Angiocytotoxic Therapy in Human Non-Small Cell Lung Cancer Cell Lines

Advantage of Combined Effects of TNP-470 and SN-38

Hiroaki Satoh, Hiroichi Ishikawa, Mitsuo Fujimoto, Masachika Fujiwara, Yuko T. Yamashita, Takuya Yazawa, Morio Ohtsuka, Shizuo Hasegawa and Hiroshi Kamma

From the Department of Respiratory Medicine, Institute of Clinical Medicine (H. Satoh, H. Ishikawa, Y. T. Yamashita, M. Ohtsuka, S. Hasegawa), and the Department of Pathology, Institute of Basic Medical Sciences (M. Fujimoto, M. Fujiwara, T. Yazawa, H. Kamma), University of Tsukuba, Tsukuba-city, Japan

Correspondence to: Dr Hiroaki Satoh, Department of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba-city, Ibaraki 305, Japan. Tel: +81 298 53 3210. Fax: +81 298 53 3320. E-mail: hirosato@md.tsukuba.ac.jp

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The combined effects of TNP-470, a promising antiangiogenic agent, and SN-38, a camptothecin derivative, were evaluated in four human cultured cell lines derived from non-small cell lung cancer (NSCLC). Cytotoxicity experiments were determined by using a tetrazolium salt (MTT) assay. The inhibitory effects of TNP-470 on cell proliferation were dose related and the 50% inhibitory concentrations on these cell lines were 47.3–139.8 μM. Evaluation of drug interactions with isobologram and the combination index values showed that sequential exposure to SN-38 followed by TNP-470 produced synergistic effects in the four cell lines tested. Our findings suggest that such an angiocytotoxic chemotherapy might be promising for the treatment of NSCLC.

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Lung cancer is the leading cause of cancer death in many Western countries as well as in Japan. Platinum-containing chemotherapy is effective in the primary treatment of small cell lung cancer (SCLC), but, to date, there is no standard chemotherapy for locally advanced or metastatic patients with non-small-cell lung cancer (NSCLC). To improve the likelihood of response, response duration, and survival, it is clear that more active drugs or drug combinations are required. The clinical application of a novel agent such as angiogenic inhibitors in advanced NSCLC is therefore of increasing interest. TNP-470, a semisynthetic analogue of fumagillin, has been shown to possess potent antiangiogenic and antitumor properties (1).

Recently, there have been a number of new potentially active drugs developed for treating patients with lung cancer. CPT-11, one of the most promising candidates among these active drugs for NSCLC, is a camptothecin derivative with greater aqueous solubility and in vivo antitumor activity than camptothecin (2). CPT-11 is a prodrug of SN-38, and SN-38 is 1 000-fold as potent as CPT-11 in vitro (3). In this study, we evaluated the combined effects of TNP-470 and SN-38 in human NSCLC cell lines.

MATERIAL AND METHODS

Materials

TNP-470 induced from fumagillin, which is a naturally secreted antibiotic of *Aspergillus fumigatus* Fresenius, was generously provided by Takeda Chemical Industries Ltd. (Osaka, Japan). The structure of TNP-470 (molecular weight: 401.89) has been reported (1). SN-38 was a generous gift from Daiichi Pharmaceutical Co. (Tokyo, Japan). Dulbecco's minimal essential medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from GIBCO Laboratories (Grand Island, New York, USA). Sodium dodecyl sulfate (SDS) and 3-(4,5-dimethyl-thiazole-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Wako Pure Chemicals (Osaka, Japan).

Cancer cell lines

The human NSCLC cell lines TKB-4, 7, and 20 (squamous cell carcinomas), and TKB-30 (adenocarcinoma) were established in our laboratory (4, 5). These cell lines were maintained in DMEM containing 5% heat-inactivated FBS in a humidified 5% CO_2 atmosphere.

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Growth inhibition assay

Cells were adjusted to 5×10^4/ml, and poured into 96-well microplates (100 μl per well). The cells were preincubated for 2 days at 5% CO_2. Based on previously published studies (6, 7), TNP-470 was added at concentrations of 2.5×10^{-2}–2.5×10^3 μM for each cell line. In the SN-38 experiments, cells were exposed to SN-38 at concentrations of 1–20 μM. There were four schedules of drugs administration: a) 24-h treatment with TNP-470 followed by a 24-h exposure to SN-38; b) 24-h treatment with SN-38 followed by a 24-h exposure to TNP-470; c) 24-h administration of TNP-470 or SN-38 and d) cells in medium but no drugs. For each cell line, eight wells for each schedule were performed.

MTT assay

MTT assay (8) was used to evaluate the cytotoxicity. At the end of culture, 10 μl of 5 mg/ml MTT solution was added to the culture. After an additional 4-h incubation, 100 μl of 10% SDS solution was added. The absorbance at 570 nm was determined using a microplate reader (BIO-RAD Model 3550).

Data analysis

A previously described (9) isobologram method was used to determine the in vitro effects of combining TNP-470 and SN-38. IC50 was chosen as the primary biological endpoint for comparisons. Three isoeffect curves, referred to as Mode I, Mode IIa and Mode IIb, were obtained by making measurements from the mean dose-response curves for two agents given alone as described by Okano et al. (9); for Mode I line, the increments in dose for both TNP-470 and SN-38 starting from zero were taken; for Mode IIa line, the increments in dose for TNP-470 starting from zero and for SN-38 starting from where the effect of TNP-470 had ended were taken; and for Mode IIb line, the increments in dose for SN-38 starting from zero and for TNP-470 starting from where the effect of SN-38 had ended were taken. The area surrounded by these three lines represents the envelope of additivity (10).

We also used the combination index (CI), as previously described (11), to compare the cytotoxic effects of TNP-470, SN-38 and TNP-470 + SN-38. The CI was defined as the sum of the relative concentrations (e.g. IC50 units) of each drug which yield an isoeffect (e.g. an inhibition of 0.5) when added together:

CI = (concentration of TNP-470)/(IC value of TNP-470) + (concentration of SN-38)/(IC value of SN-38)

With this method, CI < 1 indicates synergy, whereas CI > 1 and CI = 1 represent antagonism and additivity, respectively.

The results were expressed as the surviving fraction (mean ± standard deviation) of cells from treated groups as compared with untreated controls. All cytotoxicity studies were conducted at least three times for each cell line.

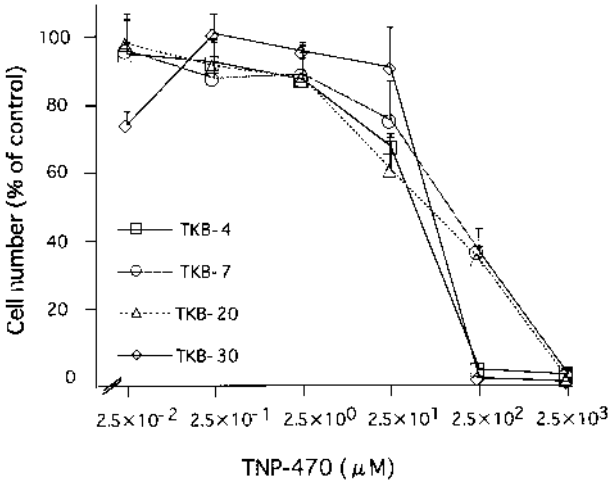

Fig. 1. Effect of TNP-470 on the cell growth in vitro.

RESULTS

Single-drug induced cytotoxicity

Initial experiments were carried out to determine the optimum drug concentration which would elicit responses in cancer cell growth inhibition. This was done in order to allow ample opportunities for combined drug effects to occur. As shown in Fig. 1, TNP-470 had an inhibitory effect of the proliferation of four TKB cell lines, in which the 50% inhibitory concentrations (IC_{50}) of TKB-4, 7, 20, and 30 were 47.3, 139.8, 57.0 and 70.9 μM, respectively. Fig. 2 shows the cytotoxic effects of SN-38 in the cell lines, which in IC_{50} were 10.6, 13.0, 11.6 and 9.4 μM, respectively.

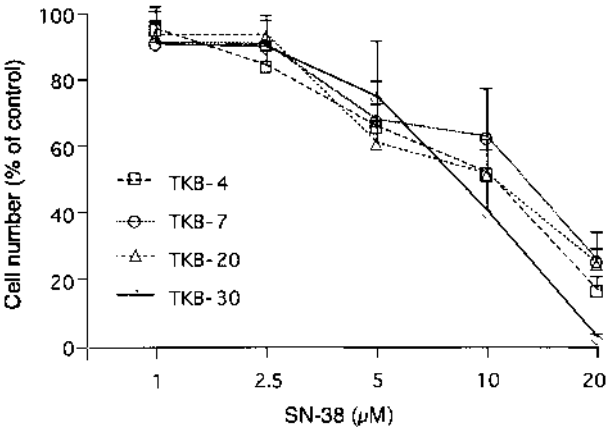

Fig. 2. Effect of SN-38 on the cell growth in vitro.

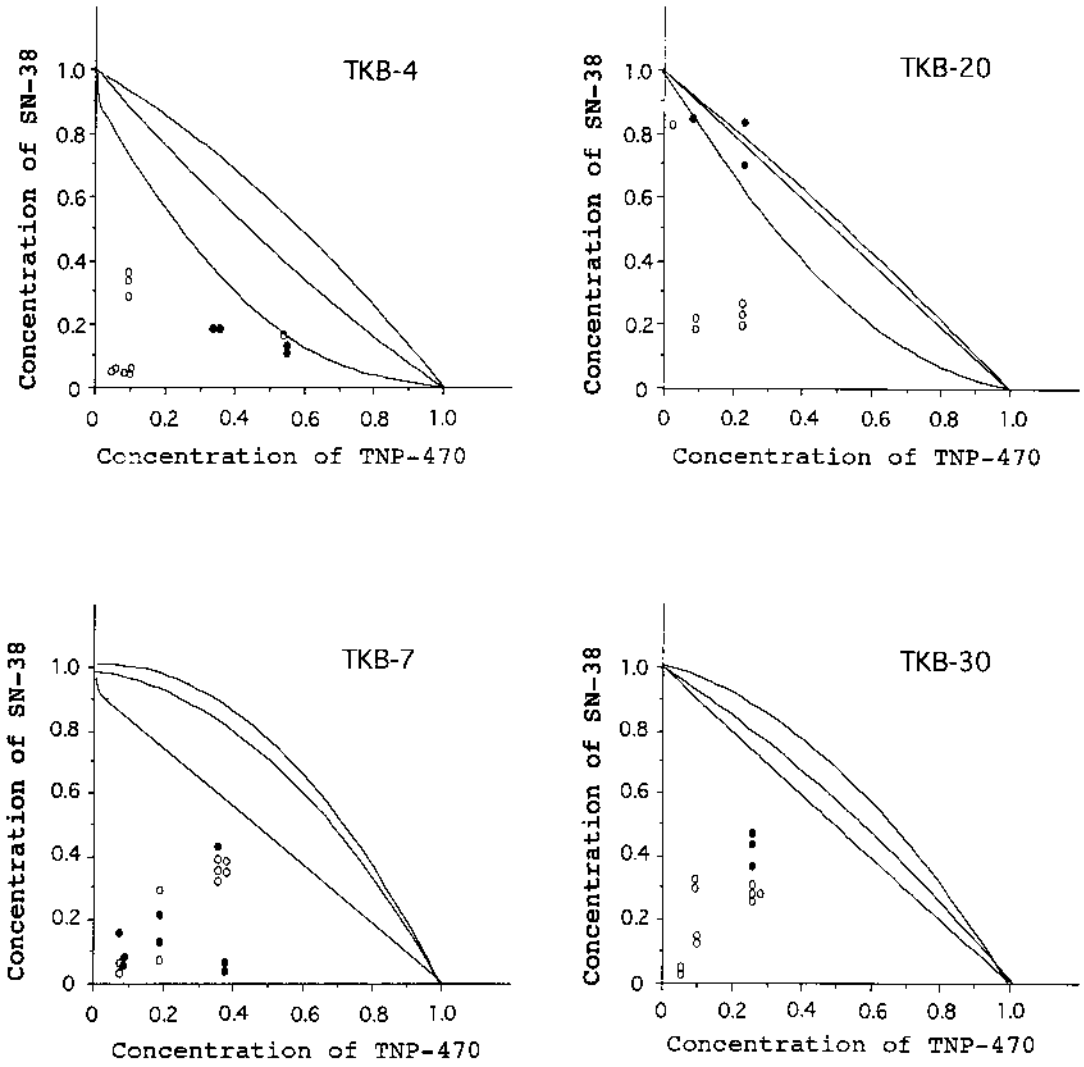

Fig. 3. Isobologram analyses of interactions between SN-38 and TNP-470 in the growth inhibition of NSCLC cell lines. Closed circle: the sequence TNP-470 before SN-38, open circle: the sequence TNP-470 after SN-38.

Effects of combination treatment with TNP-470 and SN-38

To assess the effects of combination treatment on the growth inhibition, each cell line was incubated with the two drugs at various concentrations. For the schedule TNP-470 before SN-38, the combined data points fell on the left side of the envelope or in the envelope (Fig. 3). For the schedule TNP-470 after SN-38, the combined data points fell on the left side of the envelope in all four cell lines tested. These results indicate that the combined effects of TNP-470 and SN-38 were synergistic or additive, and the latter schedule seemed to yield higher cytotoxic effects than the reversed-sequence exposure of these two drugs.

We have also analyzed all data using the combination index (CI). The means ($\pm$SD) of the CI at IC_{50} isoeffect level are listed in Table 1. The schedule TNP-470 after SN-38 was more cytotoxic than the reversed-sequence schedule in these cell lines ($p = 0.02, 0.03, 0.03, 0.05$ respectively, Mann-Whitney test).

Potentiation of SN-38 cytotoxicity with non-toxic concentration of TNP-470

Combinations of non-cytotoxic concentrations (less than 0.25 μM) of TNP-470 and SN-38 were evaluated. As shown in Fig. 1, concentrations less than 0.25 μM of TNP-470 were not toxic per se. Very recently, Figg et al.

Table 1

The combination index at IC_{50} isoeffect level for lung cancer cells

Cells	Treatment schedule	
	SN-38 followed by TNP-470	TNP-470 followed by SN-38
TKB-4	0.21 ± 0.13	0.64 ± 0.08
TKB-7	0.48 ± 0.28	0.55 ± 0.37
TKB-20	0.39 ± 0.08	0.99 ± 0.07
TKB-30	0.32 ± 0.20	0.61 ± 0.13

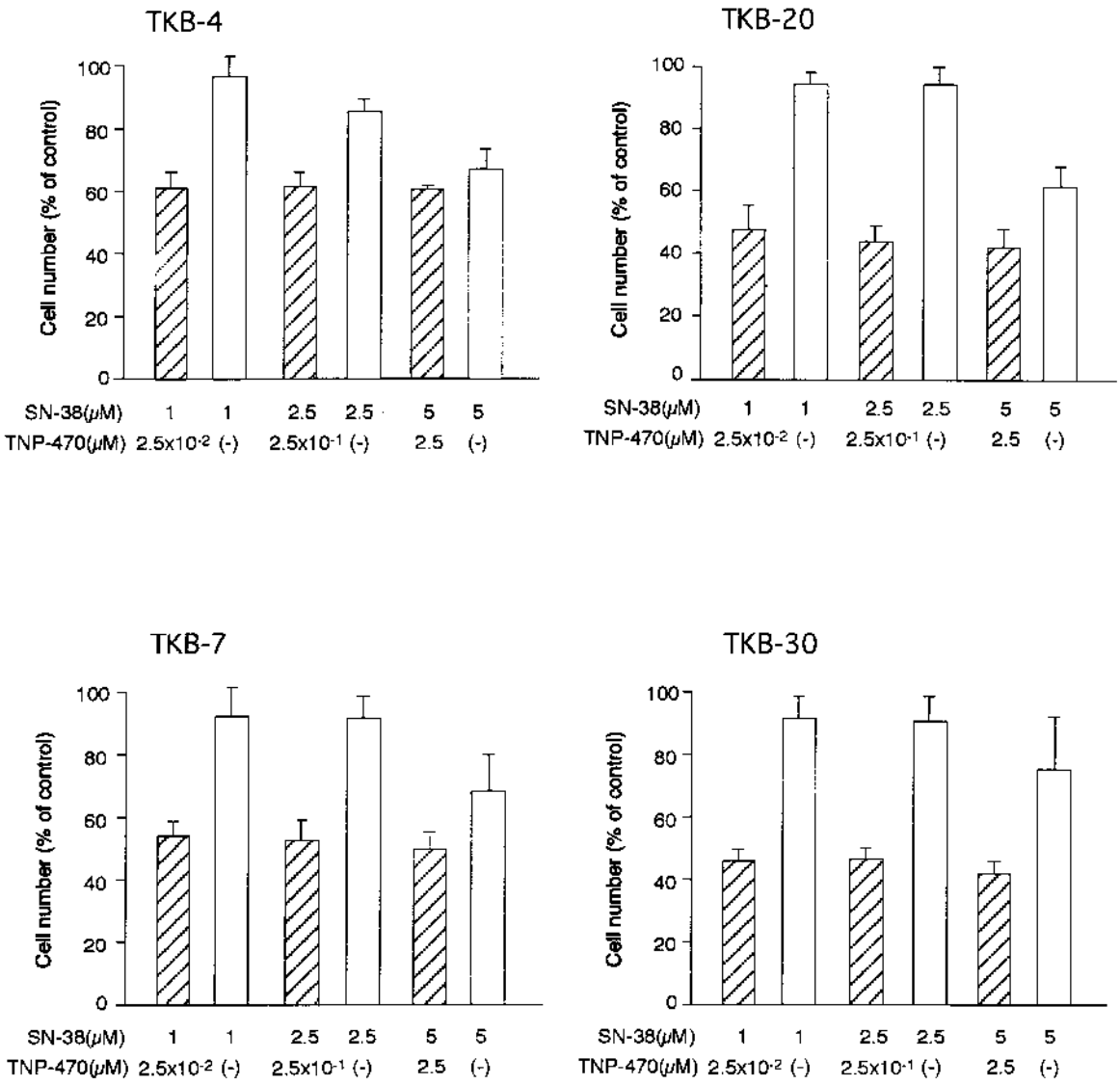

Fig. 4. Influence of TNP-470 on the cytotoxic effect of SN-38. Cytotoxicity of SN-38 with TNP-470 at non-toxic concentrations (the schedule TNP-470 after SN-38) was significantly higher than that of SN-38 alone (Wilcoxon signed rank test, $p = 0.0001$, respectively).

reported the pharmacokinetics of TNP-470 in a phase I clinical trial (12). According to the results of their study, the time to maximum concentration (Cmax) ranged between 1.7×10^{-2} and 1.5 μM. Concentrations less than 0.25 μM of TNP-470 were close to the concentrations that Figg et al. suggested as the Cmax. Fig. 4 showed the results of the enhancement of SN-38 cytotoxicity for each cell line tested. For the schedule TNP-470 after SN-38, the combined cytotoxic effect was higher than that of SN-38 alone in each cell line tested (Wilcoxon signed rank test, $p = 0.0001$, respectively). On the other hand, for the schedule TNP-470 before SN-38, the combined effect was not enhanced (data not shown).

DISCUSSION

TNP-470 is one of the promising antiangiogenic drugs to reach clinical trials based on its efficacy and the lack of major adverse effects. After successfully completing preclinical toxicological tests, phase I clinical trials (12, 13) with TNP-470 were initiated even though the exact mechanisms of action were not completely elucidated. At cytostatic concentrations, TNP-470 inhibits DNA synthesis in endothelial cells, indicating that the molecule affects one or several steps of the cell cycle (14). TNP-470 is also found to suppress tumor growth and metastasis in vivo (6, 7, 15). Interestingly, apoptotic cell death is reported to be

increased in TNP-470-treated tumors (16). The effect of TNP-470 on apoptosis is consistent with a recent study of transplantation of Lewis lung carcinoma into syngenic mice followed by treatment with TNP-470 (17). However, a much higher (around 3 logs higher) concentration of TNP-470 is required than that restraining endothelial proliferation in vitro (6, 7). In the present study, we observed that TNP-470 did not have a cytotoxic effect on the NSCLC cell lines until a concentration above 2.5 μM was achieved. This concentration is considerably higher than that required for cytostatic inhibition of endothelial cells and tumor cells, which is consistent with recent studies (6, 7). Our results showed that TNP-470 had a direct cytotoxic effect on NSCLC cells; however, the concentration was very high and the mechanisms of action by which TNP-470 inhibits tumor cell proliferation are still unclear. The results of this study together with the results of previous studies indicate that the direct cytotoxic mechanisms of TNP-470 on tumor cells may be different from the cytostatic mechanisms of TNP-470 on endothelial cells. As for the mechanisms, additional studies will be necessary.

The potential therapeutic value of combination treatment with TNP-470 and anticancer modalities including chemotherapeutic agents has been evaluated (7, 18, 19). However, few studies of combination therapies have been conducted in human tumors (7). It is likely that inhibition of angiogenesis might have led to death of tumor cells most distal from the established vasculature, thereby decreasing the tumor volume and facilitating the permeation of given cytotoxic agents throughout the tumor tissues. The combination of TNP-470 and 5-fluorouracil or doxorubicin did not enhance the cytotoxicity; however, enhanced cytotoxicity of the combination of TNP-470 and cisplatin was reported in vivo (7, 18, 19). In this study, we evaluated the effects of combining TNP-470 and SN-38, a derivative of the topoisomerase inhibitor camptothecin (CPT-11), using the isobologram analysis and the combination index method, which can concisely analyze drug interactions independently of the shape of the dose-response curves. We demonstrated the synergistic cytotoxic effects of the combination of TNP-470 and SN-38 against NSCLC cell lines. SN-38 inhibits topoisomerase I by stabilizing the enzyme-DNA complex and causing single-strand DNA breaks (20). This may influence many aspects of DNA metabolism, including replication, recombination and chromatin segregation (21). TNP-470 might render some SN-38-induced DNA damage irreparable. It is possible that these DNA inhibition-related interactions might be associated with the two drugs.

The concentrations of the TNP-470 tested in this study ranged between 2.5×10^{-2} and 2.5 μM. Very recently, Figg et al. reported the pharmacokinetics of TNP-470 in a phase I clinical study (12). According to the results of their study, the maximum concentrations (Cmax) ranged between 1.7×10^{-2} and 1.5 μM.

In conclusion, our data indicate that combination therapy with TNP-470 and SN-38 can be of interest when investigating the treatment of NSCLC. It is very important to determine whether TNP-470 potentiates the effect of SN-38 on the tumor cells without increasing multiorgan toxicity of the chemotherapeutic agents. Further investigations will elucidate the clinical availability of such an angiocytotoxic therapy.

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