Effect of a novel oral chemotherapeutic agent containing a combination of trifluridine, tipiracil and the novel triple angiokinase inhibitor nintedanib, on human colorectal cancer xenografts

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Abstract. Trifluridine/tipiracil (TFTD) is a combination drug that is used for the treatment of metastatic colorectal cancer and was formerly known as TAS-102. It is a combination of two active pharmaceutical compounds, trifluridine, an antineoplastic thymidine-based nucleoside analog, and tipiracil, which enhances the bioavailability of trifluridine *in vivo*. TFTD is used for the treatment of patients with unresectable advanced or recurrent colorectal cancer that is resistant to standard therapies. In the present study, the anticancer effects of trifluridine in combination with nintedanib, an oral triple angiokinase inhibitor, on human colorectal cancer cell lines were investigated. The cytotoxicity against DLD-1, HT-29, and HCT116 cell lines was determined by the crystal violet staining method. The combination of trifluridine and nintedanib exerted an additive effect on the growth inhibition of DLD-1 and HT-29 cells and a sub-additive effect on HCT116 cells, as determined by isobologram analyses. Subsequently, the human colorectal cancer cell lines were implanted subcutaneously into nude mice to allow the evaluation of the *in vivo* tumor growth inhibitory effects of TFTD and nintedanib combination therapy. TFTD (150 mg/kg/day) and/or nintedanib (40 mg/kg/day) were orally administered to the mice twice daily from day 1 to day 14. The tumor growth inhibition with combination therapy was 61.5, 72.8, 67.6 and 67.5% for the DLD-1, DLD-1/5-FU, HT-29, and HCT116 xenografts, respectively. This was significantly ($P<0.05$) higher than the effects of monotherapy with either TFTD or nintedanib. These results demonstrated the effectiveness of the combination of TFTD and nintedanib in the treatment of colorectal cancer xenografts. The concentration of trifluridine incorporated into DNA in the HT-29 and HCT116 tumors was determined by liquid chromatography-tandem mass spectrometry. The incorporation levels following treatment with TFTD and nintedanib for 14 consecutive days were higher than those associated with TFTD treatment alone. The preclinical findings indicate that the combination therapy with TFTD and nintedanib is a promising treatment option for colorectal cancer.

Introduction

Trifluridine/tipiracil (TFTD) (formerly used with the code name TAS-102) is a novel antitumor therapeutic agent (1,2). It comprises a mixture of two distinct chemicals, trifluridine and tipiracil (TPI), at a molar ratio of 1:0.5. Trifluridine is an analog of thymidine that exhibits two distinct mechanisms of antitumor action. It inhibits the enzyme thymidylate synthase (3) and it intercalates with DNA (4). TPI enhances the bioavailability of trifluridine by the inhibition of the *in vivo* degradation of the latter compound by the enzyme thymidine phosphorylase. Consequently, TPI can produce a more durable and sustainable response to trifluridine (5).

The antitumor effects of TFTD on colon cancer xenograft models resistant to 5-fluorouracil (5-FU) involve notably the incorporation of trifluridine in DNA (6). The primary cytotoxic mechanism of TFTD at twice-daily oral dosing (the dose of administration used clinically) is thought to cause DNA incorporation of trifluridine (7). The effect of TFTD on metastatic colorectal cancer in patients who were resistant to, and/or intolerant of, standard chemotherapies was recently evaluated in a randomized phase II clinical trial (8). The overall survival (OS) period of the patients who received TFTD with the best supportive care was significantly longer than the OS period of patients who received the corresponding placebo with the best supportive care (8). Furthermore, TFTD significantly prolonged

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Abbreviations: 5-FU, 5-fluorouracil; HIF, hypoxia inducible factor; BWC, body weight change; OS, overall survival; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PFS, progression-free survival; RTV, relative tumor volume; TGI, tumor growth inhibition; TFTD, trifluridine and tipiracil; TPI, tipiracil; VEGF, vascular endothelial growth factor

Key words: TAS-102, trifluridine, tipiracil, nintedanib, colorectal cancer

the OS period (median OS, 7.1 months; 95% CI, 6.5-7.8 months vs. median OS, 5.3 months; 95% CI, 4.6-6 months for placebo) and progression-free survival (PFS) in patients with metastatic colorectal cancer refractory to standard chemotherapies, as demonstrated by an international multi-center randomized double-blind phase III clinical study (RECOURSE study) (9). In addition, the study indicated that TFTD exhibited a favorable safety profile. These results led to the regulatory approval of the drug in the USA and recently, in Europe.

Tumor angiogenesis is a complex process that represents a perturbed balance of highly regulated proangiogenic and antiangiogenic mechanisms (10). Vascular endothelial growth factor (VEGF) is considered to be one of the most important factors involved in tumor angiogenesis (11). Bevacizumab is a monoclonal antibody that blocks angiogenesis by binding to VEGF-A (a ligand for VEGFR1 and VEGFR2). It was the first antiangiogenic agent approved for cancer therapy. The major challenges to the success of antiangiogenic therapy include the associated toxicity risks, the limitation of efficacy through the possible development of resistance, and the induction or promotion of metastatic progression (12,13). Nintedanib is an oral triple angiokinase inhibitor that simultaneously inhibits VEGFs, platelet-derived growth factor receptors, and fibroblast growth factor receptor signaling pathways (14). Nintedanib has demonstrated significant activity against several tumor types in preclinical studies. An alternating regimen of nintedanib (250 mg, twice daily) and then afatinib (50 mg, once daily) was evaluated in patients with advanced pretreated colorectal cancer in a phase II clinical study (15). The median PFS was 1.9 months and the median OS was 5.5 months. Nintedanib in combination with mFOLFOX6 showed efficacy as a first-line therapy in a phase II clinical study that included patients with metastatic colorectal cancer (16). Furthermore, nintedanib with mFOLFOX6 exhibited a favorable safety profile (16,17). A double-blind, randomized, phase III study of nintedanib vs. placebo in refractory colorectal cancer is currently ongoing (NCT02149108).

TFTD in combination with irinotecan hydrochloride (18), oxaliplatin (19), bevacizumab, cetuximab, or panitumumab (20) exhibited superior *in vivo* activity against human colorectal cancer, including 5-FU-resistant tumors, compared with any of these drugs alone, as demonstrated by previous studies. In the present study, the effects of TFTD in combination with nintedanib against human colorectal tumor xenografts in a nude mouse model were evaluated. The present study provides additional evidence for the therapeutic options for human colorectal cancer.

Materials and methods

Reagents. Trifluridine and TPI were obtained from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Nintedanib was purchased from Medchem Express (Monmouth Junction, NJ, USA). Hydroxypropyl methylcellulose (HPMC) was obtained from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan).

Cancer cell lines. The human colon cancer cell line HT-29 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human colorectal carcinoma DLD-1 and HCT116 cells were purchased from Dainippon Pharma (Osaka, Japan). The 5-FU-resistant cell line DLD-1/5-FU was established using a long-term culture in the presence of 5-FU *in vitro* (21). These cell lines were cultured in RPMI-1640 (HT-29, DLD-1 and DLD-1/5-FU) or Dulbecco's modified Eagle's cell culture media (DMEM) (HCT116) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 5% CO_2 in air. HT-29 cells possess a wild-type *kras* status, whereas DLD-1 and HCT116 cells a mutant.

Animals. Five-week-old male nude mice (BALB/c *nu/nu*) were purchased from Clea Japan (Tokyo, Japan) and were housed under specific pathogen-free conditions, with food and water provided *ad libitum*. The procedures of all animal studies were performed according to the protocol and guidelines of the Institutional Animal Care and Use Committee of Taiho Pharmaceutical Co. Ltd. Ethical approval was obtained prior to execution of the animal experimentation.

Cytotoxicity assay and evaluation of the combination effect in vitro. The drug cytotoxicity was measured with the crystal violet assay (22). The cells (2,000-4,000) were cultured in a 96-well microplate with 100 μl medium per well for 24 h. Trifluridine and nintedanib were dissolved at the concentrations of 10 mM in dimethyl sulfoxide and the corresponding solutions were prepared using the culture medium under aseptic conditions. A total of 100 μl of the drug solution (trifluridine: 0.18-10 μM; nintedanib: 0.18-10 μM) were added into the culture medium. Following incubation of the plates for 72 h, the culture medium was removed and the cells were fixed with 4% glutaraldehyde for 30 min. The fixed cells were stained with 0.1% crystal violet for 2 min and washed and dissolved in 0.05 M NaH_2PO_4/50% ethanol. The absorbance was measured at a wavelength of 540 nm using a microplate reader (Spectra MAX 190; Molecular Devices, Tokyo, Japan).

The cytotoxic effects of the trifluridine and nintedanib combination were analyzed using the isobologram method (23). A total of 3 isoeffect curves (modes I, IIa, and IIb), based on the growth inhibition curves of trifluridine alone and nintedanib alone, were drawn. The total area enclosed by the three curves represented an 'envelope of additivity'. The combination of drug treatment was considered to show a supra-additive (synergistic) interaction, when the experimentally observed IC_{50} values were included in the left side of the envelope, whereas when the IC_{50} values were included in the envelope, the combination was considered as additive. The combination was considered to be sub-additive, when the IC_{50} values were included on the right side of the envelope and were within the dotted line square. Finally, when the IC_{50} values fell outside the square, the combination was considered to be protective.

In vivo antitumor activity. The cancer cell lines (DLD-1, DLD-1/5-FU, HT-29, or HCT116) were transplanted subcutaneously into the dorsal region of each nude mouse at a density of $4x10^6$ cells/mouse. Following 1 week of cell growth, the animals were grouped so as to possess a uniform mean and a standard deviation of the tumor volume (calculated using the equation below). Each group consisted of 6 mice at day 0.

TFTD was prepared by mixing trifluridine and TPI at a molar ratio of 1:0.5 in 0.5% HPMC solution. The dose of TFTD

was expressed on the basis of the trifluridine content. TFTD was administered orally from day 1 to 14, twice a day at 6-h intervals at the reported effective dose (150 mg/kg/day) (6). Nintedanib was administered orally from day 1 to 14, twice a day at 6-h intervals at the reported effective dose (40 mg/kg/day) (14,24). The vehicle solution that consisted of 0.5% HPMC solution was administered at 10 ml/kg to the control mouse group, following the same administration schedules as for the test drugs.

The tumor diameters were measured twice a week, and the tumor volume (V) was estimated as: $V = 0.5 \times \text{length} \times \text{width}^2$. The relative tumor volume (RTV) was calculated using the following formula: RTV = (tumor volume on the measured day)/(tumor volume on day 0). The tumor growth inhibition ratio (TGI, %) was calculated using the following formula: TGI (%) = [1 - (RTV of the treated group)/(RTV of the control group)] x 100 (%). The antitumor effect of the drugs, based on the RTV measurements, was evaluated 24 h after the final drug administration (day 15).

The change in the body weight (BWC) was used for the determination of the toxicity caused by the drug treatments. BWC was calculated using the following formula: BWC (%) = [(body weight on the last day) - (body weight on day 0)]/(body weight on day 0) x 100 (%). Toxicity was defined as a BWC indicating a weight loss of >20%, or toxic death. The experimental endpoint was defined as the day on which the average tumor volume in the average body weight within each group reached more than 10%.

Extraction and quantification of trifluridine incorporated into tumor DNA. TFTD monotherapy and TFTD combined with nintedanib were administered from day 1 to 14 to the nude mice bearing HT-29 and HCT116 xenografts. On day 15 the mice were sacrificed, the tumor diameters corresponding to each mouse were measured and the tumors were stored in liquid nitrogen. The genomic DNA of the HT-29 and HCT116 tumor cells was extracted and purified using a Getpure DNA kit (Dojindo Molecular Technology, Kumamoto, Japan) following the manufacturer's instructions. The purified DNA was completely digested by DNase I and alkaline phosphatase enzymes to the deoxyribonucleoside level (including trifluridine) according to previously described methods (25,26). The samples were then prepared for LC/MS/MS analysis as follows. An aliquot that consisted of water (10 μl), 1 M hydrochloric acid (10 μl) and internal standard (20 μl) was added to a 100-μl aliquot of sample. The mixture was extracted with 1 ml of methyl t-butyl ether followed by centrifugation (15,000 x g, 5˚C, 5 min). The supernatant was dried under nitrogen at 40˚C and the residue was reconstituted with 0.1 ml of mobile phase that consisted of 0.1% acetic acid/acetonitrile (75/25, v/v). A 5-μl aliquot of the reconstituted sample was injected into an API 4000 LC/MS/MS system (AB Sciex, Foster City, CA, USA).

Statistical analysis. The differences in the mean RTV between the treated and control groups on day 15 were assessed using the Aspin-Welch two-sided t-test (27). The combinatorial antitumor effect of TFTD and nintedanib was analyzed using the Aspin-Welch two-tailed t-test. The statistical significance was determined at $P<0.05$ and the P-values were calculated using

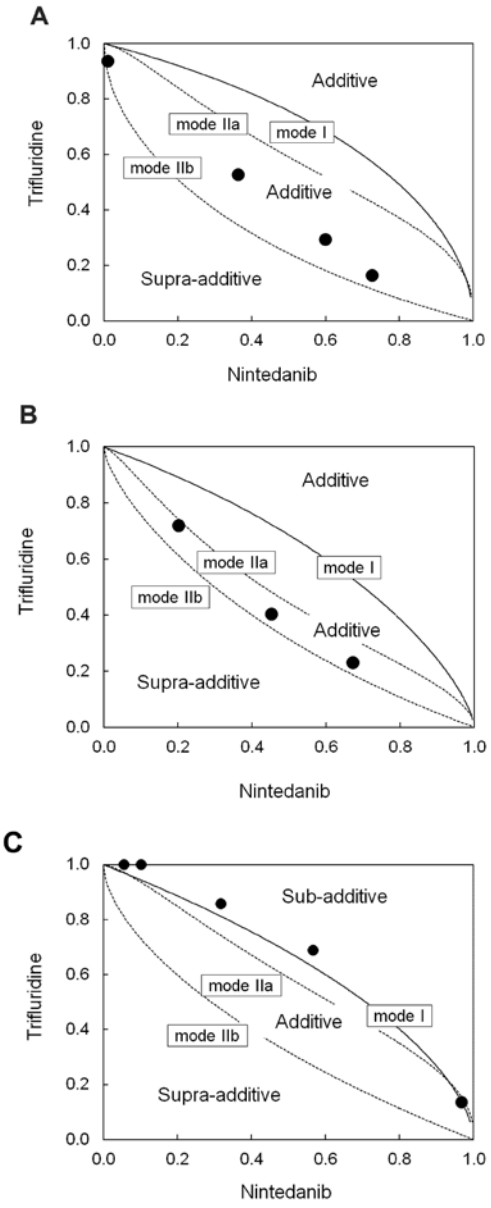

Figure 1. The isobologram plots of trifluridine (active compound of TFTD) treatment in combination with nintedanib on DLD-1, HT-29 and HCT116 cancer cells (A-C). Isobologram plots based on the IC_{50} values for the 72 h drug exposure in DLD-1, HT-29, and HCT116 cancer cells, respectively. The axes correspond to the fractional inhibitory concentrations (FIC).

EXSUS, ver. 8.1 (CAC Exicare Corp., Osaka, Japan). The differences in the trifluridine-mediated DNA incorporation between the trifluridine and nintedanib-treated group and the trifluridine group were assessed using the Student's one-sided t-test.

Results

Combination effect of trifluridine and nintedanib on colorectal cancer cell lines in vitro. The isobologram plots were drawn using three isoeffect curves (mode I, mode IIa, and mode IIb) based on the 72-h growth inhibition curves for DLD-1, HT-29, and HCT116 cells (Fig. 1A-C) with trifluridine or nintedanib alone. Based on available dose-response curves, we analyzed the combined effect of the two drugs at the points of IC_{50}. The

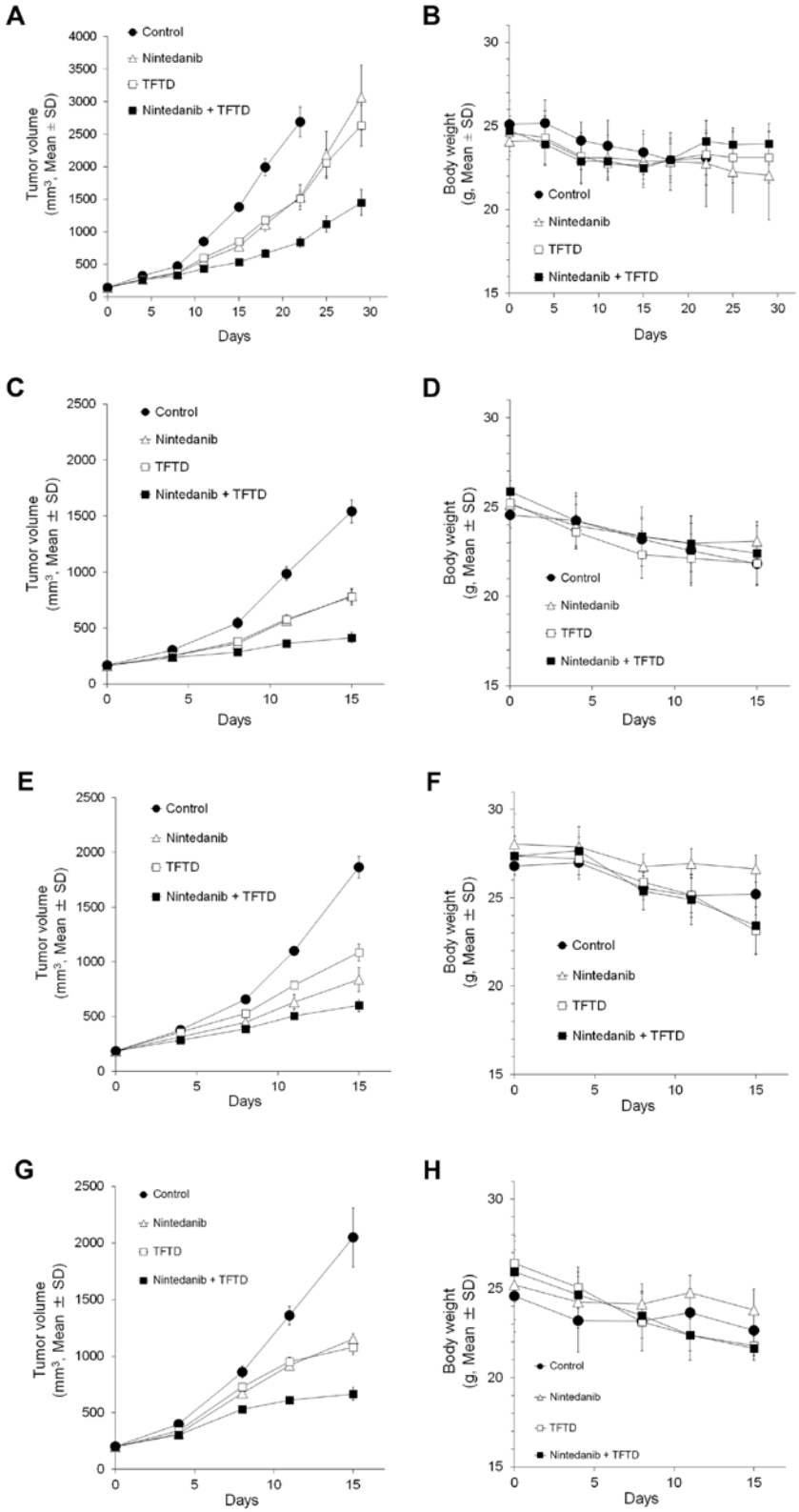

Figure 2. DLD-1 (A and B), DLD-1/5-FU (C and D), HT-29 (E and F), and HCT116 (G and H) cancer cells were transplanted subcutaneously into the dorsal region of nude mice. Tumor volume change in human colorectal tumors (A, C, E and G) and body weight change in tumor-bearing nude mice (B, D, F and H). The mice were treated with vehicle (0.5% HPMC, 10 ml/kg, orally twice daily from days 1 to 14, ●), nintedanib (40 mg/kg, orally twice daily from days 1 to 14, △), TFTD (150 mg/kg, orally twice daily from days 1 to 14, □), or a combination of TFTD and nintedanib, ■). The values indicate the mean ± SD (n=6). The tumor volume and body weight were measured twice weekly.

IC_{50} values for trifluridine in DLD-1, HT-29, and HCT116 cells were $4.3x10^{-6}$, $3.8x10^{-6}$, and $1.8x10^{-6}$ M respectively, whereas the corresponding IC_{50} values for nintedanib were $3.4x10^{-6}$, $1.4x10^{-6}$ and $2.5x10^{-6}$ M, respectively. In the DLD-1 and HT-29 cells, a 72-h exposure to the combination treatment resulted in an additive effect (Fig. 1A and B). In the HCT116 cells

Table I. BWC in mice implanted with human colorectal DLD-1, DLD-1/5-FU, HT-29, and HCT116 tumor cells after treatment with TFTD and nintedanib.

Groups	Dose (mg/kg)	Tumors			
		DLD-1	DLD-1/5-FU	HT-29	HCT116
Control		-6.7±3.6	-11.1±2.2	-6.0±3.2	-7.5±5.8
Nintedanib	40	-4.9±4.8^{ns}	-8.0±4.8^{ns}	-4.8±4.0^{ns}	-5.2±6.8^{ns}
TFTD	150	-7.8±6.2^{ns}	-13.5±3.5^{ns}	-15.4±5.8^{ns}	-17.3±5.2^{ns}
Combination	40+150	-9.0±2.4^{ns}	-13.5±4.4^{ns}	-14.4±3.8^{ns}	-16.3±5.1^{ns}

Data represent the body weight change (BMC) in mice from day 0 to day 15 (%, mean ± SD) in the different tumors. ^{ns}Not significant vs. control using two-sided Aspin-Welch t-test.

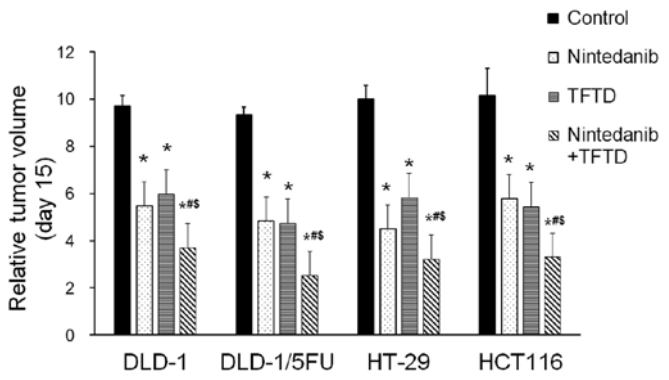

Figure 3. The relative tumor volume exhibited in the human colorectal DLD-1, DLD-1/5-FU, HT-29 and HCT116 tumors, following administration of the drug treatment. The mice were administered with control (0.5% HPMC, 10 ml/kg, orally twice daily from days 1 to 14), nintedanib (40 mg/kg, orally twice daily from days 1 to 14), TFTD (150 mg/kg, orally twice daily from days 1 to 14), or a combination of TFTD and nintedanib (150 mg/kg and 40 mg/kg, respectively. Both therapies were administered orally twice daily from days 1 to 14). The tumor volumes were measured at 24 h after the final administration of the therapies (day 15). The values indicate the mean ± SD (n=6). *P<0.01 vs. the control group. $^{\#}$P<0.01 vs. the nintedanib monotherapy group. $^{\$}$P<0.01 vs. the TFTD monotherapy group.

the aforementioned combination treatment resulted in a sub-additive effect (Fig. 1C).

Antitumor efficacy of TFTD/nintedanib combination therapy in vivo. The *in vivo* efficacy of TFTD monotherapy, nintedanib monotherapy, and TFTD and nintedanib combination in human colorectal cancer xenograft models was evaluated.

Nude mice bearing DLD-1 tumors were treated with 150 mg/kg TFTD, 40 mg/kg nintedanib, or a combination of TFTD and nintedanib for 14 consecutive days. On day 15, TFTD monotherapy and nintedanib monotherapy resulted in a significant reduction in tumor growth *in vivo* (P<0.01) (Fig. 2A). In addition, the combination therapy exhibited greater antitumor activity than both monotherapies.

The efficacy of the aforementioned treatments was evaluated in nude mice bearing tumors that were derived from 5-FU-resistant human colorectal cancer cells, DLD-1/5-FU (Fig. 2C). TFTD monotherapy and nintedanib monotherapy resulted in a significant reduction in tumor growth *in vivo* (P<0.01). The antitumor efficacy of both monotherapies was similar between the 5-FU-resistant DLD-1 cells and the parent DLD-1 cells. This indicated that no cross-resistance had occurred between DLD-1/5-FU and either of the monotherapies. The TFTD/nintedanib combination therapy exhibited greater antitumor activity against DLD-1/5-FU compared with the antitumor activity exhibited by both monotherapies. Thus, the combination therapy showed a similar antitumor effect against the DLD-1/5-FU (tumor growth inhibition rate 72.8%) and the DLD-1 (tumor growth inhibition rate 61.5%) tumors (data not shown).

The efficacy of the above treatments was further evaluated in the HT-29 (Fig. 2E) and HCT116 (Fig. 2G) xenograft models. TFTD and nintedanib monotherapies both significantly suppressed tumor growth when compared with control (P<0.01). The combination therapy significantly suppressed tumor growth when compared to each monotherapy (P<0.01).

Fig. 3 summarizes the antitumor effects of the administered therapies as evaluated by the mean RTV at day 15. The antitumor activity of the TFTD/nintedanib combination therapy, for all human colorectal cancer xenografts, was significantly greater than that of either monotherapy (P<0.01).

The tumor volume and body weight of the mice were monitored following the evaluation of the antitumor effects caused by the administration of the compounds. The tumor volume of the drug-free control DLD-1 group exceeded 10% of the body weight loss of each animal on day 22 (data not shown). All mice in the control group were immediately euthanized because the tumor burden had exceeded a human endpoint (the experimental endpoint described in Materials and methods). The evaluation and monitoring was not carried out beyond day 15 for any of the drug treatments of DLD-1 (Fig. 2A) and/or the control or drug treatments of the HT-29, DLD-1/5-FU and HCT116 tumor xenografts (Fig. 2C-H) since it was anticipated that, in these cases, the tumor burden would reach the experimental endpoint.

In the present study no severe adverse events were noted for all TFTD-treated xenograft models, including a greater than 20% reduction in the body weight, diarrhea, or death, due to toxicity. Thus, any potential toxic effects of TFTD were well-tolerated. Notably, the superior antitumor efficacy of the TFTD/nintedanib combination was not associated with any significant increase in body weight loss (Table I).

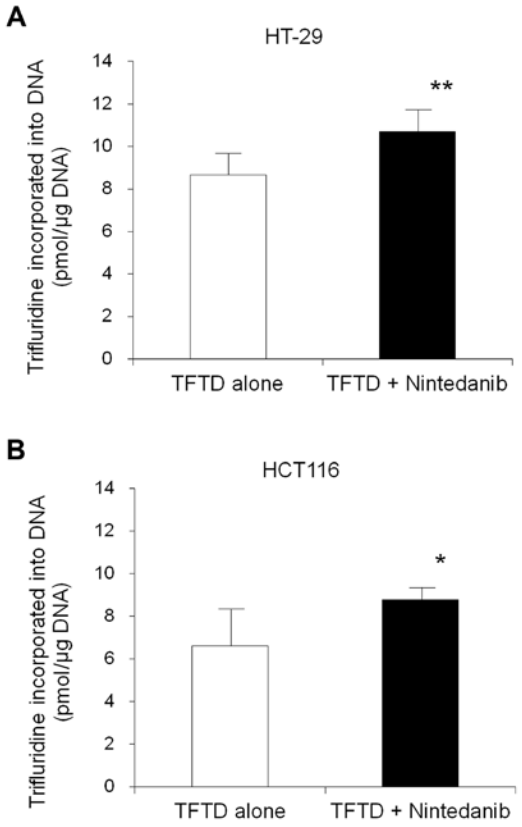

Figure 4. The concentration of trifluridine incorporated in the DNA in the human colorectal HT-29 (A) and HCT116 (B) xenografts. The mice were treated with TFTD alone (150 mg/kg, orally twice daily from days 1 to 14, open column), or a combination of TFTD and nintedanib (closed column). The HT-29 and HCT116 tumors were collected at 24 h following the final administration of the drugs (day 15). The values indicate the mean ± SD (n=6). *P<0.05, **P<0.01 by the Aspin-Welch two-tailed t-test compared to the TFTD monotherapy group.

Trifluridine-mediated DNA incorporation in HT-29 and HCT116 tumors after TFTD/nintedanib combination therapy. The amount of trifluridine that was incorporated into the DNA of HT-29 and HCT116 tumors that had been exposed to both treatments, was assessed, in order to provide insight in the mechanism underlying the efficacy noted by the TFTD/nintedanib combination therapy, compared with the TFTD monotherapy. The incorporation of trifluridine following treatment with TFTD alone for 14 consecutive days was measured as 8.7±1.0 and 6.6±1.7 (pmol/μg DNA) in the HT-29 and HCT116 tumors, respectively. The corresponding values for the TFTD/nintedanib treatment was 10.7±1.0 and 8.8±0.6 (pmol/μg DNA), respectively (Fig. 4). These values were significantly higher than those of the TFTD monotherapy group.

Discussion

The present study evaluated i) the antitumor effects of trifluridine, the antineoplastic agent of TFTD (trifluridine/tipiracil mixture), in combination with nintedanib on human colorectal tumors *in vitro* and ii) the antitumor effects of TFTD in combination with nintedanib on human colorectal tumors *in vivo*. The combination of trifluridine and nintedanib exerted an additive effect on the growth inhibition of DLD-1 and HT-29 cells, and a sub-additive effect on the growth inhibition of HCT116 cells *in vitro*. The exact cause of the sub-additive effect noted in the HCT116 cells is unknown. However, the TFTD/nintedanib combination therapy was superior to the drug monotherapies. In addition, the TFTD/nintedanib combination therapy suppressed the growth of the HT-29, HCT116, DLD-1 and DLD-1/5-FU cells, in nude mice. This suppression was significantly greater than the effects of the monotherapies. Notably, this antitumor activity occurred in the absence of any increased toxicity.

A total of three of the colorectal cancer cell lines used in the present study carry a KRAS mutation (HT-29 DLD-1 and HCT116). In the present study, the trifluridine and nintedanib monotherapies and the TFTD/nintedanib combination therapy indicated a similar anticancer activity *in vitro* and *in vivo*, irrespective of the *KRAS* status of the colorectal cancer cell lines (Figs. 1A-C and 2A, E and G). TFTD has been shown to improve overall survival in a clinical setting regardless of *KRAS* tumor status that is consistent with these results (8,9), Consequently, the combination therapy with TFTD and nintedanib may also be useful in the clinical treatment of colorectal tumors irrespective of the *KRAS* mutation status.

DLD-1/5-FU is a 5-FU-resistant clone of the DLD-1 cell line. It was developed by repeated 5-day exposures of stepwise-increasing concentrations of 5-FU *in vitro* (21). The mechanism of tumor cell resistance to 5-FU is thought to involve reduced incorporation of 5-FU into RNA. In a study conducted by our group (6), TFTD indicated a dose-dependent effect against DLD-1/5-FU and parent DLD-1 tumors *in vivo*, whereas the efficacy of the drug administration between the two cell lines was similar (tumor growth inhibition rate 73.2% at 150 mg/kg/day for DLD-1/5-FU vs. 73.4% at 150 mg/kg/day for DLD-1). This result is consistent with the findings noted in the present study.

Trifluridine exhibits higher resistance to the enzyme DNA glycosylase than 5-FU (28) and its incorporation into DNA induces instability of the DNA (29). In the present study, we showed that TFTD/nintedanib combination therapy was more effective than TFTD and/or nintedanib monotherapy in the DLD-1/5-FU cancer xenografts and that no cross-resistance occurred in the DLD-1/5-FU xenografts following administration of the drug therapy. As a result, the combination therapy with TFTD and nintedanib may be considered a promising option for the patients suffering from cancer that is refractory to 5-FU-based therapy.

The incorporation of trifluridine in the DNA in HT-29 and HCT116 tumors following treatment with TFTD combined with nintedanib for 14 consecutive days was higher than that observed for TFTD monotherapy. In a previous study (20), the combination of TFTD with bevacizumab was shown to increase the antitumor efficacy and the levels of the phosphorylated trifluridine in SW48 and HCT116 tumors. Jain proposed that blood vessels in tumors are structurally and functionally abnormal (30). An imbalance of proangiogenic and antiangiogenic factors produces irregular and leaky blood vessels. These blood vessels are poorly structured and hyper-permeable. They cause increased interstitial fluid pressure (hydrostatic and colloid osmotic pressures) in most tumors (31). This process can potentially limit the accumula-

tion of trifluridine in tumors. Nintedanib and bevacizumab inhibit angiogenesis by the competitive inhibition/antagonism of VEGF and the modulation of the tumor vasculature. The latter improves tumor blood supply and increases trifluridine accumulation and phosphorylation in tumors. Furthermore, this hypothesis is in agreement with the results demonstrated in the present study, as the combination of trifluridine and nintedanib indicated a similar antitumor efficacy between HT-29 and HCT116 xenografts *in vivo*, irrespective of the mode of drug action.

In the present study nintedanib monotherapy and nintedanib with TFTD combination therapy exhibited potent antitumor activity against HT-29 colorectal tumor cells. This antitumor potency was comparable to the effects noted on DLD-1 cells (Fig. 2A and E). It has been demonstrated that HT-29 cells display intrinsically higher HIF-VEGF signaling intensity and hypoxia tolerance than DLD-1 cells (32). Nintedanib, unlike bevacizumab, may attenuate the survival signaling that usually protects these cells from hypoxia-mediated cell death. Hypoxia is thought to play an important role in the malignant progression of colorectal cancer (33,34). Nintedanib has shown an increased cytotoxicity for bevacizumab-resistant HT-29 cells under hypoxia (32). Similarly, TFTD/nintedanib combination therapy may exert potent antitumor activity against advanced colorectal tumors.

In the present study the combination of TFTD and nintedanib exhibited superior antitumor efficacy than the TFTD or nintedanib monotherapies. The preclinical findings presented in the study indicate that the combination therapy with TFTD and nintedanib is a promising treatment option for a range of colorectal cancers. A clinical study evaluating the combined TFTD and nintedanib therapy is ongoing (no. UMIN000017114) and it is expected that the outcome of the trial will be highly informative.

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