Noninvasive Measurements of Capecitabine Metabolism in Bladder Tumors Overexpressing Thymidine Phosphorylase by Fluorine-19 Magnetic Resonance Spectroscopy

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ABSTRACT

Purpose: Previous studies have shown that tumor response to capecitabine strongly correlates with tumor thymidine phosphorylase (TP). The aims of our study were to (a) investigate the pharmacological role of TP by measuring the pharmacokinetics (PK) of capecitabine in a human bladder tumor model that was characterized by the overexpression of TP and (b) develop the use of PK measurements for capecitabine by fluorine-19 magnetic resonance spectroscopy as a noninvasive surrogate marker for determining TP levels in tumors and for predicting tumor response to capecitabine in patients.

Experimental Design: TP overexpressing (2T10) and control tumors were grown s.c. in nude mice. Mice were given a dose of capecitabine or 5'-deoxy-5-fluorouridine (5'DFUR). 19F tumor spectra were acquired for determination of rate constants of capecitabine breakdown and buildup and subsequent breakdown of intermediates, 5'-deoxy-5-fluorocytidine (5'DFCR) and 5'DFUR. The rate constant of 5'DFUR breakdown was doubled compared with controls (P < 0.001).

Results: The rate constant of breakdown of intermediates was significantly faster in 2T10 tumors than controls (P < 0.003). No significant differences in the rate of capecitabine breakdown or intermediate buildup were observed.

The rate constant of 5'DFUR breakdown in the 2T10 tumors was doubled compared with controls (P < 0.001).

Conclusions: This study confirmed the expected pathway of capecitabine metabolism and showed that the level of TP was related to the rate of 5'DFUR conversion. Using in vivo fluorine-19 magnetic resonance spectroscopy to measure the PK of capecitabine and its intermediate metabolites in tumors may provide a noninvasive surrogate method for determining TP levels in tumors and for predicting tumor response to capecitabine in patients.

INTRODUCTION

Capecitabine (N-pentyloxy carbonyl-5'-deoxy-5-fluorocytidine, Xeloda) is a novel fluropyrimidine carbamate (1) and a pro-drug of 5-fluorouracil (5-FU). It is activated and preferentially converted into 5-FU within tumors by exploiting the differences in thymidine phosphorylase (TP) activity in tumor and normal tissue (2). 5-FU, selectively converted at the tumor site, will enhance antitumor activity.

Capecitabine is first converted to 5'-deoxy-5-fluorocytidine (5'DFCR) by hepatic carboxylesterase and then to 5'-deoxy-5-fluorouridine (5'DFUR) by cytidine deaminase in the liver and tumor tissue (1). TP, which is highly active in tumor tissue, converts 5'DFUR to 5-FU in the final conversion step (Fig. 1; Refs. 3–5). Therefore 5-FU can be generated preferentially in tumor tissue compared with normal tissue. 5-FU is then activated via a number of different biochemical pathways and results in anabolite production of cytotoxic metabolites, which are ultimately responsible for tumor cell death. In addition to anabolism, 5-FU could be catabolised by dihydropyrimidine dehydrogenase (DPD) into biologically inactive metabolites, principally α-fluoro-β-alanine (FBAL), that are excreted in the urine and bile (Fig. 1; Ref. 6, 7).

TP is structurally identical to the platelet-derived endothelial cell growth factor and a major angiogenic stimulatory factor (8, 9). It is highly active in most solid tumors, and levels correlate with fast malignant growth and aggressive invasion potential (10, 11). In addition, TP prevents tumor cells from entering apoptosis (12, 13). High levels of TP expression in many tumors (e.g., breast, colorectal, gastric, bladder, etc.) have been correlated with increased angiogenesis as well as poor prognosis (14–21).

Previous studies have shown that the efficacy of capecitabine is correlated with TP levels in tumors (2, 5, 22). A recent clinical study suggested that high TP expression is a good indication of disease-free survival for those patients taking 5-FU prodruk-based chemotherapy (23). Induction of TP has been suggested as a potential method of increasing the efficacy of capecitabine. Irradiation and anticancer drugs such as paclitaxel, docetaxel, cyclophosphamide, have all been shown to increase TP expression with improved capecitabine efficacy (24–28).
It would be very useful to have a noninvasive predictive marker for response to chemotherapeutic drugs. Such a marker would minimize the unnecessary exposure of patients to cytotoxic drugs, and clinicians could opt for an alternative or combination therapy at an earlier time, if a patient is found to be unlikely to be responsive to capecitabine. However, the methodologies for determining TP levels involve obtaining invasive biopsies from patients. A noninvasive pharmacokinetic marker for monitoring the uptake and breakdown of capecitabine and potentially for predicting treatment response would be a more desirable end point for use in clinical trials and eventually in the clinic.

The use of noninvasive in vivo fluorine-19 magnetic resonance spectroscopy ($^{19}$F MRS) to study the metabolism of capecitabine may have the potential to be developed into such a marker. In vivo $^{19}$F MRS has been used extensively as an investigative tool for 5-FU pharmacology. It has been used to study the distribution and metabolism of 5-FU in normal and tumor tissues both in xenografts and in patients, and it has provided significant information on the fate and the potential pharmacodynamic effects of this drug (29–35). This technique has also been extended to study other fluorinated drugs such as 5-FU analogs (36), fluorinated nitroimidazoles (37, 38), gemcitabine (39), and antifolates (40).

In this study, we have used in vivo $^{19}$F MRS to study capecitabine and its metabolites. First, we investigated the pharmacological roles of TP by measuring the pharmacokinetic parameters of these drugs in a human bladder cell xenograft that was characterized by the overexpression of TP and compared it with wild-type and empty vector controls (41). Second, we developed the use of pharmacokinetic measurements for capecitabine as a potential noninvasive surrogate marker for determining TP levels in tumors and for use in predicting tumor response to capecitabine in patients.

MATERIALS AND METHODS

Materials. Capecitabine, 5’DFCR and 5’DFUR were provided by Roche (Tokyo, Japan). RPMI 1% medium, FCS, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Paisley, United Kingdom). Hypnorm was obtained from Jansen Pharmaceuticals (Buckinghamshire, United Kingdom) and Hypnovel and 5-FU from Roche (Welwyn Garden City, United Kingdom). DMSO was purchased from Sigma Chemical Company Ltd. (Poole, United Kingdom).

Cell Lines. RT112 is a human bladder tumor cell line. 2T10 cell lines were prepared from RT112 cells transfected with full cDNA for human TP and characterized by overexpression of the TP level. An empty-vector control cell line, EV11, was also prepared in the same way as the 2T10 but without the TP cDNA in the vector (41). RT112 cells were cultured in RPMI 1% medium supplemented with 10% FCS and glutamine at 37°C in a 5% CO₂ atmosphere. 2T10 and EV11 cells were cultured in the same way as the RT112 cells but also with 80 µg/ml penicillin and 80 µg/ml streptomycin added into the medium.

Animal Models. MF1 nude mice received injection s.c. in the flank with 0.1 ml of a suspension of human bladder (RT112, EV11, or 2T10) tumor cells (1 x 10⁶ cells/ml in RPMI medium) that had been grown as a monolayer in cell culture (32). Tumor volume was calculated by measuring length, width, and depth using calipers and the following formula: $l\times w\times d^2/6$. When tumors reached 568 ± 35 mg, mice were given a single dose of capecitabine (360 mg/kg in DMSO i.p) or 5’DFUR (200 mg/kg in DMSO i.p). The capecitabine injected group comprised ten 2T10, two EV11, and five RT112 tumors, and the 5’DFUR group included four 2T10, three EV11, and three RT112 tumors. Animals were treated in accordance with local and national ethical requirements and with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia (42).

In Vivo $^{19}$F MRS of a Phantom. A phantom was prepared with a solution of 6 mM 5-FU in saline. $^{19}$F MR spectra of the phantom were then obtained as described in the section below. Capecitabine, 5’DFCR and 5’DFUR in DMSO were each in turn added to the phantom, and $^{19}$F MRS was performed after each addition. A total 7% DMSO was present in the phantom. This phantom study was designed to determine the $^{19}$F chemical shifts of capecitabine and its intermediate metabolites (i.e., 5’DFCR, 5’DFUR and 5-FU) to assign the resonances in in vivo $^{19}$F MR spectra of a tumor after capecitabine or 5’DFUR treatment.

In Vivo $^{19}$F MRS of Tumors. Animals were anesthetized with a single i.p. injection of a Hypnovel-Hynnorm mixture as described previously (32). After the capecitabine or 5’DFUR injection, animals were placed in the bore of a Varian
4.7T spectrometer at 37°C, and tumors were positioned in the center of a 12-mm double-tuned (19F)/31P two-turned surface coil. Nonlocalized 19F spectra were acquired immediately in 10 min blocks (1500 transients with TR of 0.2 s, spectral width of 30 kHz, and 45° pulse at coil center) for up to 180 min (31).

The 19F MRS data were quantified using VARPRO (31). Two 10-min blocks were summated (20 min) and transformed with 40 Hz broadening. Spectra were then curve-fitted using VARPRO, and the area under the curve value for each metabolite was obtained at each time point. Log area under the curve of each metabolite was plotted against time for each tumor, and linear regressions were used to fit the plots. The rate constant (−k) of capecitabine breakdown, the buildup of the intermediate molecules (5’DFCR/5’DFUR), and the subsequent breakdown of the molecules were then obtained from the slope of the plot. The half-life (t1/2) of the capecitabine and the intermediate

Fig. 2 Immunostaining of thymidine phosphorylase overexpression in (A and B) 2T10 tumors. Brown staining demonstrates the areas where tumor cells are overexpressing TP: C: RT112 tumor (wild-type control); D: EV11 tumor (empty-vector control), no brown staining.

Fig. 3 19F MR spectra of a phantom for capecitabine and its metabolites in 7% DMSO and 93% saline. 5’DFUR, 5’-deoxy-5-fluorouridine; 5’DFCR, 5’-deoxy-5-fluoro-cytidine; 5’FU, 5-fluorouracil; PPM, parts per million.
molecules are estimated using the following equation: \( t_{1/2} = 0.693/k \). The \( t_{1/2} \) of 5′DFUR was assessed in the same way.

**Immunohistochemistry.** Slides were pretreated with peroxidase-blocking reagent for 5 min (part of DAKO Envision-Plus kit, DakoCytomation, K4006). The primary mouse monoclonal antihuman thymidine phosphorylase antibody PGF-44c (NCL-PDECGF; Novocastra Laboratories), at a dilution of 15 \( \mu \)g/ml, was incubated on sections for 30 min at room temperature in a humid chamber. After washing in PBS, staining was visualized using a DAKO Envision-Plus Mouse Horseradish Peroxidase System (DakoCytomation, K4006) and diamino-benzidine yielding a dark brown reaction product. Sections were counterstained with hematoxylin and coverslipped using Glycergel aqueous-mounting medium (DakoCytomation, C0563).

**Statistical Analysis.** All data are presented as the mean ± SE of mean (SE), using a 2-tailed \( t \) test with \( P < 0.05 \) considered significant.

**RESULTS**

**Immunostaining of the Cell Line.** An immunoblot displaying the relative TP expression in the cell lines used to
generate the tumors has been published previously using the same cells (41) showing a higher level of TP expression in the 2T10 cells when compared with the control lines (RT112 and EV11). When sections of 2T10 tumors were stained for TP expression (Fig. 2, A and B) specific brown staining demonstrated areas where the tumor cells were overexpressing TP. This was in distinct contrast to the control tumors (RT112 and EV11) where negligible brown staining was observed (Fig. 2, C and D). TP overexpression was observed in only 35% of the cells in 2T10 tumors relative to the controls, which had levels of ≤1%. This probably represents uneven distribution of TP within the tumor where secondary mechanisms regulating TP expression in vivo may be operating (e.g., effects of hypoxia or acidic micro-environment). However, the results confirmed previous findings that in 2T10 tumors (41), a significantly higher percentage of cells overexpress TP when compared with the wild-type (RT112) or empty-vector controls (EV11).

In Vivo 19F MRS of Phantom. The 19F MR spectrum of the phantom solution is shown in Fig. 3 with the resonances from capecitabine, 5-FU, 5’DFCR, and 5’DFUR assigned. 5-FU was referenced as 0 parts/million. The spectra show that the capecitabine intermediates (5’DFCR and 5’DFUR) resonate close to each other between the capecitabine and 5-FU peaks. Hence it may not be possible to resolve the 5’DFCR and

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**Fig. 5** 19F MR spectra of a RT112 tumor at various time points after capecitabine injection. 5’DFCR, 5’-deoxy-5-fluorocytidine; 5’DFUR, 5’-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine; PPM, parts per million.
PK Endpoints of Capecitabine in Tumor Xenografts

5'-DFUR peaks in the in vivo situation where all of the metabolite peaks will be broadened because of tissue susceptibility.

**In Vivo 19F MRS of Tumors.** In vivo 19F MR spectra of a 2T10 and a RT112 tumor at various time points after capecitabine injection are shown in Fig. 4 and 5, respectively, where signals from capecitabine, capecitabine intermediates (5'-DFCR and predominately 5'-DFUR), 5-FU, and FBAL can be readily observed.

A maximum peak value (C\text{max}) for capecitabine was found 12–32 min after injection in both 2T10 and control tumors (RT112/EV11). The time course of the capecitabine breakdown in 2T10 and control tumors is plotted in Fig. 6, where the level of capecitabine (relative to C\text{max}) was plotted against time. The rate constant (k) of capecitabine breakdown in not significantly different between the two tumor groups (Table 1). The time course of the accumulation and breakdown of the intermediate molecules (5'-DFCR/5'-DFUR) in both tumor groups is illustrated in Fig. 7, where the level of 5'-DFCR/5'-DFUR (relative to C\text{max}) is plotted against time. The C\text{max} for the intermediates was found 92–112 min after injection in both groups. The k value of the breakdown of the intermediates was significantly faster in the 2T10 tumors than in the control group (P < 0.003).

No significant difference in the rate of accumulation of the intermediates was observed (Table 1). The t\text{1/2} of 5'-DFCR/5'-DFUR in the 2T10 and control groups is estimated as 58 and 116 min, respectively. The maximum level of FBAL was observed from the early stage of the time course (12–32 min onwards in most animals) in both tumor groups. This indicated that a high level of DPD was present in this bladder tumor model.

Another group of 2T10 (n = 4) and control tumors (three EV11 and three RT112) was also treated with 200 mg/kg of 5'-DFUR, and the rate of 5'-DFUR breakdown was measured. The time course is shown in Fig. 8, where the level of 5'-DFUR (relative to C\text{max}) is plotted against time. The C\text{max} for 5'-DFUR was found 10–20 min after injection in both animal groups. The k value of the breakdown of 5'-DFUR in the 2T10 tumors was found to be doubled when compared with the controls (P < 0.001), as summarized in Table 2. The t\text{1/2} of 5'-DFUR in 2T10 and control groups are estimated as 33 and 69 min, respectively. These data confirmed that the rate of 5'-DFCR converting to 5'-DFUR is slower than the conversion of 5'-DFUR to 5-FU and that the rate of 5'-DFUR breakdown is TP dependent.

**DISCUSSION**

Characterization of 2T10, EV11, and RT112 tumor lines have been carried out and reported by us previously (41), and TP-overexpressing cells were found to be more sensitive to 5'-DFUR than wild-type and empty vector cells. Our present study confirmed the previous observation that the growth rate of 2T10 tumor xenografts in nude mice was significantly faster.

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**Table 1** Rate constants (k) of capecitabine and intermediary metabolites

<table>
<thead>
<tr>
<th></th>
<th>5'-DFUR/5'-DFUR\text{a}</th>
<th>5'-DFUR/5'-DFUR\text{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Capcitabine (breakdown)</td>
<td>Capcitabine (breakdown)</td>
</tr>
<tr>
<td>2T10</td>
<td>0.028 ± 0.003</td>
<td>0.028 ± 0.003</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>±0.003</td>
<td>±0.003</td>
</tr>
<tr>
<td>Control</td>
<td>0.027 ± 0.003</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>(2 EV11, 5 RT112)</td>
<td>±0.003 ± 0.004</td>
<td>±0.004 ± 0.001</td>
</tr>
<tr>
<td>\text{t1/2}</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>&lt;0.003\text{b}</td>
<td>&lt;0.003\text{b}</td>
</tr>
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</table>

\text{a} 5'-DFCR, 5'-deoxy-5-fluorocytidine; 5'-DFUR, 5'-deoxy-5-fluorouridine.

\text{b} Statistically significant.
of TP expression in tumors. This study is a starting point to show that noninvasive in vivo 19 F MRS can be used for measuring the pharmacokinetics of capecitabine and its intermediate metabolites in tumors and that such a measurement may have potential as a surrogate marker for determining TP levels in tumors. However, it has been shown that a measure of the DPD level may be required to optimize the prediction of response to treatment (2, 5, 22, 23). Genetically modified animal tumor models give insight into the rates of capecitabine metabolism in the competing anabolic and catabolic pathways. This noninvasive technique could be used to help predicting the response to capecitabine in individual patients.

ACKNOWLEDGMENTS
We thank Roche (Tokyo, Japan) for the provision of capecitabine, 5’DFCR, and 5’DFUR.

REFERENCES

Table 2 Rate constants (k) of 5’DFUR* metabolism

<table>
<thead>
<tr>
<th>5’DFUR* (breakdown)</th>
<th>2T10 (n = 4)</th>
<th>Control</th>
<th>(3 EV11 and 3 RT112) (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥0.001</td>
<td>0.001</td>
<td>≥0.003</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td></td>
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* 5’DFUR, 5’-deoxy-5-fluorouridine.
Statistically significant.

than the control tumors (data not shown) and that high levels of TP expression were present in the 2T10 tumors when compared with controls (41).

The conversion rate of 5’DFUR into 5-FU, which is TP dependent, was faster in the TP-overexpressing tumors (2T10) when compared with controls, as demonstrated by the capecitabine and 5’DFUR studies. This confirms the expected pathway of capecitabine metabolism and also shows that the level of TP expression is related to the rate of 5’DFUR conversion. The level of 5-FU remained low, probably attributable to either further metabolism of 5-FU in the tumor or fast removal by the blood and further metabolism in the liver.

In this study, we have concentrated on examining the role of 19 F MRS as a noninvasive in vivo tool to measure the levels of TP expression in tumors. This “proof of concept” investigation shows that it is possible to obtain a noninvasive in vivo surrogate marker for TP expression, which could then be used as a predictive marker for capecitabine efficacy in patients (2, 5, 22). Ideally, this noninvasive methodology could be further evaluated by examining xenografts generated from TP transfectants expressing variable amounts of protein. However, such TP transfectants are not currently available, and the 2T10 model used in this study has TP levels similar to the median level found in human bladder tumors.

Several previous studies evaluating capecitabine using invasiveness tumor biopsies have demonstrated that intratumoral levels of TP and DPD (expressed as a TP/DPD ratio) are the best indicators of tumor response, with increased efficacy characterized by high TP expression (which promotes increased conversion of capecitabine into 5-FU) and low DPD expression (which decreases conversion of 5-FU into inactive products; Refs. 2, 5, 22). A recent clinical study suggested that high-TP expression is a good indicator of disease-free survival for those patients taking 5-FU prodrug-bacetherapy, with patients expressing high TP and low DPD demonstrating the best disease-free survival (23). To extend the current investigation, future studies should include examining the role of DPD in capecitabine metabolism, which would further complement the use of TP expression in predicting tumor response to capecitabine in patients. Previous pharmacokinetic studies have shown that the amount of 5-FU available for anabolism is also influenced by the extent of its catabolism (6, 7). These studies also showed that apart from tumor TP level, the DPD level plays a part in the efficacy of capecitabine in tumors. Because the DPD level has also been found to correlate with catabolism, for capecitabine to work efficiently, the TP level should be high, and the DPD level should be low to maximize the production of active cytotoxic metabolites from 5-FU and minimize the conversion of 5-FU into inactive metabolites (Fig. 1; Refs. 2, 5, 22).

The role of DPD in capecitabine metabolism could be confirmed in a pair of DPD overexpressed or knockout xenograft models (when available) using 19 F MRS. It should be possible to extend the current 19 F MRS methodology used in this study to develop a noninvasive in vivo surrogate marker of DPD expression. This marker for DPD level could be obtained by measuring the rate of the catabolite (FBAL) buildup using in vivo 19 F MRS (29–35). Furthermore, these measurements could be obtained at the same time as the TP measurement. Hence, it might be possible to obtain a ratio of 5’DFUR breakdown rate to FBAL buildup rate as a surrogate ratio for TP and DPD. For an optimal response to capecitabine, the rate of 5’DFUR breakdown should be high for an indication of a high-TP expression, and the rate of the FBAL buildup should be low for an indication of a low-DPD expression.

This study is a starting point to show that noninvasive in vivo 19 F MRS can be used for measuring the pharmacokinetics of capecitabine and its intermediate metabolites in tumors and that such a measurement may have potential as a surrogate marker for determining TP levels in tumors. However, it has been shown that a measure of the DPD level may be required to optimize the prediction of response to treatment (2, 5, 22, 23). Genetically modified animal tumor models give insight into the rates of capecitabine metabolism in the competing anabolic and catabolic pathways. This noninvasive technique could be used to help predicting the response to capecitabine in individual patients.


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