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Preclinical report

Docetaxel alone or orally combined with 5-fluorouracil and its derivatives: effects on mouse mammary tumor cell line MM_2 *in vitro* and *in vivo*

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Although docetaxel (Taxotere; TXT), a taxoid anticancer drug, is clinically and experimentally very effective against breast cancer, its antitumor effect is of very short duration. We addressed whether 5-fluorouracil (5-FU) and its derivatives can act synergistically with TXT against mammary tumors, with placing particular stress on their use by oral route. Mouse mammary tumor cell line, MM_2, was propagated in culture and as ascites in mice. Carmofur (HCFU) and doxifluridine (5′-DFUR) were used as 5-FU derivatives. *In vitro*, the cytotoxic effects of antitumor drugs on MM_2 cells were examined by MTS assay. *In vivo*, mice inoculated i.p. with MM_2 cells were treated with i.p. injection of TXT and/or oral administration of 5-FU or its derivatives, and observed for curing tumor. *In vitro*, the synergistic effects were observed in the combination of TXT and 5-FU or HCFU, but not in that of TXT and 5′-DFUR. *In vivo*, all of these combinations cured tumors far more effectively than TXT alone. The discrepant result of the combination of TXT and 5′-DFUR between *in vitro* and *in vivo* was ascribed to up-regulation of pyrimidine phosphorylase in tumor cells *in vivo* by TXT. Thus, 5-FU, its masked compounds like HCFU and its prodrugs like 5′-DFUR can act synergistically with TXT in the therapy of cancer even when administered by the oral route.

Key words: 5-Fluorouracil, 5-fluorouracil derivatives, docetaxel, mouse mammary tumor, oral administration.

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Introduction

Docetaxel (Taxotere™; TXT), a taxoid anticancer drug, is currently under investigation for its therapeutic efficacy against advanced, recurrent and metastatic breast cancers as a neoadjuvant or adjuvant agent in many clinical trials.[1–3] This drug has been proved to promote tubulin assembly in microtubules and inhibit their depolymerization. In a pharmacokinetic study, the plasma curve obtained after i.v. injecting TXT at a highly active dose of 111 mg/m^2 showed a biphasic profile with a very short distribution phase ($t_{1/2}$ = 7 min) and an elimination half-life of about 1.1 h.[4] The overall response rate to TXT ranged from 55 to 68% in phase II studies using it as first-line chemotherapy against metastatic breast cancer and also from 53 to 57% even in patients with anthracycline-resistant cancer.[5] However, the median durations of response and survival were as short as 8.3 and 16.4 months, respectively.[6] Therefore, combination regimens of TXT with other chemotherapeutic agents including anthracyclines, cisplatin, cyclophosphamide, 5-fluorouracil (5-FU) and vinorelbine, are currently being explored with the aim of improving objective tumor response rates and prolonging survival, with regard to complete response rates, response duration, and both progression-free and overall survival.[7–10]

5-FU is an antimetabolite synthesized by Duschinsk.[11] It acts as a pyrimidine antagonist, and has demonstrated significant antitumor activity both as a single agent and as a component of CAF (cyclophosphamide, doxorubicin and 5-FU), the most active combination regimen currently used for metastatic breast cancer.[12] A pharmacokinetic study showed that an i.v. injection of 5-FU gave a plasma curve with a very

short distribution phase ($t_{½}$ = 21 min). 5-FU is a good candidate for evaluation in combined therapy with TXT, since both agents are effective against metastatic breast cancer but different in action mechanisms and tolerability profiles.[13] In a tumor-bearing mouse model, therapeutic synergy was observed between TXT and 5-FU, and in this combination each agent could be even infused at 70% of its highest non-toxic dose without additional toxicity.[4] On the other hand, oral 5-FU treatment has been re-evaluated recently because of the development of new 5-FU derivatives according to the novel concept of biochemical modulation.[14] 5-FU and its derivatives have been proved to manifest anticancer effects on breast cancer in single as well as combined therapy.[15]

In this study, we addressed whether the cytotoxic effect of TXT on mouse mammary tumor cell line, MM_2, could be enhanced by combination with 5-FU or its derivatives *in vitro*. In addition, we investigated whether these combinations could cure ascites MM_2 tumors *in vivo* more effectively than their single use, with placing particular stress on oral use of 5-FU and its derivatives.

Materials and methods

Mice

Female C3H/He (C3H) mice were purchased from SLC (Hamamatsu, Japan) and used for experiments at 8–10 weeks of age. They were housed five to eight in a cage with *ad libitum* F-2 pellets (Funabashi Nojo, Funabashi, Japan) and tap water in a temperature- and humidity-controlled room with a 12-h light/12-h dark cycle. All animal experiments were performed in accordance with the guidelines of the Animal Research Ethics of the Institute of Medical Science, University of Tokyo, Japan.

Antitumor drugs

TXT was provided from Aventis Pharma (Vitry-Alfortville, France). The stock solution (20 mg/ml) in ethanol was prepared according to the supplier's instructions, aliquoted and stored at −20°C. For *in vitro* experiments, the test solutions were prepared freshly at the time of use by diluting the stock solution with sterilized distilled water or tissue culture medium. For *in vivo* experiments, the test solutions were prepared by diluting the stock solution by 20 times with 1 volume of polysorbate 80 and 18 volumes of 5% glucose solution and i.p. injected into mice.

5-FU and its derivatives, carmofur (HCFU) and doxifluridine (5′-DFUR), were gifts from Kyowa Hakko (Tokyo, Japan), Mitsui Pharmaceuticals (Tokyo, Japan) and Japan Roche (Tokyo, Japan), respectively. The stock solutions of these drugs (10–50 mg/ml) were prepared with tissue culture medium, aliquoted and stored at −20°C. The test solutions for *in vitro* experiments were prepared at the time of use by diluting the stock solution with culture medium. In the *in vivo* experiments, 5-FU and its derivatives were administered orally using a cannula with a bulb on the end after dissolving 5-FU and 5′-DFUR in saline solution, and HCFU in 10 mM acetic acid–0.5% methylcellulose.

Tumor cells

MM_2 mouse mammary tumor cells, syngeneic to C3H mice, were used. MM_2 cells were transferred in either ascites form in C3H mice *in vivo* or culture *in vitro*. Culture was routinely performed in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Schott, Mainz, Germany), 2 mM glutamine (Gibco, Carlsbad, CA), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Gibco) using 25-cm^2 tissue-culture flasks at 37°C in an atmosphere of 95% air and 5% CO_2 in a humidified incubator. MM_2 cells were semi-floating, and culture medium was changed every 3–4 days by aspiration.

Cytotoxicity assay *in vitro*

To prepare a tumor cell suspension, MM_2 cells growing in exponential phase in culture were treated with 0.25% tripsin, passed through mesh, washed twice with culture medium, pelleted and resuspended in culture medium at a density of 1.25×10^5/ml. An aliquot of 80 μl of the suspension was placed in each well on a 96-well microplate (10^4 cells/80 μl/well) and incubated at 37°C overnight. After 24 h, TXT, 5-FU, HCFU or 5′-DFUR in a volume of 10 μl was added to wells in triplicate at the final concentration from 0 to 2 μg/ml. The plate was incubated at 37°C for 72 h, and then cell viability was determined with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, WI) assay. A volume of 20 μl of the reagent was added to each well and incubated at 37°C for 1 h, and the absorbance was measured at 490 nm with an ELISA reader using the medium alone as a blank. The cytotoxic activity against tumor cells was estimated by the percentage of the absorbancy of the drug-treated cells of that of control cells.

Hoechst 33258 staining

MM_2 tumor cells were treated with 0.05 μg/ml TXT and 0.5 μg/ml HCFU in combination at 37°C for 72 h

and investigated for apoptosis as described elsewhere.[16] Briefly, cells were fixed with 1% glutalaldehyde in calcium and magnesium-free PBS (PBS) for 30 min at room temperature. After washing twice with PBS, cells were resuspended in 20 μl PBS and added with 4 μl of 1 mM Hoechst 33258 in PBS. The stained cell suspension was observed immediately under a fluorescent microscope.

Isobolographic analysis

The isobologram is used as a convenient graphical method introduced by Loewe and Muischnek[17] to analyze the interaction of two combined drugs. In this method, the equieffective pairs of doses of drugs X and Y are represented using rectangular coordinates (x, y) for the respective doses. In this format, the dose of X alone (a) and Y alone (b) are represented as the axial points (a, 0) and (0, b). The straight line connecting these points (the 'line of additivity') provides a convenient means for visually discriminating additive from non-additive interactions on the basis of whether or not the coordinate of the IC_{80} value of the combination falls on (additive), below (synergistic) or above (subadditive) the line of additivity.

Antitumor effects *in vivo*

Mice were i.p. inoculated with 2×10^5 MM_2 cells and divided at random into four groups containing five mice each. From day 1 of tumor implantation, a group of mice were i.p. injected with 20 mg/kg of TXT in 0.4 ml 3 times at intervals of 3–4 days or orally administered with 50 mg/kg of 5-FU in 0.1 ml, 100 mg/kg of HCFU in 0.2 ml or 100 mg/kg of 5′-DFUR in 0.2 ml 5 times weekly for 3 weeks. Another group of mice were treated with TXT and 5-FU, HCFU or 5′-DFUR in combination in the same way or remained untreated as control. All mice were followed up to day 40 of tumor inoculation, followed by autopsy.

Assay of pyrimidine phosphorylase (PyNPase) activity

Mice were inoculated i.p. with 2×10^5 MM_2 cells and divided at random into two groups containing five mice each. One group was i.p. injected with 20 mg/kg of TXT on day 4 of tumor inoculation and the other group was untreated. Six days later, the liver, intestine and ascites tumor cells were obtained from each mouse in both groups. Tumor cells were sonicated, and the liver and intestine were homogenized with a glass homogenizer in 10 mM Tris buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$ and 50 μM potassium phosphate. The sonicated cells and homogenized tissues were centrifuged at 10 000 g for 20 min to isolate the supernatant. For the measurement of PyNPase activity, the supernatant was further centrifuged at 105 000 g for 90 min, dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and used as a crude enzyme solution. The protein concentration was determined by the method of Lowry *et al.*[18] All procedures were performed at 4°C. The reaction mixture (120 μl) for the enzyme activity assay contained 183 mM potassium phosphate (pH 7.4), 10 mM 5′-DFUR and the crude enzyme solution. The reaction was done at 37°C for 60 min and then terminated by addition of 360 μl methanol. After removal of the precipitate by centrifugation, 100 μl of the reaction mixture was added with 20 μM 5-chlorouracil as the internal standard and then applied to the high-performance liquid chromatograph (HPLC) column (ERC-ODS-1171). The solvent system used was as follows: 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM 1-decane sulfonic acid:methanol (85:15, v/v). The amount of 5-FU produced was measured with a UV monitor (280 nm). PyNPase activity converting 5′-DFUR into 5-FU was measured as reported elsewhere.[19,20] This enzyme activity is carried by both dThdPase and uridine phosphorylase (UrdPase). To examine their contribution to it, an UrdPase-specific inhibitor, 2,2′-anhydro-5-ethyluridine (ANEUR), was synthesized according to a method reported elsewhere[21] and used.

Statistical analysis

Student's t-test was used for statistical evaluation between the control and treated groups. The difference was defined as significant at $p < 0.05$.

Results

Cytotoxic effect *in vitro* on MM_2 cells of TXT alone or combined with 5-FU and its derivatives

TXT was added to the culture of MM_2 tumor cells at various concentrations alone or in combination with 5-FU, HCFU or 5′-DFUR of various concentrations to investigate whether the cytotoxic effect of TXT was enhanced by 5-FU and its derivatives. As shown in Figure 1(a and b), the cytotoxicity of TXT was enhanced by 5-FU and HCFU in a concentration-dependent fashion. However, it was not affected at all by 5′-DFUR at any concentration.

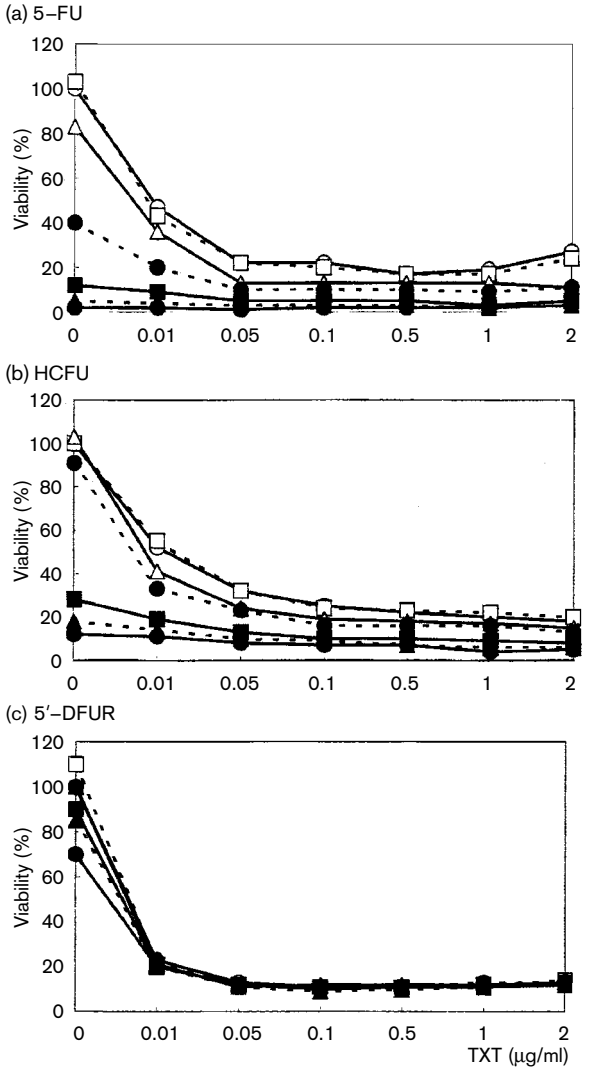

Figure 1. Cytotoxic effect *in vitro* on MM_2 cells of TXT alone or combined with 5-FU (a), HCFU (b) and 5′-DFUR (c). MM_2 cells (10^4) were placed in wells and added with TXT at the indicated concentrations: 0 (○), 0.01 (□), 0.05 (△), 0.1 (●; dotted line), 0.5 (■), 1.0 (▲) and 2.0 (●; solid line) μg/ml of 5-FU (a), HCFU (b) or 5′-DFUR (c).

Table 1. IC_{80} values in combination of TXT and 5-FU or HCFU

5-FU or HCFU/TXT	10/1[a]	5/1	1/1	1/5	1/10
5-FU (μg/ml)	0.1[b]	0.28	0.04	0.02	0.01
TXT (μg/ml)	0.01	0.056	0.04	0.1	0.1
HCFU (μg/ml)	0.36	0.355	0.075	0.076	0.013
TXT (μg/ml)	0.036	0.071	0.075	0.113	0.133

[a]Ratio.
[b]IC_{80} value.

The IC_{80} value (the concentration required to reduce the cell survival by 80%) was calculated for these combined treatments (Table 1). This value could not be computed for the combination of TXT and 5′-DFUR, because these drugs did not interact with each other. The IC_{80} of TXT ranged from 0.01 to 0.1 μg/ml and 0.036 to 0.133 μg/ml, respectively, when combined with 5-FU and HCFU. On the other hand, the IC_{80} of 5-FU and HCFU ranged from 0.01 to 0.28 μg/ml and 0.013 to 0.36 μg/ml, respectively, in combination with TXT. These values corresponded to 76.8–2152.5 and 50.5–1399.3 pmol/ml, respectively. Therefore, 5-FU and HCFU were similar in the cytotoxic activity against MM_2 mammary tumor cells *in vitro* when combined with TXT.

Analysis of cytotoxic activity by isobologram

As mentioned above, 5-FU and HCFU had a significant influence on the cytotoxic effect of TXT. Therefore,

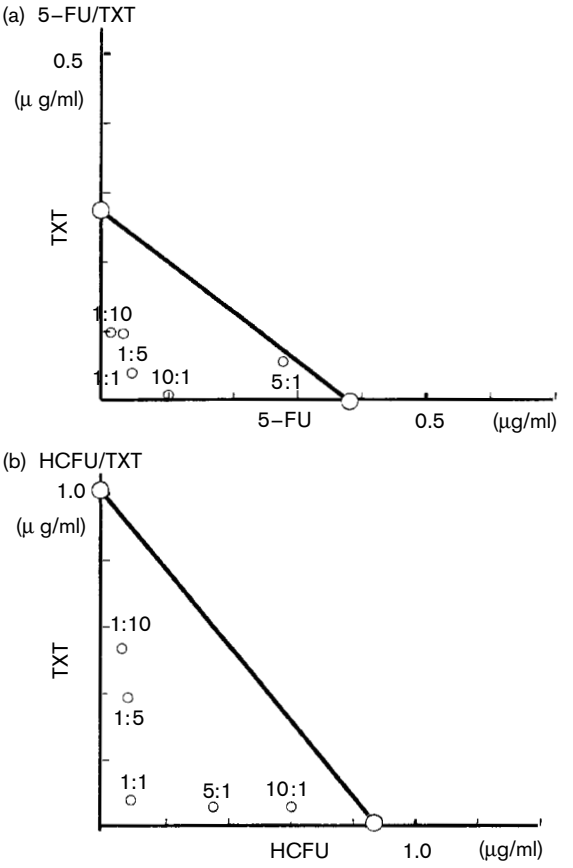

Figure 2. Analysis of the cytotoxic effect *in vitro* on MM_2 cells by isobologram analysis in combination of TXT and 5-FU (a) or HCFU (b).

the data of these combined treatments were analyzed by isobologram. As depicted in Figure 2(a), all the points obtained in various combinations of TXT and 5-FU were plotted under the line of additivity. Moreover, almost the same tendency was observed in the combination of TXT and HCFU (Figure 2b). It was thus concluded that 5-FU and HCFU act in a synergistic manner with TXT to exhibit cytotoxic activity against MM_2 mammary tumor cells. In addition, it appeared that the synegism was more typical in the combination of TXT and HCFU than that of TXT and 5-FU.

Induction of apoptosis by combination of TXT and HCFU

Since the synergism was more conspicuous in the combination of TXT and HCFU, we determined whether or not it causes apoptosis of MM_2 cells. Apoptotic bodies were observed in MM_2 cells treated with 0.05 μg/ml TXT and 0.5 μg/ml HCFU in combination (Figure 3). This proved that MM_2 cells underwent apoptosis when exposed to the combination of TXT and HCFU. It was thus suggested that apoptosis may play an important role in the cytotoxicity or tumor cell death induced by combination of TXT with HCFU or 5-FU.

Antitumor effects *in vivo* of TXT alone or combined with 5-FU and its derivatives

Mice bearing MM_2 ascites tumors were i.p. injected with 20 mg/kg TXT alone or in combination with oral administration of 50 mg/kg 5-FU, 100 mg/kg HCFU or 100 mg/kg 5′-DFUR as described in Materials and methods, and observed for illness up to 40 days after tumor inoculation. At the end of observation, all surviving mice were autopsied and those macroscopically free from tumor cells were considered complete cure. The results are depicted in Figure 4. The mean survival of mice was 15.0 ± 2.3, 21.0 ± 1.3, 20.2 ± 2.6 and 16.4 ± 1.7 days (mean $\pm$ SD) in control, and 5-FU-, HCFU- and 5′-DFUR-treated groups, respectively. Therefore, 5-FU and HCFU equivalently prolonged the survival of mice, but 5′-DFUR had no influence on it. Noticeably, TXT alone prolonged the survival far longer and achieved complete cure in one of five mice. This tumor-curing effect of TXT was significantly enhanced when combined with 5-FU, HCFU or 5′-DFUR: tumors were completely cured in all (100%), four (80%) and four (80%) of five mice, respectively. Thus, the combination of TXT and 5-FU exhibited the strongest tumor-curing activity, although it caused significant body weight loss supposedly due to side effects. It was noteworthy that 5′-DFUR which had no antitumor activity in single use was as effective as HCFU in curing MM_2 tumors when combined with TXT. This might be relative to the up-regulation of PyNPase by TXT as mentioned below.

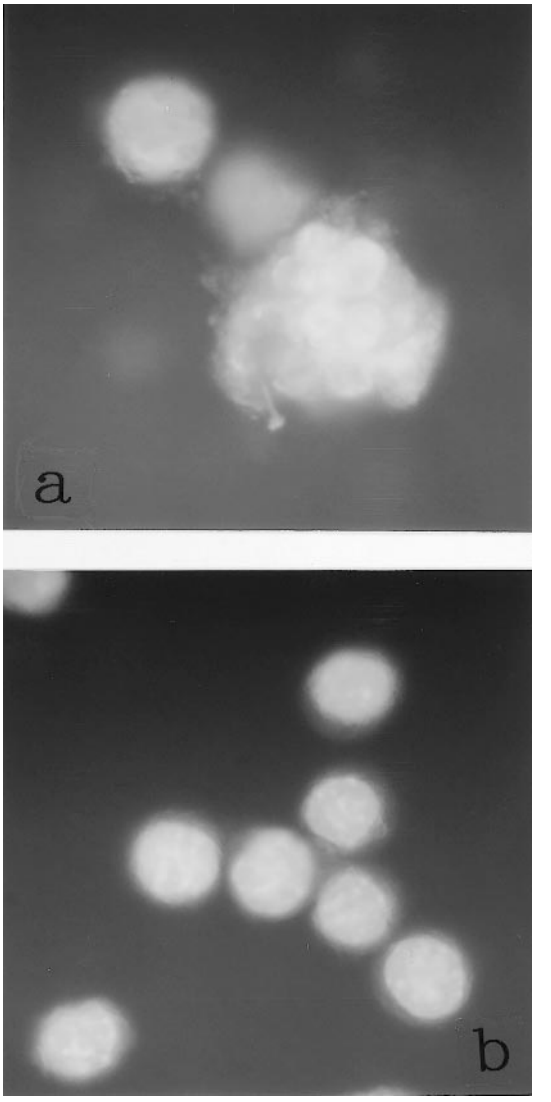

Figure 3. Hoechst 33258 staining. MM_2 cells were treated with 0.05 μg/ml TXT and 0.5 μg/ml HCFU in combination for 72 h (a) or untreated (b). Note the formation of apoptotic bodies in treated cell (a).

Up-regulation of PyNPase by TXT

5-FU but not 5′-DFUR displayed an antitumor effect on MM_2 tumors in single use (Figure 4). As PyNPase converts 5′-DFUR into 5-FU, this enzyme plays a critical role for 5′-DFUR to manifest an anti-tumor effect. Therefore, TXT was i.p. injected into mice bearing MM_2 tumors to investigate its effect on the production

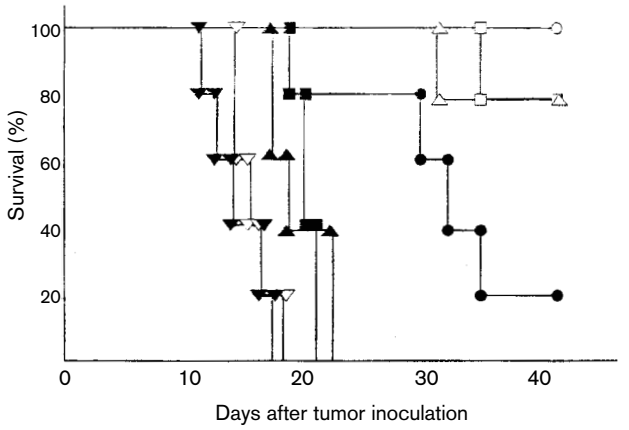

Figure 4. Antitumor effects *in vivo* of TXT alone or combined with 5-FU and its derivatives. Mice were i.p. inoculated with 2×10^5 MM_2 cells, and administered with 20 mg/kg TXT by the i.p. route 3 times at intervals of 3–4 days and/or with 50 mg/kg 5-FU, 100 mg/kg HCFU or 100 mg/kg 5′-DFUR by the oral route 5 times weekly for 3 weeks from the day after tumor inoculation. (○) TXT+5-FU, (□) TXT+HCFU, (△) TXT+5′-DFUR, (●) TXT alone, (■) 5-FU alone, (▲) HCFU alone, (▽) 5′-DFUR alone and (▼) control.

of PyNPase by tumor cells *in vivo*. The average PyNPase activity (mean ± SD) was 47.88 ± 14.72 and 85.24 ± 8.97 μg 5-FU/mg protein/h in MM_2 tumor cells from untreated and TXT-treated mice, respectively. Therefore, TXT stimulated the production of PyNPase by tumor cells. In addition, the average enzyme level decreased to 0.24 ± 0.31 and 0.24 ± 0.05 μg 5-FU/mg protein/h, respectively, when the activity was determined in the presence of ANEUR, a selective inhibitor of UrdPase. Since the PyNPase activity is carried by dThdPase and UrPase, the increased PyNPase level by TXT could be explained by up-regulation of UrdPase. The PyNPase levels in the liver and intestine were not different between untreated and TXT-treated mice (data not shown). Thus, it was likely that TXT may up-regulate this enzyme in tumors but not in normal tissues.

Discussion

TXT and paclitaxel are taxoid anticancer drugs. It is generally accepted that TXT is 1.3- to 12-fold stronger in cytotoxic activity than paclitaxel *in vitro*.[22] This is ascribed to the ability of TXT to attain a higher intracellular concentration, its stronger affinity to microtubules and its slower efflux from cells.[23] However, TXT has been clinically used in combination with 5-FU for several reasons. First, TXT and the fluoropyrimidines have overlapping antitumor spectra including breast, esophageal, and head and neck cancers, and the utility of taxane–fluoropyrimidine regimens as first-line treatment in patients with metastatic breast cancer may progressively increase as the anthracyclines are more frequently used in the adjuvant setting. Second, their principal toxicities do not completely overlap. Neutropenia is the principal toxicity of TXT, whereas stomatitis and diarrhea are the predominant toxicities of fluoropyrimidines on the most commonly used schedule. Finally, the principal mechanisms of antineoplastic activity of taxanes and fluoropyrimidines are different.[5] 5-FU arrested tumor cells in the G_1 and S phases of the cell cycle, and therapy prevented tumor cells from entering G_2/M phases when cells are most prone to taxane-induced cytotoxicity.[5] In general, the combination therapy with TXT and 5-FU is performed by i.v. administration of both agents in several kinds of cancer.[5,24–27] On the other hand, in several experimental studies, the therapeutic efficacy of this combination was also well documented by the i.v. route[8,28] and 5-FU has been rarely combined by the oral route.

In the current *in vitro* experiment, the combination of TXT and 5-FU or HCFU acted synergistically in the cytotoxicity against MM_2 mammary tumor cells (Figures 1 and 2). The synergism appeared to be stronger in the combination of TXT and HCFU (Figure 2). MM_2 tumor cells treated with TXT and HCFU in combination underwent apoptotic changes characterized by formation of apoptotic bodies (Figure 3). However, the ladder formation could not be proved (data not shown). Since epithelial tumor cells could often undergo apoptosis without ladder formation, the tumor cell death induced by TXT combined with 5-FU or its derivatives appeared to be due to apoptosis. HCFU is a masked compound of 5-FU and can penetrate into cells more efficiently than 5-FU.[29] Unexpectedly, however, it did not achieve a higher potential in the synergism with TXT, compared with 5-FU. On the other hand, the combination of 5′-DFUR had no influence on the cytotoxic activity of TXT. This derivative is a prodrug of 5-FU and manifests its antitumor effect after being converted into 5-FU by PyNPase. In addition, this enzyme level was very low in cultured MM_2 tumor cells (data not shown), which was probably the reason why 5′-DFUR could not enhance the cytotoxicity of TXT in combination.

In the current *in vivo* experiment, 5-FU or its derivatives were combined with TXT by the oral route. It was noticed that 5′-DFUR was equivalent to 5-FU and HCFU in curing tumors in combination with TXT, although it did not influence the cytotoxicity of TXT *in vitro* (Figure 1c). This might be explained by the up-

regulation of PyNPase by treatment with TXT in MM_2 cells. The measurement of the enzyme activity in the presence of ANEUR, a specific inhibitor of UrdPase, revealed that up-regulation of PyNPase in MM_2 was ascribable mainly to the induction of UrdPase. Several anti-cancer drugs including TXT[30] and cyclophosphamide[31] are reported to up-regulate PyNPase in tumor cells. Similar enzyme induction was observed with some cytokines.[19,20] The doses of 5-FU and its derivatives were determined on the molar basis. The maximal tolerance doses (MTD) are different among 5-FU, HCFU and 5′-DFUR: 30, 200 and 185 mg/kg, respectively. Therefore, the doses of 5-FU, HCFU and 5′-DFUR used in this experiment are 167, 50 and 54% of MTD, respectively. 5-FU was administered at an excessive dose compared to HCFU and 5′-DFUR, as reflected in the acute body weight loss in mice treated with TXT and 5-FU in combination (data not shown). Although the tumor-curing rate was slightly lower in HCFU and 5′-DFUR than 5-FU when combined with TXT (Figure 4), it will be expected that these derivatives can achieve at least the equivalent or stronger antitumor effect compared to 5-FU in the combination with TXT.

Conclusion

Anticancer effects of the combination of TXT with 5-FU have been investigated in many experimental studies in which 5-FU was administered by the i.v. or i.p. route, but not by the oral route. Moreover, its combination with 5-FU derivatives has not been tested. Thus, it can be stressed from our results that 5-FU, its masked compounds like HCFU and its prodrugs like 5′-DFUR can act synergistically with TXT in the therapy of cancer even when they are administered by the oral route. Oral administration of 5-FU or its derivatives may enable combination therapy with TXT in outpatients and bring a higher quality of life in clinical application.

Acknowledgments

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