Differential Expression of Uridine Phosphorylase in Tumors Contributes to an Improved Fluoropyrimidine Therapeutic Activity

Deliang Cao1, Amy Ziemba2, James McCabe3, Rulian Yan1, Laxiang Wan2, Bradford Kim2, Michael Gach4, Stuart Flynn1, and Giuseppe Pizzorno2,4

Abstract

Abrogation of uridine phosphorylase (UPase) leads to abnormalities in pyrimidine metabolism and host protection against 5-fluouracil (5-FU) toxicity. We elucidated the effects on the metabolism and antitumor efficacy of 5-FU and capecitabine (N4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) in our UPase knockout (UPase−/−) model. Treatment with 5-FU (85 mg/kg) or capecitabine (1,000 mg/kg) five days a week for four weeks caused severe toxicity and structural damage to the intestines of wild-type (WT) mice, but not in UPase−/− animals. Cepacitabine treatment resulted in a 70% decrease in blood cell counts of WT animals, with only a marginal effect in UPase−/− mice. UPase expressing colon 38 tumors implanted in UPase−/− mice revealed an improved therapeutic efficacy when treated with 5-FU and cepacitabine because of the higher maximum tolerated dose for fluoropyrimidines achievable in UPase−/− mice. 19F-MRS evaluation of cepacitabine metabolism in tumors revealed similar activation of the prodrug in UPase−/− mice compared with WT. In WT mice, approximately 60% of cepacitabine was transformed over three hours into its active metabolites, whereas 80% was transformed in tumors implanted in UPase−/− mice. In UPase−/− mice, prolonged retention of 5'dFUR allowed a proportional increase in tumor tissue. The similar presence of fluorinated catabolic species confirms that dihydropyrimidine dehydrogenase activity was not altered in UPase−/− mice. Overall, these results indicate the importance of UPase in the activation of fluoropyrimidines, the effect of uridine in protecting normal tissues, and the role for tumor-specific modulation of the phosphorolytic activity in 5-FU or cepacitabine-based chemotherapy. Mol Cancer Ther; 10(12); 2330–9. ©2011 AACR.

Introduction

Uridine phosphorylase (UPase), a phosphorolytic enzyme ubiquitously expressed, has been shown to be induced in various human solid tumors compared with surrounding normal tissues (1–2). This is possibly due to frequent mutations of p53 (a suppressor of UPase gene expression; ref. 3) or higher expression of various cytokines (inducers of UPase expression) in tumor tissues (4, 5). Because of its increased expression in tumors, it is important to determine the role of UPase in the activation and antitumor activity of fluoropyrimidines, such as 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5'-dFUR), and cepacitabine (N4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine). UPase knockout (UPase−/−) mice provide an ideal model for this study (6).

The antitumor activity of 5-FU stems from its proximal metabolites, 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), 5-fluorouridine-5'-triphosphate (FUTP), and 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP). These active metabolites either inhibit the activity of thymidylate synthase or incorporate into RNA or DNA, leading to nucleic acid dysfunction and cell death (7, 8). Several studies have indicated the role of UPase in fluoropyrimidine activation, including the anabolism of 5-FU into 5-fluorouridine (FUr) with subsequent phosphorylation to 5-fluorouridine monophosphate (FUMP) and the phosphorolysis of the prodrug 5'dFUR into 5-FU (9–11). Using human and murine cancer cell lines, Peters and colleagues (12) reported that FUr synthesis was directly correlated to the intracellular UPase activity. In colon 26 tumor cells, a mixture of TNF-α, interleukin-1α (IL-1α), and IFN-γ efficiently enhanced 5-FU cytotoxicity 2.7-fold and 5'dFUR cytotoxicity 12.4-fold due to induction of UPase activity (5). However, in most experimental models the contribution of UPase to fluoropyrimidine activity has been controversial due to the coexistence of other related metabolic enzymes, thymidine phosphorylase (TPase).
and orotate phosphoribosyltransferase (OPRTase). OPRTase participates in the de novo pyrimidine synthesis pathway, directly converting 5-FU to FUMP in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP; ref. 13), whereas TPase contributes to the formation of fluorodeoxyuridine (dFURd; ref. 14). In addition, TPase is also involved in the phosphorolysis of 5′-dFUR into 5-FU (15). We have investigated the effect of UPase on the antiproliferative activity of fluoropyrimidines using a gene-targeted cell model, UPase gene-knockout murine embryonic stem cells, and found that the abrogation of UPase in these cells resulted in an 8- and 16-fold increase in the IC50 values of 5-FU and 5′-dFUR, respectively (9). The unique genetic modification of UPase activity directly confirmed the role of UPase in fluoropyrimidine metabolism and activation. However, this in vitro system does not allow the investigation of the metabolic kinetics and tissue distributions of fluoropyrimidines. In addition, the cultured cells are also limited for the study of capecitabine. A prodrug of 5-FU, because its activation is precluded by the lack of carboxylesterase (16). In this article, we investigated the metabolic kinetics and tissue distributions of 5-FU in UPase−/− mice and evaluated the histologic basis of host toxicity of capecitabine. A major drawback of fluoropyrimidine-based chemotherapy are the severe dose-limiting side effects at the level of the bone marrow and gastrointestinal tract, often resulting in therapeutic failure (17, 18). Therefore, the development of prodrugs or modulatory strategies to increase tumor selectivity is an important effort to improve the therapeutic efficacy of fluoropyrimidines. Using UPase−/− mouse-based colon tumor models, we assessed the effect of the tumor-specific modulation of UPase activity on tumor selectivity and antitumor efficacy of 5-FU and capecitabine. The results provide important information for clinical approaches to improve the therapeutic outcomes of fluoropyrimidine-based regimens.

Materials and Methods

Animals and cell lines

Wild-type (WT) and UPase−/− mice were produced and maintained as previously described (6). All experiments were carried out according to Yale University and NVCI guidelines for the humane treatment of animals. Colon 38 murine adenocarcinoma cells (MC38) were originally obtained from the Southern Research Institute, Birmingham, AL (19) and authenticated by flow cytometry before implantation.

Table 1. Enzymatic activities in tissues of WT and UPase−/− mice (nmol/mg/h)

<table>
<thead>
<tr>
<th></th>
<th>UPase</th>
<th>TPase</th>
<th>UK</th>
<th>OPRTase</th>
<th>DPD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>UPase−/−</td>
<td>WT</td>
<td>UPase−/−</td>
<td>WT</td>
</tr>
<tr>
<td>Liver</td>
<td>5.6 ± 1.5</td>
<td>1.3 ± 1.0</td>
<td>25.9 ± 6.2</td>
<td>26.7 ± 7.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Small intestine</td>
<td>696.8 ± 80.0</td>
<td>ND</td>
<td>1.9 ± 0.5</td>
<td>2.0 ± 0.5</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Colon 38</td>
<td>28.5 ± 4.7</td>
<td>27.9 ± 5.3</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>6.6 ± 1.5</td>
</tr>
</tbody>
</table>

Abbreviations: TPase, thymidine phosphorylase; UK, uridine kinase; OPRTase, orotate phosphoribosyltransferase. +/+ , UPase WT; −/−, UPase knockout; ND, not detectable. a, P < 0.05 compared with WT mice.
blood collected by retro-orbital bleeding. Plasma was separated by centrifugation at 2,000 × g, 4°C for 5 minutes, extracted with 2 volumes of 15% trichloroacetic acid (TCA) at 14,000 rpm for 10 minutes and neutralized with an equal volume of trictylamine-freon (45:55, v:v). Aqueous phase was used for high-performance liquid chromatography (HPLC) analysis (19). Tissues frozen in liquid nitrogen were weighed and homogenized in 2 volumes of 15% TCA and the supernatant extracted for HPLC analysis as described for plasma. 5-FUrd and 5-FU were extracted using TRIzol reagent (Invitrogen; ref. 9). DNA and tumor, and intestinal tissues were weighed and total RNA digested by scintillation counter (Beckman). For RNA incorporation of 5-FU, liver, tumor, and intestinal tissues were weighed and total RNA extracted using TRIzol reagent (Invitrogen; ref. 9). DNA was isolated by suspending the precipitated nucleic acids in lysis buffer, then incubating the suspension at 37°C overnight and subsequently for 1 hour at 37°C with RNase A/T1. To precipitate DNA, an equal volume of 2-propanol was added and mixed until precipitation was complete and further by extractions with phenol/chloroform/iso-amylalcohol (49/1 v/v). DNA was precipitated with an equal volume of 2-propanol and reconstituted in 0.5 mL digestion buffer at 55°C. For measurement of concentration and purity, optical density was measured at 260 and 280 nm. The radioactivity in DNA/RNA was determined by measuring the conversion of thymidine to uracil as described previously (9). TPase activity was determined by measuring the conversion of uridine into UMP in the presence of ATP (6). The reaction was done in a 100 μL volume with 200 μmol/L [14C]-uridine, 1 mmol/L ATP, 100 μmol/L MgCl2, 50 mmol/L Tris-Cl (pH 7.6), and 100 μmol/L BAU. The resultant [14C]-UMP was separated on TLC plates and determined by scintillation counting. Dihydropyrimidine dehydrogenase (DPD) activity was measured by determining the conversion of uracil into dihydouracil. The assay mixture contained 200 mmol/L NADPH and 8.25 mmol/L [14C]-uracil in a solution constituted by 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L magnesium chloride, 10 mmol/L 2-mercaptoethanol. The formed [14C]-dihydouracil was separated on TLC plates and radioactivity determined (22).

MRS evaluation of capecitabine metabolism

WT or U/Pase−/− mice bearing colon 38 tumors were treated orally with 1,000 mg/kg capecitabine. Animals were anesthetized with isoflurane (2%–3% induction and 1%–2% maintenance) along with 0.5 LPM O2. MRI and MRS were done using a Bruker 7T/20 Biospin MRI, equipped with a dual-tuned 7 cm ID 1H/19F volume coil (Bruker), and a 15-mm ID detachable receive-only 19F surface coil (Doty Scientific). The surface coil was placed over the region of interest (ROI) and the animal secured to minimize the effects of motion on the MR data. A 1H T2-weighted TurboRae sequence [repeat time (TR): 2,330 milliseconds, echo time (TE): 36 milliseconds, flip angle: 90 degree; ref. 23].

19F spectra were acquired and averaged during 27.5 minutes blocks [3,300 free induction decays (FID), TR: 0.5 seconds, spectral width: 60 kHz, points/FID: 890, flip angle: 60 degree, RF pulse duration: 50 μseconds] for 3 hours. The 19F MRS data were analyzed using Bruker’s Topspin application. The rate of capecitabine activation and buildup of the intermediate molecules were evaluated.

Peripheral blood cells counting

Peripheral blood samples were collected from mice treated with capecitabine at 1,000 mg/kg for 4 weeks. Number of erythrocytes per microliter and hematocrit values were determined using a Coulter counter (Model ZF, Coulter Electronics). Hemoglobin was measured by a hemoglobinometer (Coulter Electronics) using the cyanmethemoglobin method. Numbers and subtypes of white blood cells were determined on 50 μL of peripheral blood, after red blood cells were osmotically lysed, using a flow cytometer (Becton Dickinson). Total and differential white blood cell counts were determined. To determine the
platelet counts, the white blood cell preparations were diluted at 1:10 and the counts were obtained by appropriately adjusting gains and threshold (24).

**Histologic examination of small and large intestine**

The intestinal tract, the small intestine, and colon were excised from mice treated with 5-FU (85 mg/kg) or capecitabine (1,000 mg/kg) for 4 weeks. The isolated tissues were then fixed in formal fixative and transverse sections (5 μm) were prepared for staining with hematoxylin and eosin. Histologic changes of the tissues were evaluated under light microscopy.

**Results**

In a previous study, we reported that the abrogation of UPase in mice led to a decrease in 5-FU host toxicity (6). However, it was not clear whether this host protection resulted from a reduced activation of 5-FU through the pyrimidine salvage pathway or from the protection of increased uridine in the plasma and tissues, due to the elimination of UPase activity. In this study on the UPase<sup>−/−</sup> mouse model, we investigated metabolism and tissue distribution of 5-FU and examined the pathologic basis of 5-FU and capcitabine toxicity, and we evaluated the effect of UPase abrogation on the antitumor activity of 5-FU and capcitabine.

Plasma clearance of 5-FU displayed a faster rate in UPase<sup>−/−</sup> mice than in WT with a t<sub>1/2</sub> of 26.3 minutes versus 37.9 minutes and clearance of 29.4 versus 18.1 mL/min/kg, respectively, for UPase<sup>−/−</sup> and WT C57 BL6 mice (Table 2). This indicates that alterations in the anabolic pathway of 5-FU due to UPase abrogation significantly affected its clearance rate, consequently reducing exposure to the drug as indicated by a significantly smaller systemic exposure with an AUC of 84.5 mmol/L-min in WT compared with 52.3 mmol/L-min for the knockout mice. Normally, plasma 5-FU is mainly removed via the DPD-initiated degradation pathway (25). When the drug is administered orally or intraperitoneally, liver represents the major site of 5-FU metabolism, with more than 85% of a given dose of the fluoropyrimidine eliminated through the rapid formation of dihydrofluorouracil (26). Although the expression and the enzymatic activity of DPD in the liver of both mice are virtually similar (Table 1), the elevated 5-FU clearance in UPase<sup>−/−</sup> mice possibly suggests a reduced competition of uracil for DPD, in which uracil formation from uridine degradation is blocked because of the lack of UPase. However, the plasma pharmacokinetics of FUrd, following 5-FU delivery, displayed a significant difference in UPase<sup>−/−</sup> mice (Table 2). In WT mice, FUrd appeared in plasma within 5 minutes following intraperitoneal administration of 5-FU (200 mg/kg), reaching nearly 4 μmol/L at 10 minutes. Thereafter, FUrd concentration quickly declined to an undetectable level at 4 hours. Plasma FUrd in UPase<sup>−/−</sup> mice was barely detectable at 10 minutes, and its peak appeared 1 hour after administration of the same dose of 5-FU. The clearance of plasma FUrd was significantly slower in UPase<sup>−/−</sup> mice than in WT, and a considerable amount of FUrd was still detected 4 hours after 5-FU administration resulting in a significantly higher AUC (316 vs. 105 μmol/L-min; Table 2). In WT mice, UPase rapidly catalyzed the formation of FUrd from administered 5-FU. However, in UPase<sup>−/−</sup> mice, plasma FUrd was possibly originated from the degradation of FUMP synthesized by OPRTase-catalyzed de novo pathway (13, 27) and eventually cleared by kidney excretion (6). As a result, a delay occurred in plasma peak concentration of FUrd, accompanied with a prolonged half-life in UPase<sup>−/−</sup> mice.

The liver is the major metabolic organ for 5-FU, and the gastrointestinal system represents one of the major toxicity targets for fluoropyrimidines. Therefore, the 5-FU metabolic pharmacokinetics and distribution in these tissues were examined. Overall, 5-FU and FUrd showed similar metabolic patterns in these tissues unlike that in

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>WT</th>
<th>5-FU</th>
<th>5-FUrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>min</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>μmol/L</td>
<td>3,175.4</td>
<td>2,500.7</td>
<td>3.55</td>
</tr>
<tr>
<td>C&lt;sub&gt;0&lt;/sub&gt;</td>
<td>μmol/L</td>
<td>5,333.1</td>
<td>5,074.6</td>
<td>—</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>mmol/L-min</td>
<td>85,177</td>
<td>52,385</td>
<td>0.104</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-240&lt;/sub&gt;</td>
<td>mmol/L-min</td>
<td>84,523</td>
<td>52,338</td>
<td>0.105</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>min</td>
<td>37.9</td>
<td>26.3</td>
<td>—</td>
</tr>
<tr>
<td>CL</td>
<td>mL/min/kg</td>
<td>18.1</td>
<td>29.4</td>
<td>—</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>mL/kg</td>
<td>633</td>
<td>595.7</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE: Estimated using WinNonlin V5.2, noncompartmental IV bolus model. Linear up, log down trapezoidal integration rule. AUC<sub>inf</sub> value is predicted from terminal elimination rate constant. Treated the 0 concentration at 240 min for FURD WT as missing.
plasma (Fig. 1A and B), 5-FU was cleared slightly faster in both liver and intestines of UPase\(^{-/-}\) mice, but with no statistical significance (\(P > 0.05\)) compared with WT animals; although the formation and metabolism of FUrd varied with UPase expression status and tissue types. In WT intestinal tissues, a high level of FUrd was detected at 10 minutes after intraperitoneal administration of 5-FU (200 mg/kg) but declined rapidly within 30 minutes and still maintained detectable levels after 4 hours. In UPase\(^{-/-}\) intestinal tissues, however, FUrd appeared at 30 minutes and peaked at 60 minutes after 5-FU delivery. However, in UPase\(^{-/-}\) liver tissues, FUrd was present at 10 minutes and reached a peak concentration at 30 minutes, 3-fold higher than that in the intestines, indicating an active 5-FU anabolism through pyrimidine de novo synthesis in liver. Similar to that in the intestines, the clearance of FUrd was much faster in WT liver than that in UPase\(^{-/-}\) (Fig. 1B). Taken together, these data indicate the important role of UPase in 5-FU/FUrd metabolism in normal intestinal and liver tissues, supporting our previous observation that ribose-1-phosphate is not a rate-limiting factor in the 5-FU anabolic metabolism catalyzed by UPase (9).

The incorporation of 5-FU into RNA is important for its antiproliferative activity (28) but has also been linked to the toxic effect of fluoropyrimidines (29-30). Therefore, we included in our investigation the measurement of FUXP levels and 5-fluoro-RNA amounts in liver, gastrointestinal tract and transplanted colon 38 tumor. As shown in Fig. 1C and D, the levels of FUXP and 5-fluoro-RNA were significantly higher in WT liver and intestines than in UPase\(^{-/-}\), 5-FU incorporated into RNA of liver tissue started to be cleared within the first hour, although we observed a protracted accumulation in the RNA of the intestinal tissues of WT mice for more than 4 hours, providing the pharmacokinetic basis for the intestinal lesions caused by the fluoropyrimidines in the WT mice. The lower incorporation of 5-FU in the normal tissues of the UPase\(^{-/-}\) mice, likely due to the competition of high uridine levels, confirms our previous observation of reduced in vivo host toxicity to 5-FU and a 75% higher MTD of 5-FU in this strain (6).

We observed significant differences in the concentration of uridine in plasma and normal tissues of WT mice compared with the UPase\(^{-/-}\) (Table 3). Uridine concentration was elevated 3-fold in the intestine and kidney and up to 15-fold in spleen of UPase\(^{-/-}\) mice compared with the corresponding WT tissues. In colon 38 tumors transplanted in both WT and UPase\(^{-/-}\) mice, we observed a reduced uridine concentration compared with the other tissues with levels similar to plasma concentrations. The elevation in tumor uridine concentration we determined in UPase\(^{-/-}\) was highly significant (\(P < 0.01\)) compared with the tumor in WT mice (7 vs. 1 \(\mu\)mol/L); however, the
Uriddine concentration still remained almost 10-fold below the normal tissue concentrations (Table 3).

When we measured the incorporation of 5-FU in colon 38 tumors, we observed 69.2 ± 28.4 nmol/g into the RNA of tumors in WT mice and 64.6 ± 26.9 nmol/g in tumors implanted in UPase−/− 4 hours after the administration of a 200 mg/kg dose of 5-FU. Also the incorporation of 5-FU into DNA was not significantly different between tumors grown in WT mice compared with UPase−/−, 367 ± 57 fmol/μg of DNA and 431 ± 83 fmol/μg of DNA, respectively.

The concentration of FdUMP present in colon 38 tumors following a 200 mg/kg administration of 5-FU did not show any significant difference between the tumors implanted in the 2 different strains, 66 ± 21 pmol/g of tissue of FdUMP in WT versus 72 ± 13 in UPase−/− mice.

The role of UPase in the activation of 5′-dFUR to 5-FU has also been determined in this study. Capecitabine is a prodrug of 5-FU, approved for the treatment of advanced breast and colon cancers (31, 32). The carbamate modification facilitates its passage through the gastrointestinal mucosa without activation, leading to almost 100% oral bioavailability (33). In liver, capcitabine is hydrolyzed by carboxylesterase to 5′-deoxy-5-fluorocytidine (5′-dFCR) then converted to 5′-dFUR by cytidine deaminase. 5′-dFUR has no inherent cytotoxicity; to exert its antiproliferative activity, it must be hydrolyzed to 5-FU by TPase and UPase (17, 33). In this study, we assessed the effect of TPase on the host toxicity of capecitabine using UPase and TPase (17, 33). In this study, we assessed the effect of UPase on the host toxicity of capecitabine using UPase−/− mice. A daily 1,000 mg/kg oral dose of capcitabine caused nearly 20% weight loss in WT mice within 4 weeks; and 1,375 mg/kg led to the first death immediately preceding the third dose due to gastrointestinal bleeding (data not shown). In UPase−/− mice, however, no significant host toxicity was observed at doses up to 1,250 mg/kg, and the higher dose of 1,375 mg/kg caused weight loss (15%) only comparable to that in WT mice at 1,000 mg/kg, further clarifying the role of UPase in the activation of 5′-dFUR/capcitabine. Possibly due to the significant TPase activity reported in mouse liver (6), which also contributes to conversion of 5′-dFUR into 5-FU (15, 34), a larger MTD dose was not achieved in the UPase−/− mice.

As previously indicated, bone marrow and the gastrointestinal tract are the 2 major targets of fluoropyrimidines lesions (17, 18). To understand the histologic basis of this host protection by UPase abrogation, we further examined the pathologic changes in the intestines and bone marrow of mice treated with 5-FU and capcitabine. Peripheral blood cell counts were used to evaluate the hematologic toxicity, and intestinal transverse sections were examined following hematoxylin and eosin staining to evaluate the intestinal lesions. The treatment with capecitabine (1,000 mg/kg) for 4 weeks caused a decrease of up to 70% in peripheral blood cells of WT animals. The alterations occurred in the hematocrit level (−40%), red blood cells (−44%), white blood cells (−72%) and subtypes, and platelets (−66%), indicating an overall effect on the regeneration and differentiation of bone marrow cells. These decreases in cell counts were minimal and not significant in the peripheral blood of UPase−/− mice, indicating that the abrogation of UPase activity and the high concentration of circulating uridine protected the bone marrow from capcitabine-induced toxicity. A similar result was also observed in mice treated with 5-FU (data not shown).

The UPase abrogation also protected murine intestine from capcitabine lesions. We observed a complete destruction of the villous architecture, crypts, and muscle layer in the intestines of WT mice treated with 1,000 mg/kg of capcitabine for 4 weeks, but not in the intestines of UPase−/− mice exposed to the same treatments. This protection by UPase abrogation confirms the critical role of UPase in 5′-dFUR/capcitabine activation and toxicity.

To evaluate the clinical significance of host protection provided by UPase abrogation, we further assessed the antitumor efficacy of 5-FU and capcitabine (Fig. 2A). Treatments were initiated when the implanted tumors grew to an average size of 100 to 150 mg. 5-FU was used at 85 mg/kg (the MTD for WT mice) and 150 mg/kg (a dose shown to be tolerated only by UPase−/− mice; ref. 7); and capcitabine was administered at 1,000 mg/kg (the MTD for WT mice) and 1,375 mg/kg (a dose shown to be tolerated only by UPase−/− mice). As presented in Fig. 2B, a higher dose of 150 mg/kg of 5-FU efficiently controlled and reduced tumor size, whereas the standard 85 mg/kg dose of 5-FU only partially slowed down tumor growth in both strains. In the capcitabine treatments, a 30% dose increase from 1,000 mg/kg to 1,300 mg/kg resulted in dramatic tumor growth inhibition in UPase−/− mice (Fig. 2C), with complete disappearance of tumors in 10 of 11 mice. These data indicate that the tumor-specific modulation of UPase activity can greatly improve the antitumor efficacy of 5-FU and capcitabine by allowing dose escalation without causing significant host toxicity. To better evaluate the activation of capcitabine and the potential differences in metabolism between colon 38

| Table 3. Plasma and tissue concentrations of endogenous uridine in WT and UPase−/− mice |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Plasma (μmol/L) |                 |                 | Tissues (pmol/mg) |                 |                 |                 |                 |                 |
|                 | sd              | Gut sd           | Kidney sd       | Liver sd         | Spleen sd       | Tumor sd        |                 |                 |                 |
| WT              | 1.5             | 0.6              | 29.3            | 3.4              | 24.6            | 3.6             | 6.4             | 2.5             | 4.9             |
| UPase−/−        | 7.2             | 2.5              | 89.3            | 2.3              | 71.0            | 12.6            | 42.8            | 7.0             | 75.2            |

Published OnlineFirst September 27, 2011; DOI: 10.1158/1535-7163.MCT-11-0202
tumors implanted in WT mice and in UPase<sup>−/−</sup> mice, we conducted a series of in vivo<sup>19</sup>F-MRS experiments using a 1,000 mg/kg oral dose of capecitabine administered as a bolus. The in vivo spectra (Fig. 3A) resolve 5 main fluorinated species, with the administered drug capecitabine at 7 ppm upfield from the 5-FU signal, 5<sup>0</sup>-deoxy-5-fluorocytidine at 5 ppm, 5<sup>0</sup>-deoxy-5-fluorouridine at 3.5 ppm, and the 2 main fluorinated metabolites fluoroureaepropionic acid and α-fluoro-β-alanine at −17 and −19 ppm, respectively. As reported in Fig. 3A, we detected all 5 fluorinated species mentioned, starting 30 minutes after the oral administration of capecitabine, and were able to follow their presence over 3 hours of spectra acquisition. We observed in tumors implanted in WT mice that capecitabine represented approximately 35% of the total fluorine signal, 5<sup>0</sup>-dFCR corresponded to a similar percentage, whereas 5<sup>0</sup>-dFUR ranged from 25% to 30% of total fluorine. The 2 catabolites, FUPA and FβAL were approximately 5% of the total (Fig. 3B). The tumors implanted in the UPase<sup>−/−</sup> mice showed that capecitabine was approximately 15% of the total fluorine signal, 5<sup>0</sup>-dFCR 45%, 5<sup>0</sup>-dFUR 35%, and the sum of the 2 catabolites ranged from 4% to 7% of the total (Fig. 3C).

We were unable to capture the 5-FU signal consistently, likely due to the rapid transformation of 5-FU into 5-FUrd and its fluoronucleotides in colon 38 tumors expressing WT UPase. 5-FUrd and FUXP with their signals at 3.6 and 5.1 ppm, respectively, were impossible to quantitate, given the overwhelming presence of 5<sup>0</sup>-dFCR and 5<sup>0</sup>-dFUR in the 3.5 to 5 ppm region.

Our data indicates that the genetically induced changes and the elevation in circulating uridine did not interfere with the carboxylesterases and cytidine deaminase in the activation of capecitabine. Also, the comparable presence of 5-FU catabolites FUPA and FβAL in liver from WT and UPase<sup>−/−</sup> mice confirms the similar DPD enzymatic activity (Table 1) and quantitative real-time RT-PCR data (WT 1.0 ± 0.1 vs. 0.8 ± 0.2 in UPase<sup>−/−</sup>) and, therefore, a similar rate of catabolic degradation for 5-FU in the 2 murine strains.

Discussion

UPase activity and expression has been shown to be elevated in many tumors including colorectal carcinomas, breast cancer, melanomas, and lung adenocarcinomas (11). Several mechanisms have been uncovered, all leading to an increased expression in human tumors. We have previously reported that the expression of UPase is induced by TNF-α through the NF-κB pathway (11). Other groups have shown that EWS/ETS fusion proteins, playing a dominant oncogenic role in cell transformation in Ewings family tumors, induce UPase gene expression through interaction with the UPase promoter (35).
recently, PGC-1α/ERR-dependent upregulation of UPase was shown to contribute to an increased enzymatic activity in colon and breast cancer cells (36). These results show that the elevation of UPase in tumor is a key contributor to the tumor selectivity of 5-FU and capecitabine. Unlike TPase, UPase is not associated with any identified angiogenic activity because of its limited catalytic activity on deoxynucleosides. It has been shown that 2-deoxyribose-1-phosphate released from the deoxynucleoside by TPase activity can act as an endothelial cell chemoattractant and angiogenic factor (37). This feature allows for a convenient modulation of the phosphorolytic activity in tumors by UPase gene transfer or delivery of specific inducers of UPase gene expression, such as cytokines. This modulation would not be complicated by possible tumor growth stimulation due to simultaneously induced angiogenesis, as is the case with TPase (38).

The data here presented establish once more the role of uridine in protecting normal tissues from the toxicity of fluoropyrimidines. In the normal tissues of UPase/−/− mice, we observed a constitutive uridine concentration above 50 μmol/L with the gut, the major target of 5-FU toxicity at 90 μmol/L. These concentrations have been found previously to be sufficient to provide adequate protection against 5-FU–based chemotherapy regimens (39–40). Similarly, the concentration of uridine in colon 38 tumors implanted in UPase/−/− mice approximated the plasma uridine concentration of 7 μmol/L, indicating that the inability of some tumors to accumulate this nucleoside is likely due to the loss of the concentrative transport mechanism (41).

Several studies have shown the capacity of large doses of uridine to reduce 5-FU toxicity, without affecting its antitumor activity, if properly sequenced (42). Unfortunately, the administration of large doses of uridine, because of its rapid half-life, results in moderate to severe toxicity. Our laboratory has shown that this problem could be overcome by using inhibitors of UPase, such as BAU, to conserve endogenous uridine with consequent elevation of its concentration in plasma and tissues. A phase I clinical trial of oral BAU administered as a single agent has shown the ability of this inhibitor to elevate the
plasma uridine concentration 2- to 3-fold without significant host toxicity (43).

The combination “rescue regimens” of 5-FU plus uridine were initially proposed to evaluate the hypothesis that the antitumor effect of 5-FU is primarily due to the inhibition of thymidylate synthase and the host toxicity mostly caused by the incorporation of the fluoropyrimidine into RNA (44). In vivo studies in a murine model and in vitro data (45, 46) have clearly indicated that the incorporation of 5-FU into RNA seems to be the major cause of gastrointestinal toxicity that uridine inhibited the incorporation and avoided the cytotoxic effect, whereas thymidine did not prevent 5-FU toxicity.

Clinical studies of 5-FU in combination with methotrexate and PALA have shown that patients tolerated combination therapy with delayed uridine up to a weekly dose of 750 mg/m² of 5-FU, with 25% experiencing moderate mucositis (grade II). In previous clinical trials without uridine, 4 of 6 patients could not tolerate a 600 mg/m² dose of 5-FU because of mucositis, diarrhea, and a decrease in performance status. Another study of high-dose 5-FU with doxorubicin, high-dose methotrexate and leucovorin, and oral uridine administration allowed for dose intensification of 5-FU and ensured rescue from 5-FU-induced hematologic toxicity without adverse impact on tumor response (47). As indicated in preclinical studies, properly delayed uridine rescue results in a faster clearance of 5-FU from RNA of bone marrow and enhancement of the rate of recovery of DNA synthesis (39).

Nucleoside transporters are expressed in a variety of cells and tissues with different substrate specificity and selectivity. Most cell types coexpress concentative nucleoside transporters (CNT) and equilibrative nucleoside transporters (ENT) to maintain their nucleoside supply. Uridine is mainly accumulated in cells of different origin by the Na⁺-dependent CNT1 (SLC28A1) but also by CNT3 (SLC28A3) that has broader substrate specificity. ENTs, particularly ENT1 (SLC29A1) and ENT2 (SLC29A2), mediate facilitated diffusion transport with broader selectivity but relatively low affinity. Recent studies have indicated that ENT1 is a sort of housekeeping transporter, and its expression is often high and is highly retained in tumors (48).

A clinical study using a tissue array on 300 paraffin-embedded gynecologic tumors (endometrium, ovary, and cervix) showed that hENT1 and hENT2 protein expression were highly retained, but a significant number of tumors were hCNT1 negative (49). A more recent breast cancer study has shown that the percentage of hCNT1-positive cells correlates positively with a reduced long-term survival in patients treated with cyclophosphamide/methotrexate/5-FU chemotherapy (50). It is reasonable to speculate that high expression of hCNT1 could be linked to high nucleotide salvage efficiency interfering with 5-FU nucleoside metabolism and, ultimately, with its antitumor activity.

In summary, this study determined the metabolism and tissue distribution of 5-FU in UPase⁻/⁻ mice and proved the role of UPase in the activation and antitumor activity of 5-FU and capecitabine. This study also showed the pathologic basis for host protection by UPase abrogation from 5-FU and capecitabine lesions, the ability of uridine to protect the normal tissues, and exhibited the effect of tumor-specific expression of UPase on the therapeutic efficacy of these agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported in part by grants from the National Cancer Institute CA67035 and CA102121 (G. Pizzorno), the Charlotte Geyer Foundation (G. Pizzorno), and the United States Army Medical Research Breast Cancer Research Program DAMD17-00-1-0508 (D. Cao).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 15, 2011; revised September 2, 2011; accepted September 5, 2011; published OnlineFirst September 27, 2011.

References


Molecular Cancer Therapeutics

Differential Expression of Uridine Phosphorylase in Tumors Contributes to an Improved Fluoropyrimidine Therapeutic Activity

Deliang Cao, Amy Ziemba, James McCabe, et al.

Mol Cancer Ther 2011;10:2330-2339. Published OnlineFirst September 27, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0202

Cited articles
This article cites 49 articles, 20 of which you can access for free at:
http://mct.aacrjournals.org/content/10/12/2330.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/10/12/2330.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.