Induction of Thymidine Phosphorylase Activity and Enhancement of Capecitabine Efficacy by Taxol/Taxotere in Human Cancer Xenografts

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ABSTRACT
Thymidine phosphorylase (dThdPase) is an essential enzyme for the activation of the cytostatics capecitabine (N'-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) and its intermediate metabolite [5'-deoxy-5-fluorouridine (5'-dFUrU)] to 5-fluorouracil in tumors. We have tried to identify the best partners of capecitabine in combination therapy, such as dThdPase up-regulators, which may enhance the efficacy of this compound. Among various cytostatics studied with the WiDr human colon cancer xenograft model, Taxol, Taxotere, and mitomycin C greatly increased levels of human dThdPase in tumors, and cyclophosphamide slightly increased the enzyme level. These cytostatics simultaneously increased the levels of human tumor necrosis factor α (TNFα), which is an up-regulator of dThdPase. In cultures of the WiDr cells, however, Taxol did not up-regulate TNFα to a detectable level and only slightly enhanced levels of dThdPase. These results suggest that Taxol might indirectly elevate TNFα in tumor cells, which in turn up-regulated dThdPase in the tumor cells in the WiDr cancer xenograft. In the combination therapy, the efficacy of Taxol and Taxotere with either capecitabine or 5'-dFurU was more than just additive. In contrast, Taxol and either 5-fluorouracil or UFT (a mixture of tegafur and uracil) in combination showed only additive activity. Taxol and Taxotere might enhance the efficacy of capecitabine and 5'-dFUrU, probably by modulating dThdPase activity in tumor tissues.

INTRODUCTION
Capecitabine generates 5-FUra selectively in tumors by three enzymes located in the liver and in tumors; the final step is the conversion of the intermediate metabolite 5'-dFUrU to 5-FUra by dThdPase in tumors (1, 2). The enzyme is now known to be an angiogenic factor (3, 4), and its activity is higher in various tumor tissues than in normal tissues adjacent to the tumors (5, 6). dThdPase is essential for the efficacy of capecitabine and 5'-dFUrU. The susceptibility of tumor cells to 5'-dFUrU is reported to be enhanced by the transfection of the dThdPase gene (7, 20), whereas the activity of 5'-dFUrU was inhibited by dThdPase inhibitors (8). Therefore, it is expected that dThdPase up-regulators would enhance the efficacy of capecitabine and 5'-dFUrU. In fact, the efficacy of 5'-dFUrU has been reported to be enhanced through the up-regulation of this enzyme activity by various factors, such as IL-1α, TNFα, IFNγ (9), basic fibroblast growth factor, platelet-derived growth factor (10), and IFNα (11, 12). In addition, we preliminarily reported that IL-12 enhanced the efficacy of 5'-dFUrU and capecitabine in the in vivo tumor models indirectly through the up-regulation of IFNγ, a dThdPase up-regulator (13, 14).

Capecitabine is being assessed in clinical trials worldwide, and 5'-dFUrU is being marketed in Japan and in other Asian countries for the treatment of breast, colorectal, gastric, and other cancers. It is therefore of interest to know what factors and drugs used in cancer treatment other than cytokines could either directly or indirectly affect the levels of dThdPase. Among cytostatics, Taxol has been reported to elevate levels of TNFα, a potential dThdPase up-regulator, in macrophages (15–17). In the present study, we examined various cytostatics, including Taxol, to determine whether they affected dThdPase levels and enhanced the efficacy of capecitabine and 5'-dFUrU in combination therapy. We found that several cytostatics, including Taxol and Taxotere, up-regulated the enzyme level in tumors, and these drugs in combination with either capecitabine or 5'-dFUrU showed additive to the synergistic activity. The mechanism of the up-regulation is also discussed.

MATERIALS AND METHODS
Animals. Four- or 5-week-old male and female BALB/c nu/nu mice were obtained from SLC Japan, Ltd. (Shizuoka, Japan).

Tumors. The human cancer lines used were obtained from the following institutions: colon cancer WiDr from the American Type Culture Collection (Rockville, MD) and MX-1 from Dr. T. Tashiro (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). WiDr was maintained in vitro cultures with Eagle’s MEM containing 10%
Enhancement of Capecitabine Efficacy by Taxanes

Table 1  Effect of cytostatics on up-regulation of dThdPase and tumor growth inhibition in the WiDr human colon cancer xenograft

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>dThdPase (units/mg protein)</th>
<th>Human TNFa* (pg/g tissue)</th>
<th>Mouse TNFa (ng/g tissue)</th>
<th>Tumor volume changea (mm³), mean ± SD</th>
<th>Carcass body weight changea (g), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Not treated</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td>2.2 ± 0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taxol</td>
<td>100</td>
<td>17.6 ± 2.3</td>
<td>&lt;80</td>
<td>0.81 ± 0.15</td>
<td>328 ± 77</td>
<td>-0.7 ± 0.5</td>
</tr>
<tr>
<td>Taxotere</td>
<td></td>
<td>13.5 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDDP</td>
<td>10</td>
<td>5.0 ± 2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DXR</td>
<td>7.5</td>
<td>2.7 ± 0.1</td>
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<td></td>
<td></td>
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<tr>
<td>MMC</td>
<td>5.0</td>
<td>16.9 ± 3.6</td>
<td></td>
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</tr>
<tr>
<td>VCR</td>
<td>1.0</td>
<td>3.2 ± 1.0</td>
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<tr>
<td>VLB</td>
<td>3.0</td>
<td>4.5 ± 0.8</td>
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<tr>
<td>VDS</td>
<td>5.0</td>
<td>4.7 ± 0.8</td>
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<tr>
<td>Experiment 2</td>
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<tr>
<td>Not treated</td>
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<tr>
<td>Taxotere</td>
<td>15</td>
<td>13.9 ± 1.5</td>
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<tr>
<td>CPA</td>
<td>200</td>
<td>9.2 ± 1.3</td>
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<tr>
<td>MTX</td>
<td>50</td>
<td>2.9 ± 0.1</td>
<td></td>
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</tr>
<tr>
<td>MMC</td>
<td>5.0</td>
<td>11.0 ± 7.3</td>
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* The detection limit of human TNFα level is 80 pg/g tissue.
* Measured tumor volume on day 8 minus that on day 0.
* Measured as carcass body weight on day 8 minus that on day 0.
* Significantly different from not treated; *P < 0.05.

FCS and nonessential amino acids. MX-1 was maintained by continuous passage in BALB/c nu/nu mice.

**Human Cancer Xenograft Model.** A single-cell suspension (7–8 × 10⁶ cells/mouse) of WiDr or small pieces of MX-1 was inoculated s.c. into nude mice. To evaluate the antitumor effect of the cytostatics tested, we measured tumor size and body weight when tumor tissues were excised for measuring the levels of dThdPase, human TNFα, and mouse TNFα.

**Chemicals.** Capecitabine was synthesized by the method described elsewhere (19). 5'-dFUr was synthesized at Hoffmann-La Roche (Basel, Switzerland). The other cytostatic drugs were purchased from various suppliers: 5-FUra, MMC, and DXR from Kyowa Hakko Co. (Tokyo, Japan); UFT (a mixture of tegafur and uracil) from Taiho Pharmaceutical Co. (Tokyo, Japan); Taxol and Cremophor EL from Sigma; Taxotere from Rhône-Poulenc Rorer (Antony, France); CPA, VCR, and VDS from Shionogi Pharmaceutical Co. (Osaka, Japan); VLB from Kyorin Pharmaceutical Co. (Tokyo, Japan); CDDP from Nippon Kayaku Co. (Tokyo, Japan); and MTX from Takeda Pharmaceutical Co. (Osaka, Japan).

Capecitabine, 5'-dFUr, and UFT were dissolved or suspended in 40 mM citrate buffer (pH 6.0) containing 5% gum arabic as the vehicle and then administered p.o. to mice. 5-FUra, CPA, CDDP, DXR, MMC, VLB, VCR, VDS, and MTX were dissolved in saline and given intraperitoneally. Taxol was dissolved in equal volumes of Cremophor EL and ethanol; this dissolution was facilitated by sonication for 3–5 min. The stock solution of Taxol prepared in this manner was kept refrigerated. The stock solution of Taxol was further diluted 1:9 as needed dissolution was facilitated by sonication for 3–5 mm. The stock solution of Taxol was further diluted 1:9 as needed dissolution was facilitated by sonication for 3–5 mm. The stock solution of Taxol was further diluted 1:9 as needed dissolution was facilitated by sonication for 3–5 mm.
weight. Taxotere was dissolved in saline containing 2.5% ethanol and 2.5% polysorbate 80 and given i.v. To evaluate the antitumor effect of the cytostatics tested, the cytostatics were given at doses ranging from 1/4 to 1/3 of the single LD50, except for Taxol.

dThdPase Assays. Tumor tissues were homogenized in 10 mM Tris buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl2, and 50 mM potassium phosphate with a glass homogenizer, whereas tumor cells cultured were sonicated in 10 mM PBS solution (pH 7.4). The homogenate was then centrifuged at 10,000 × g for 20 min at 4°C, and the supernatants were stored at −80°C until use. For the evaluation of Taxol and Taxotere on WiDr in the time course experiments (see Fig. 3), the homogenate was centrifuged at 105,000 × g for 90 min at 4°C. The supernatants were then dialyzed overnight against 20 mM potassium phosphate buffer containing 1 mM β-mercaptoethanol and stored at −80°C until use. The protein concentration was determined by the methods of Lowry et al. (21). The dThdPase level was measured by ELISA with monoclonal antibodies specific to human dThdPase as described previously by Nishida et al. (5). The monoclonal antibodies were not cross-reacted with mouse dThdPase. One unit corresponds to the dThdPase enzyme level. In contrast, other cytostatics, such as CDDP, DXR, VCR, VLB, VDS, and MTX, did not enhance dThdPase activity of the cytostatics, and that of mouse TNFa, which is produced by host stromal cells. Taxol, Taxotere, and MMC greatly enhanced the levels of human dThdPase in the tumors, whereas CPA slightly enhanced the dThdPase levels in tumor tissues, which were measured 8 days after the test drugs were administered to mice bearing the WiDr human colon cancer xenograft. Because the dThdPase levels were detected by the ELISA with monoclonal antibodies specific to human dThdPase, the levels of dThdPase measured corresponded to those of the enzyme produced in the human cancer cells. Taxol, Taxotere, and MMC greatly enhanced the levels of dThdPase in the tumors, whereas CPA slightly enhanced the enzyme level. In contrast, other cytostatics, such as CDDP, DXR, VCR, VLB, VDS, and MTX, did not enhance dThdPase levels.

Positive Correlation between dThdPase and Human TNFa Up-Regulations. To obtain an insight to mechanisms of the dThdPase elevation by the cytostatics, we compared the degree of the dThdPase elevation with that of the antitumor activity of the cytostatics and with that of the human TNFa elevation in each mouse treated. There appears to be no correlation between the dThdPase up-regulation and the efficacy of the cytostatics, we compared the degree of the dThdPase elevation with that of the antitumor activity of the cytostatics and with that of the human TNFa elevation in each mouse treated. There appears to be no correlation between the dThdPase up-regulation and the efficacy of the cytostatics, we compared the degree of the dThdPase elevation with that of the antitumor activity of the cytostatics and with that of the human TNFa elevation in each mouse treated. 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measured at several time points after Taxol and Taxotere were given to mice bearing the WiDr human cancer xenograft (Figs. 3 and 4). Similar time course kinetics of dThdPase elevation was observed in mice bearing other human cancer xenograft line, MX-1 breast cancer (data were not shown).

**dThdPase and TNFα Levels in Cell Cultures.** To investigate whether Taxol directly up-regulates TNFα and dThdPase, the WiDr human cancer cells were exposed to Taxol in vitro for 4 h, and levels of TNFα and dThdPase were measured after cultures of 3 days. The level of dThdPase in the cultured WiDr cells was much lower than that observed in the WiDr cancer xenograft (0.19 versus 2.2 units/mg of protein). In addition, Taxol at concentrations of 150 nM (IC50 in 4 h drug-exposure) and 1000 nM enhanced the level of dThdPase by only 2.0- and 2.1-fold and did not induce TNFα up to a detectable level. Taxol might indirectly induce TNFα and dThdPase in the human cancer xenograft model.

**Combination Therapy.** The efficacy of fluoropyrimidines in combination with Taxol or Taxotere was compared in the WiDr human colon cancer xenograft, which is refractory to fluoropyrimidines, and the MX-1 human breast cancer xenograft. Fluoropyrimidines and the MX-1 human breast cancer xenograft. Fluoropyrimidines were given at their MTD (WiDr) or ½ MTD (MX-1), whereas taxanes were given over a range of ½ MTD to MTD. Fig. 5, a and b, shows that the efficacy of Taxol in combination with either capecitabine or 5'-dFURd was more than just additive, and the tumor regressed. In contrast, Taxol and either 5-FUra or UFT in combination showed only additive activity (Fig. 5, c and d). On the other hand, toxicity in terms of weight loss does not appear to be synergistic (Fig. 5). The additive to synergistic activity was also observed in combination therapy with Taxotere and capecitabine in the same tumor model (Fig. 6) and in the MX-1 human breast cancer xenograft model (Fig. 7). No increase or additive effect in the toxicity was observed in the combination with the taxanes and capecitabine, and the degree of the increase was similar to that of the combination with the taxanes and 5-FUra (Figs. 6 and 7).

**DISCUSSION**

dThdPase, which converts 5'-dFURd to 5-FUra, is essential for the activation of capecitabine and 5'-dFURd in human cancer cells (1, 2). The present study showed that several cytostatics, such as Taxol, Taxotere, MMC, and CPA, elevated this enzyme level in tumor cells in a human cancer xenograft. In addition, both Taxol and Taxotere have thus far showed better efficacy in combination therapy with 5'-dFURd or capcitabine than with 5-FUra or UFT. The major difference between 5'-dFURd/capcitabine and 5-FUra is that the efficacy of the former compounds is greatly modulated by dThdPase. Elevating these enzyme levels by gene transfection or by dThdPase up-regulators made tumor cells more susceptible to 5'-dFURd or capcitabine by up-regulating the dThdPase levels in tumors.

Taxol and Taxotere up-regulated dThdPase levels at doses inhibiting tumor growth; however, the enzyme up-regulation does not always appear to correlate with tumor growth inhibition. CDDP, DXR, VCR, VALB, VDS, and MTX did not enhance the enzyme level, although some of them inhibited tumor growth. Levels of mouse TNFα produced in host stromal cells in tumor tissues were not changed by the cytostatics. They were elevated without Taxol or Taxotere probably by tumor environ-
Fig. 5  Antitumor activity of the combination of Taxol and fluoropyrimidines on the growth of WiDr colon cancer xenograft in nude mice. Taxol was administered i.v. weekly at the optimal dose (15 mg/kg), and fluoropyrimidines were administered p.o. or i.p. five times a week at the MTD. Data points, mean values of tumor volume change and carcass body weight; bars, SD. *, significantly different from vehicle; P < 0.05. ◦, control; ○, Taxol; △, capecitabine; ▲, capecitabine + Taxol; ▼, 5'-dFUr + Taxol; ▼, 5-FUra + Taxol; ◦, UFT; ●, UFT + Taxol.

mental stimuli or by infiltration of host cells producing TNFα to the tumor tissues, such as macrophages. In contrast, the elevation of dThdPase levels in tumors by the cytostatics accompanied by increased levels of human TNFα, which is produced by human tumor cells, in the human cancer xenograft. We previously observed that TNFα, as well as IL-1α and IFNγ, up-regulated dThdPase activity in tumor cells and made the cells more susceptible to 5'-dFUrd (9). Taxol and Taxotere might increase dThdPase levels indirectly through up-regulation of TNFα or by another dThdPase up-regulator though unknown mechanisms.

Taxol and Taxotere enhanced the levels of TNFα and dThdPase in the WiDr human cancer xenograft starting at day 4 or 6 after treatment started. However, Taxol is known to induce TNFα in 1 day in cultures of macrophages (16), whereas we observed that TNFα up-regulated dThdPase in the WiDr tumor cells also in 24 h (9). It is not yet clarified why there was a long delay between the single dose of Taxol/Taxotere and the induction of dThdPase in the tumors. The present study in vitro indicates that Taxol, however, did not induce any detectable levels of TNFα in the WiDr cells. In addition, dThdPase levels in the tumor cells in cultures were very low as compared with those in the human cancer xenograft, and Taxol enhanced dThdPase levels only 2-fold in 3-day cultures. These results indicate that the taxanes might indirectly enhance human TNFα and dThdPase in the tumor cells in tissues. Taxanes might affect host stromal cells in tumor tissues, resulting in the up-regulation of TNFα in tumor cells and consequently that of dThdPase. These processes may take a certain number of days.
In the present study with human cancer xenografts, we measured tumor levels of human dThdPase only by using an ELISA with monoclonal antibodies specific to human dThdPase. It is likely that Taxol and Taxotere also enhance the toxicity of capecitabine and 5'-dFUrd by up-regulating the enzyme in host organs in the tumor model. However, the toxicity of Taxol/Taxotere and capecitabine does not appear to be synergistic, although the efficacy of these compounds in combination was additive to synergistic, and the degree of the increase was similar to that of the combination with the taxanes and 5-FUra. In addition, we have observed that neither Taxol nor Taxotere elevated the enzyme activity, which converts 5'-dFUrd to 5-FUra, in the intestinal tract of mice thus far tested (data were not shown). Therefore, it is unlikely that Taxol or Taxotere greatly enhances the enzyme activity in normal organs. The advantage of the combination with either Taxol or Taxotere and capecitabine should be pursued further. If Taxol or Taxotere enhances the enzyme activity mainly in tumors in patients, clinical assessment of the combination would be warranted.
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REFERENCES


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