. In other words, indisulam enhances the antitumor effect of CPT-11 by suppressing the compensatory escape pathway, and CPT-11 may also enhance the antitumor effect of indisulam by increasing cellular dependency on the highly sensitive indisulam signal pathway.

Indisulam and CPT-11 combination therapy has the potential to be safe. One principle for the successful combination of chemotherapies in the clinical setting is to choose drugs that have different toxicity spectra [30]. The toxicity that limits the dose of CPT-11 in the clinic is grade 4 diarrhea [31], whereas the toxicity that limits the dose of indisulam is myelosuppression [32–34]. Additionally, the level of topoisomerase IIα expression may be a sensitive predictor for synergy in the clinical setting. Studies performed with specimens obtained from patients treated with topoisomerase I inhibitors indicate that topoisomerase IIα is upregulated in tumor tissue and peripheral blood cells [35–37]. Therefore, topoisomerase IIα expression could be a useful biomarker of indisulam and CPT-11 synergy in the clinical setting.

In summary, indisulam and CPT-11 administered in combination demonstrated synergistic interaction both in vitro and in vivo with a rationalized mechanism. There was no overlap in toxicities between indisulam and CPT-11. There was no metabolic drug–drug interaction between indisulam and CPT-11 in mice. These findings suggest that the combination of indisulam and CPT-11 is promising for cancer therapy. Clinical studies of this combination are ongoing.

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Conflict of interest None.

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