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PHARMACOKINETICS

Exploring the intracellular pharmacokinetics of the 5-fluorouracil nucleotides during capecitabine treatment

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Keywords 5-fluorouracil, 5-fluorouridine triphosphate (FUTP), capecitabine, nucleotides, peripheral blood mononuclear cells (PBMCs), pharmacokinetics

AIM

Three intracellularly formed metabolites are responsible for the antineoplastic effect of capecitabine: 5-fluorouridine 5′-triphosphate (FUTP), 5-fluoro-2′-deoxyuridine 5′-triphosphate (FdUTP), and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). The objective of this study was to explore the pharmacokinetics of these intracellular metabolites during capecitabine treatment.

METHODS

Serial plasma and peripheral blood mononuclear cell (PBMC) samples were collected from 13 patients treated with capecitabine 1000 mg QD (group A) and eight patients receiving capecitabine 850 mg m^{-2} BID for fourteen days, every three weeks (group B). Samples were collected on day 1 and, for four patients of group B, also on day 14. The capecitabine and 5-fluorouracil (5-FU) plasma concentrations and intracellular metabolite concentrations were determined using LC–MS/MS. Pharmacokinetic parameters were estimated using non-compartmental analysis.

RESULTS

Only FUTP could be measured in the PBMC samples. The FdUTP and FdUMP concentrations were below the detection limits (LOD). No significant correlation was found between the plasma 5-FU and intracellular FUTP exposure. The FUTP concentration–time profiles demonstrated considerable inter-individual variation and accumulation of the metabolite in PBMCs. FUTP levels ranged between <LOD and 1.0 μM on day 1, and from 0.64 to 14 μM on day 14. The area under the FUTP concentration–time curve was significantly increased on day 14 of the treatment compared to day 1 (mean ± SD: 28 ± 19 μM h vs. 2.0 ± 1.9 μM h).

CONCLUSIONS

To our knowledge, this is the first time that intracellular FUTP concentrations were measured in patients treated with capecitabine. During 14 days of treatment with capecitabine twice daily, intracellular accumulation of FUTP occurs.

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Three intracellularly formed metabolites are held responsible for the antineoplastic effect of capecitabine
- The concentrations of these intracellularly formed 5-fluorouracil (5-FU) nucleotides have never been measured in patients receiving capecitabine, because there was no sufficiently sensitive assay.
- Insight into the pharmacokinetics (PK) of the intracellular 5-FU nucleotides would be useful to optimize capecitabine dosage regimens.

WHAT THIS STUDY ADDS

- This is the first time that the intracellular PK of the three pharmacologically active 5-FU nucleotides have been explored during capecitabine treatment.
- Important findings are the long intracellular residence of 5-fluorouridine 5′-triphosphate (FUTP), and its consequent intracellular accumulation after dosing capecitabine twice daily for 14 days.

Introduction

Capecitabine is a widely used chemotherapeutic agent, which has an important place in the treatment of several malignancies, including colorectal, gastric, pancreatic, breast and head and neck cancer. It was developed as a tumour-selective prodrug of 5-fluorouracil (5-FU). After oral administration, capecitabine is extensively absorbed from the gastrointestinal tract, and then converted into 5-FU by an enzymatic cascade involving three steps (Figure 1). First, capecitabine is converted to 5′-deoxy-5-fluorocytidine (5′-dFCR) by carboxylesterase, an enzyme located primarily in the liver. 5′-dFCR is then converted to 5′-deoxy-5-fluorouridine (5′-dFUR) by cytidine deaminase, which is principally located in the liver and in tumour tissue. The third step, the conversion of 5′-dFUR to 5-FU is catalysed by thymidine phosphorylase. This enzyme is present at higher concentrations in solid tumour tissue than in normal tissues [1]. Therefore, the third activation step preferentially takes place in tumour tissue rather than normal tissue [2].

5-FU is further activated intracellularly by ribosylation and sequential phosphorylation. Ultimately, three intracellularly formed metabolites (nucleotides) are held responsible for the antineoplastic effect of capecitabine. These are 5-fluorouridine 5′-triphosphate (FUTP), 5-fluoro-2′-deoxyuridine 5′-triphosphate (FdUTP), and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). In brief, **FUTP** is incorporated into RNA and interferes with normal RNA processing and function. **FdUTP** is incorporated into DNA, leading to DNA damage and ultimately cell death. **FdUMP** inhibits thymidylate synthase, the enzyme that catalyses the transformation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Inhibition of thymidylate synthase by FdUMP leads to accumulation of deoxyuridine triphosphate (dUTP) and depletion of deoxythymidine triphosphate (dTTP). This imbalance has deleterious consequences for DNA synthesis and repair, ultimately leading to cell death (Figure 1) [3, 4].

As the anticancer activity of capecitabine depends on these three 5-FU nucleotides, it would be interesting and clinically relevant to monitor their intracellular concentrations during capecitabine treatment. This would provide insight into the amounts of activated drug reaching the site of action. This information could ultimately be very useful to optimize current treatment regimens.

Until now, information about the intracellular metabolism of the 5-FU nucleotides originated mainly from *in vitro* experiments and animal studies [5–8]. Little is known about the formation of 5-FU nucleotides in patients who are treated with 5-FU due to the long lack of a suitable bioanalytical assay.

Several studies were conducted that examined the FdUMP concentrations in tumour tissues of patients who were treated with an intravenous 5-FU bolus injection (500 mg m^{-2}) [9–11]. These studies used a competitive-binding assay with thymidylate synthase, isolated from Lactobacillus casei, as a binding protein. The quantification of FdUMP was based on the displacement of a known amount of radiolabelled [^{3}H]-FdUMP, measured by scintillation counting [12].

However, for the quantification of FUTP and FdUTP in cells of patients who were treated with 5-FU, a sufficiently sensitive assay was missing. Therefore, we recently developed an ultrasensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the quantification of the active 5-FU nucleotides in peripheral blood mononuclear cells (PBMCs) [13]. PBMCs were selected as a cell model for the intracellular activation, because they are easy to collect at various time points after drug administration. With the advent of this assay, it has become possible to get insight into the intracellular 5-FU nucleotide concentrations in samples of patients who are treated with 5-FU or capecitabine.

The aim of the current study was to explore the intracellular pharmacokinetics (PK) of the three pharmacologically active 5-FU nucleotides during capecitabine treatment. Except for a small pilot in our previous publication on the development of a bioanalytical assay [13], this is, to our knowledge, the first time that intracellular 5-FU nucleotides were quantified during capecitabine treatment.

Methods

Study design and treatment schedule

The intracellular PK of the active 5-FU nucleotides was assessed in two groups of patients. For group A, the intracellular PK of the 5-FU nucleotides was studied only on day 1 of the treatment. Based on the results in this group, we wondered if intracellular accumulation would occur during a treatment cycle, in which capecitabine is administered twice daily for 14 consecutive days. Therefore group B was added to the study. For this group the intracellular PK was examined on day 1 and also on day 14 of the treatment with capecitabine.

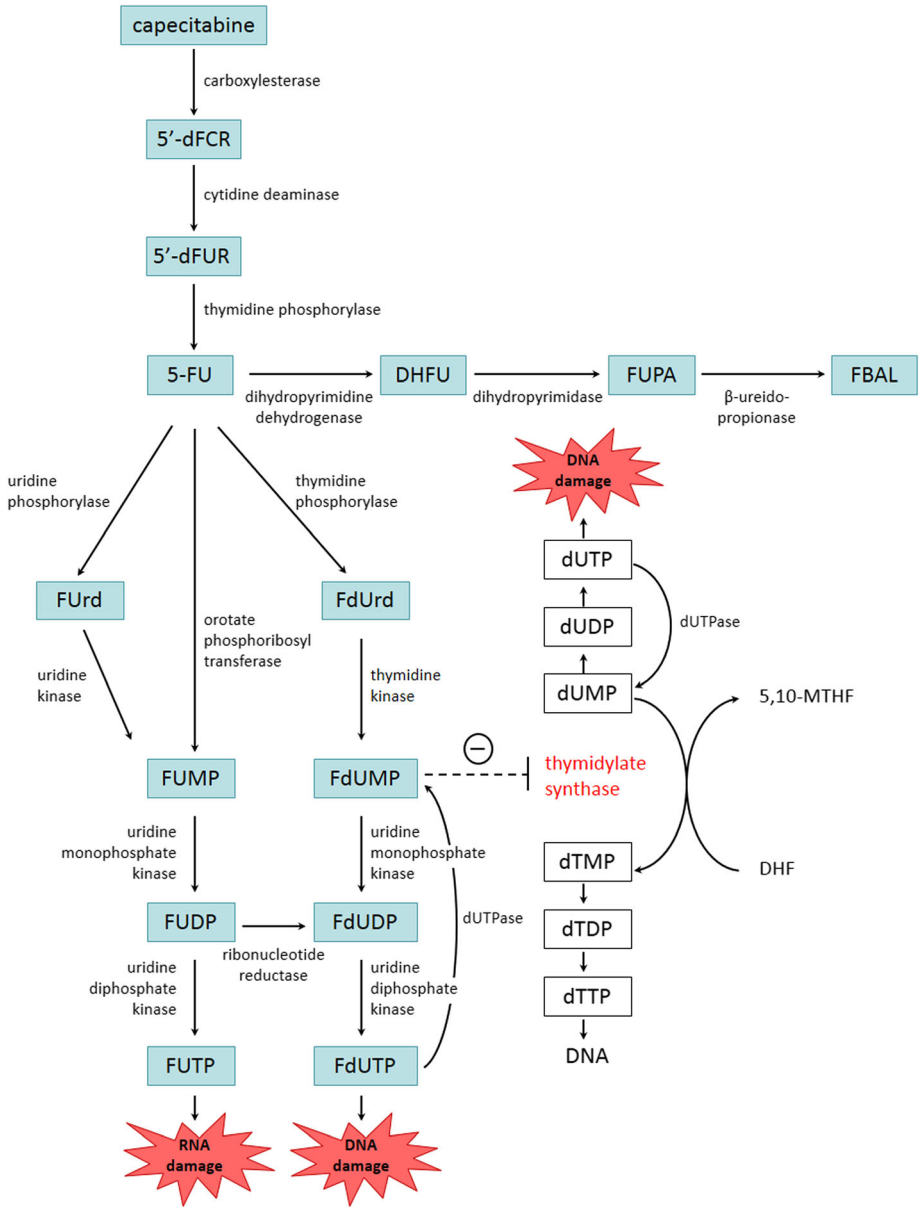

Figure 1

Capecitabine metabolism and mechanisms of action. Abbreviations: 5′-dFCR, 5′-deoxy-5-fluorocytidine; 5′-dFUR, 5′-deoxy-5-fluorouridine; DHFU, dihydrofluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FBAL, α-fluoro-β-alanine; 5-FU, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluoro-2′-*deoxy*uridine; FUMP, 5-fluorouridine 5′-monophosphate; FUDP, 5-fluorouridine 5′-diphosphate; FUTP, 5-fluorouridine 5′-triphosphate; FdUMP, 5-fluoro-2′-*deoxy*uridine 5′-monophosphate; FdUDP, 5-fluoro-2′-*deoxy*uridine 5′-diphosphate; FdUTP, 5-fluoro-2′-*deoxy*uridine 5′-triphosphate; dUMP, 2′-*deoxy*urdine 5′-monophosphate; dUDP, 2′-*deoxy*urdine 5′-diphosphate; dUTP, 2′-*deoxy*urdine 5′-triphosphate; dTMP, 2′-*deoxy*thymidine 5′-monophosphate; dTDP, 2′-*deoxy*thymidine 5′-diphosphate; dTTP, 2′-*deoxy*thymidine 5′-triphosphate; DHF, dihydrofolate; 5,10-MTHF, 5,10-methylene-tetrahydrofolate

Group A included 13 patients treated with capecitabine for one of its approved therapeutic indications (e.g. colon, breast, pancreatic and gastric cancer). All patients received a one-time capecitabine dose of 1000 mg QD to study the PK, and were then treated with a standard dose. Patients were instructed not to eat or drink (except a small amount of water <50 ml) from 11 h before the drug intake until 1 h after drug intake. PBMC samples were collected on day 1 of the first treatment cycle, just before oral administration of capecitabine (pre-dose) and 1, 2, 4, 6 and 24 h after capecitabine administration. To monitor the capecitabine and 5-FU plasma concentrations, plasma samples were collected pre-dose and 0.5, 1, 1.5, 2, 3, 4, 6, 12 and 24 h after capecitabine intake.

Group B consisted of eight patients who participated in a phase I/II study in advanced gastro-oesophageal cancer. The primary objective of this study was to explore the safety and preliminary activity of the combination of docetaxel, oxaliplatin and capecitabine. Patients received capecitabine 850 mg m^{-2}

twice daily for 14 days, every three weeks. Capecitabine tablets were taken with water within 30 min after a meal. PBMC samples were collected at day 1 of the first treatment cycle (for five patients) and at day 14 of this cycle (for four patients). PBMC samples were collected pre-dose and 2, 4, 6 and 8 h after oral capecitabine administration. For one patient an additional sample was taken 10 h after capecitabine administration. Plasma samples, to monitor the capecitabine and 5-FU plasma concentrations, were collected pre-dose and 0.5, 1, 2, 3, 4, 6 and 8 h after capecitabine intake. Sample collection at day 14 of the treatment was included in the protocol to examine whether intracellular accumulation of 5-FU nucleotides occurs during a treatment cycle.

The study was approved by the Medical Ethics Committee of our Institute and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before enrolment.

Quantification of capecitabine and 5-FU in plasma

Capecitabine and 5-FU were quantified in plasma by the two validated LC–MS/MS assays described by Deenen *et al.* [14]. In brief, stable labelled isotopes of the analytes were used as internal standards and added to 100 μl of plasma, followed by a protein precipitation step. The capecitabine assay was based on reversed-phase chromatography using an XBridge C18 column (50 mm × 2.1 mm ID, particle size 5 μm; Waters Corporation, Milford, MA, USA) and a mobile phase gradient, in which mobile phase A consisted of 0.05% formic acid in water and mobile phase B was 0.05% formic acid in methanol. For the quantification of 5-FU, the chromatography was performed on a Luna HILIC column (150 mm × 2.1 mm ID, particle size 3 μm; Phenomenex, Torrance, CA, USA) using isocratic elution with 10 mM formic acid in water (pH 4.0) mixed with acetonitrile (20:80, v/v). Detection of the analytes was performed on an API4000 triple quadrupole mass spectrometer equipped with an electrospray ionization probe (AB Sciex, Framingham, MA, USA). Capecitabine was detected in the positive ion mode and 5-FU in the negative ion mode. The lower limits of quantification (LLQs) of the assays were 139 nM for capecitabine and 384 nM for 5-FU [14].

Quantification of the 5-FU nucleotides in PBMCs

PBMCs were isolated and FUTP, FdUTP and FdUMP concentrations were determined as previously described using our validated LC–MS/MS assay [13]. In brief, 16 ml of blood was collected and PBMCs were isolated immediately using cold Ficoll-Paque PLUS density gradient (GE Healthcare, Pittsburgh, PA, USA). The collected PBMCs were resuspended in 70 μl PBS resulting in a homogeneous cell suspension with a total volume of approximately 100 μl. A 30 μl aliquot of this cell suspension was used to perform a cell count using a haematology analyser (Cell-Dyn Sapphire; Abbott Diagnostics). A 60 μl aliquot of the cell suspension was used for determination of the 5-FU nucleotide concentrations. To this end, cells were lysed by the addition of 100 μl methanol and extensive vortex mixing. After centrifugation, the supernatant (PBMC lysate) was collected and stored at −70 °C until analysis. Directly prior to LC–MS/MS analysis, the PBMC lysates were spiked with internal standard. Chromatographic separations were performed by weak anion exchange chromatography using a Biobasic AX column (50 mm × 2.1 mm ID, particle size 5 μm, Thermo Scientific). A stepwise gradient was used, in which the eluent pH was increased and the NH_4Ac concentration decreased. Detection was performed using a QTrap 5500 mass spectrometer equipped with an electrospray ionization probe operating in the negative ion mode (AB Sciex). The LLQs *in PBMC lysate* were 0.488 nM for FUTP, 1.66 nM for FdUTP and 0.748 nM for FdUMP. Accuracies were between −2.2 and 7.0% deviation for all analytes, and the coefficient of variation values were ≤4.9% [13].

The analytical results, expressed as nM in PBMC lysate, were multiplied with the lysate sample volume to obtain the absolute 5-FU nucleotide amounts in a sample. These amounts were then divided by the number of cells present in the sample to obtain the 5-FU nucleotide amounts *per* 10^6 *PBMCs*. Intracellular concentrations are generally expressed as the amount of drug per 10^6 cells. However, in order to compare the intracellular concentrations with the plasma concentrations, the intracellular concentrations were also converted to the amount of drug *per volume unit (μM)*. To this end, we measured the number of lymphocytes, monocytes and (residual) granulocytes in each individual sample and used the mean cell volumes of lymphocytes (174 fL), monocytes (339 fL) and granulocytes (302 fL) as determined by Sharma *et al.* [15] and later confirmed by Simiele *et al.* [16].

Pharmacokinetic and statistical analysis

The individual non-compartmental PK parameters in plasma and in PBMCs were determined using validated scripts in the software package R (version 3.1.2). The mean and coefficient of variation (CV) of the following PK parameters were reported: the maximum observed concentration (C_{max}), the time to reach C_{max} (t_{max}) and the area under the concentration–time curve between $t = 0$ and the time point of the last quantifiable data point ($AUC_{0\text{-}t}$).

For the plasma samples, also the terminal elimination half-life ($t_{1/2}$) was calculated, using the terminal elimination rate constant (k_e) based on the last three data points. For the calculation of the mean $t_{1/2}$, only curves that contained sufficient data points to assess this parameter were included. This meant that curves lacking a clear elimination phase were excluded from the calculation of this specific PK parameter. For the PBMC samples, the $t_{1/2}$ could not be calculated due to the limited number of data points in the descending part of the FUTP curves. The intracellular FUTP concentrations after 24 h were reported as a percentage of the C_{max}.

The relationship between the plasma 5-FU exposure and intracellular FUTP exposure was explored. Pearson's correlation was used to assess whether there was a linear relationship between the $AUC_{0\text{-}t}$ of 5-FU in plasma and the $AUC_{0\text{-}t}$ of FUTP measured in PBMCs. The correlation was considered statistically significant if the P value was less than 0.05.

To assess whether intracellular accumulation occurred during 14 days of capecitabine treatment twice daily, the $AUC_{0\text{-}8h}$ values for FUTP determined on day 1 and on day 14 of the treatment were compared. A one-sided Mann–Whitney U test was performed to assess (for group B) whether there was a significant increase of the $AUC_{0\text{-}8h}$ values for intracellular FUTP at day 14

compared to day 1. The result was considered statistically significant if the *P* value was less than 0.025. Statistical analyses were performed using SPSS Statistics version 23 (IBM Corp.).

Results

Plasma pharmacokinetics

The mean plasma concentration–time curves for capecitabine and 5-FU are shown in Figure 2. The different shapes of the curves for group A and group B are probably related to whether or not the patients took food around the time of drug intake [17]. The results of the non-compartmental PK analysis are shown in Table 1. On average, the C_{max} for capecitabine was reached after 0.7 h in patient group A and after 3.3 h in group B. This difference is probably also related to the effect of food. The mean terminal elimination half-lives of capecitabine and 5-FU were 0.4 and 0.6 h for group A and 0.7 and 0.8 h for group B, respectively. These PK data are in line with previously published data in the literature [18]. The plasma concentration–time curves determined on day 14 demonstrate (despite the limited number of sampling times and missing the C_{max}) that there was no accumulation of 5-FU or capecitabine in plasma (Figure 2B).

Intracellular pharmacokinetics

In the PBMC samples, only the intracellular FUTP concentrations could be quantified. The FdUTP and FdUMP concentrations in

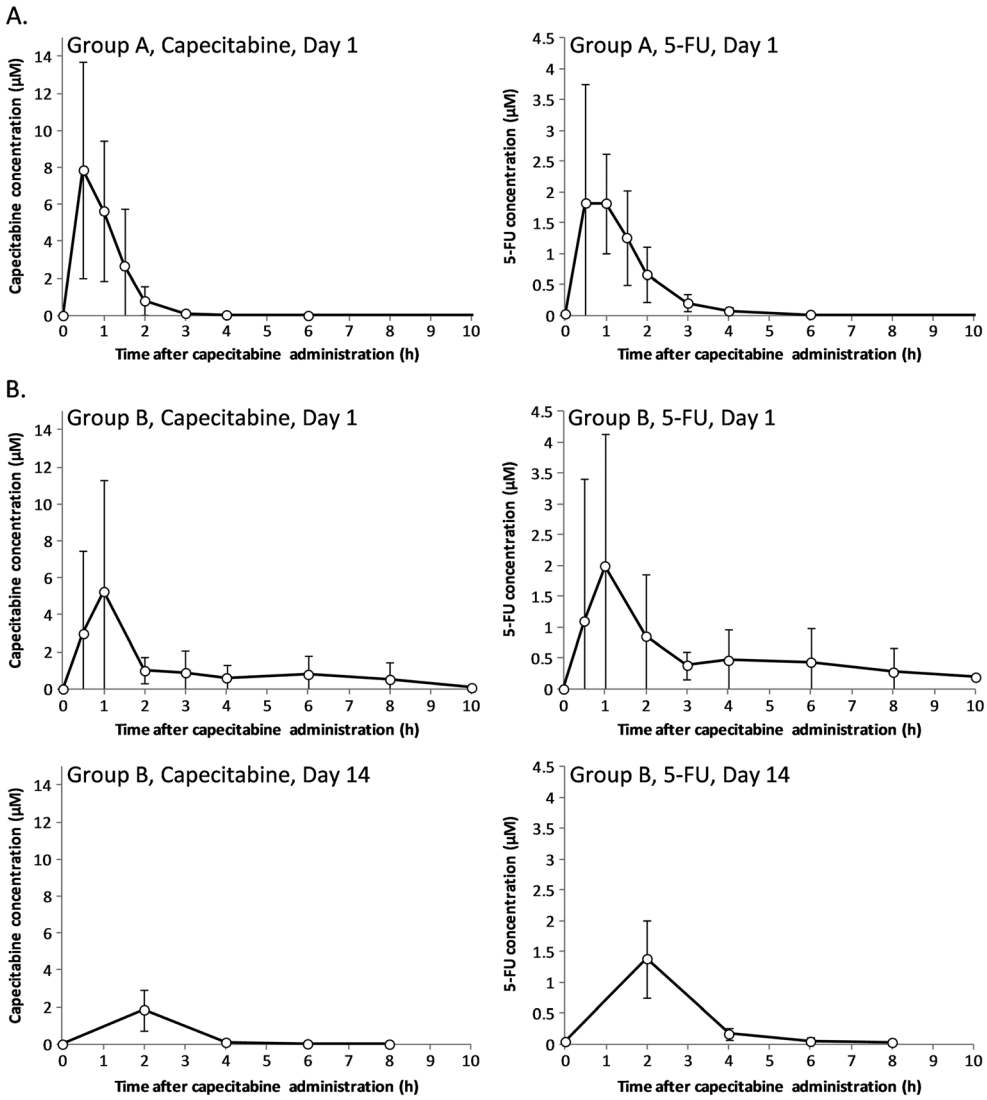

Figure 2

Plasma concentration–time curves of capecitabine and 5-FU for patient group A (receiving capecitabine 1000 mg QD) measured at day 1 of the first treatment cycle (A), and for patient group B (receiving capecitabine 850 mg m^{-2} BID) measured at day 1 and at day 14 of the first treatment cycle (B). Despite the limited number of sampling times on day 14 and the missing C_{max}, the plasma concentration–time curves determined on day 14 demonstrate that there was no accumulation of capecitabine or 5-FU in plasma. Curves were depicted up to 10 h after capecitabine intake, although more samples were collected for group A. The data are shown as mean values (symbols) with standard deviations (error bars)

Table 1

Summary statistics for pharmacokinetic parameters of capecitabine and 5-FU in plasma and FUTP in PBMCs, on day 1, after a single capecitabine dose. The data are shown as mean values (and coefficients of variation %)

	Patient group A (receiving capecitabine 1000 mg QD)			Patient group B (receiving capecitabine 850 mg m^{-2} BID)		
	Capecitabine	**5-FU**	**FUTP**	**Capecitabine**	**5-FU**	**FUTP**
n	13	13	13	8	8	5
C_{max}	11 μM (43%)	2.6 μM (58%)	0.38 μM (62%) 0.086 pmol/10^6 PBMCs (57%)	7.3 μM (71%)	3.0 μM (69%)	0.54 μM (69%) 0.13 pmol/10^6 PBMCs (69%)
t_{max}	0.7 h (45%)	0.9 h (34%)	3.5 h (61%)	3.3 h (89%)	3.4 h (86%)	7.2 h (25%)
$t_{1/2}$ [1]	0.4 h (19%)	0.6 h (24%)	—	0.7 h (70%) *n* = 7	0.8 h (48%) *n* = 7	—
$AUC_{0\text{-}t}$	8.8 μM h (31%)	3.0 μM h (45%)	5.3 μM h (68%) 1.2 pmol h/10^6 PBMCs (64%)	11 μM h (46%)	5.3 μM h (30%)	2.1 μM h (84%) 0.51 pmol h/10^6 PBMCs (83%)

5-FU, 5-fluorouracil; $AUC_{0\text{-}t}$, the area under the concentration–time curve from time zero to the time point of the last quantifiable data point; BID, twice a day; C_{max}, the maximum observed concentration; FUTP, 5-fluorouridine 5′-triphosphate; *QD*, one a day; $t_{1/2}$, terminal elimination half-life; t_{max}, the time to reach the maximum observed concentration. [1]For the calculation of the mean $t_{1/2}$, only curves of patients containing sufficient data points to assess this parameter were included. Where not all patients were included, the number of evaluable patients (*n*) is indicated.

the collected PBMC lysates were all below the detection limits of the assay. The LLQs of the LC–MS/MS assay (in PBMC lysate), mentioned in the methods section, are not directly translatable to the lowest measurable intracellular FdUTP and FdUMP concentrations in PBMCs, because the latter also depends on the number of collected lymphocytes, monocytes and granulocytes in the sample. Clinical samples derived from 16 ml whole blood using the described isolation method were found to contain 0.5–20 × 10^6 PBMCs.

The intracellular concentration–time curves for FUTP are shown in Figure 3 and demonstrate considerable inter-individual variation. The results of the non-compartmental PK analysis are given in Table 1. Intracellular FUTP circulates much longer than the corresponding plasma levels of capecitabine and 5-FU. On day 1 of the treatment, the C_{max} for FUTP was reached on average after 3.5 h in patient group A. As for the plasma concentrations, also for the intracellular levels, C_{max} was achieved much later in group B, after 7.2 h. Remarkable is the long presence of FUTP in the cell. Twenty-four hours after the intake of capecitabine, the intracellular FUTP concentration was on average still 41% of the C_{max} (range: 21–89%).

Relationship between plasma 5-FU and intracellular FUTP exposure

No significant linear correlation was found between the $AUC_{0\text{-}t}$ of 5-FU in plasma and the $AUC_{0\text{-}24h}$ of intracellular FUTP determined on the first treatment day (Figure 4). The Pearson correlation coefficient (*r*) was 0.107 (P = 0.727, n = 13) for group A. The sample size of group B was too small to make a reliable statement about possible linear correlation.

Intracellular FUTP accumulation

After 14 days of capecitabine treatment (twice daily), intracellular FUTP concentrations are clearly higher than after the first capecitabine administration on day 1 (Figure 3B). The $AUC_{0\text{-}8h}$ for intracellular FUTP was significantly increased on day 14 of the treatment compared to day 1 (Mann–Whitney U = 20, P = 0.008, one-sided, Figure 5). The mean $AUC_{0\text{-}8h}$ (± SD) on day 1 was 2.0 ± 1.9 μM h *vs.* 28 ± 19 μM h on day 14. Apparently, during these 14 days of consecutive treatment, intracellular accumulation of FUTP occurs.

Discussion

To our knowledge, this is the first time that intracellular FUTP concentrations were measured in patients who were treated with capecitabine. Our measurements show that FUTP is present in higher concentrations than FdUTP and FdUMP (which were both below the detection limits of the assay). Apparently, capecitabine is mainly converted into FUTP and to a lesser extent into FdUTP and FdUMP. However, this does not necessarily mean that the RNA pathway has a more prominent role in the mechanism of action than the DNA pathway. It is known that the *deoxy*ribonucleotides are naturally present within cells in much lower concentrations than the ribonucleotides [19, 20]. This means that the low FdUTP and FdUMP concentrations may still have a very important role.

A second important finding of this exploratory intracellular PK study is the long intracellular residence of FUTP, and its consequent intracellular accumulation after dosing capecitabine twice daily for 14 days. The prolonged intracellular retention of FUTP is an important finding, especially in view of the rapid plasma kinetics of capecitabine and 5-FU.

It was already known that 5-FU plasma levels do not reflect the 5-FU concentrations in tumour tissue. Peters *et al.* found that 5-FU was retained for a much longer period of time in tissues than in plasma, and that 4–48 h after an IV 5-FU bolus of 500 mg m^{-2}, the 5-FU tissue concentrations were at least 10 times higher than the plasma concentrations [11].

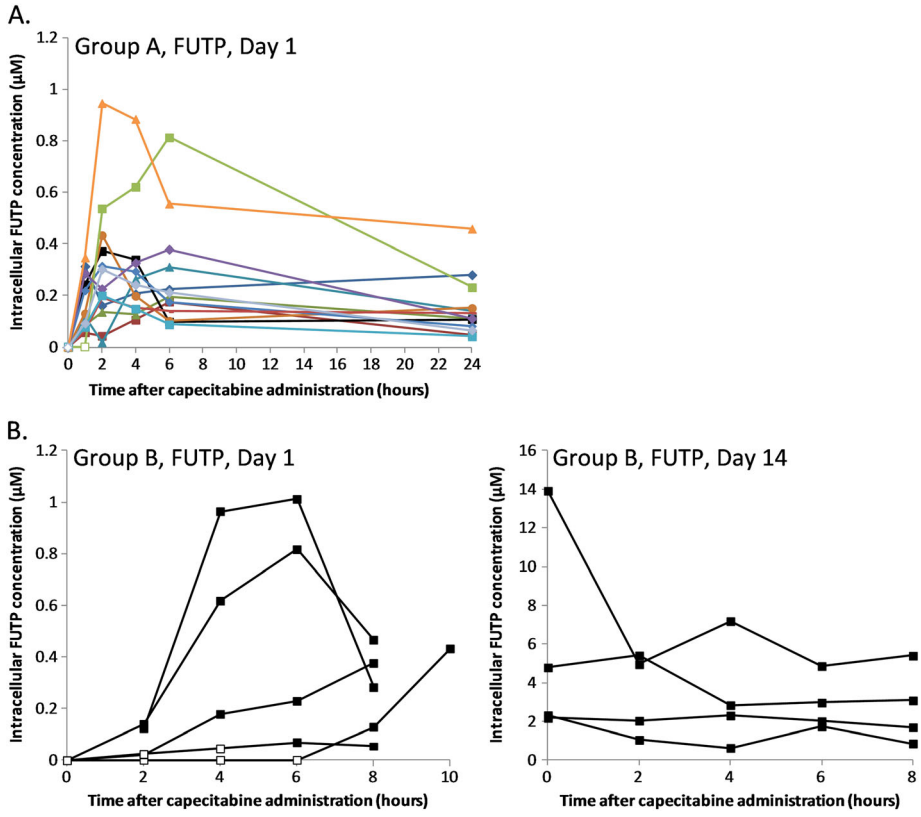

Figure 3

Intracellular concentration–time curves of 5-fluorouridine 5′-triphosphate (FUTP) for patient group A (receiving capecitabine 1000 mg QD) measured at day 1 of the first treatment cycle (A), and for patient group B (receiving capecitabine 850 mg m^{-2} BID) measured at day 1 and at day 14 of the first treatment cycle (B). Concentrations within the range of the assay are indicated by solid markers and concentrations below the LLQ by open markers

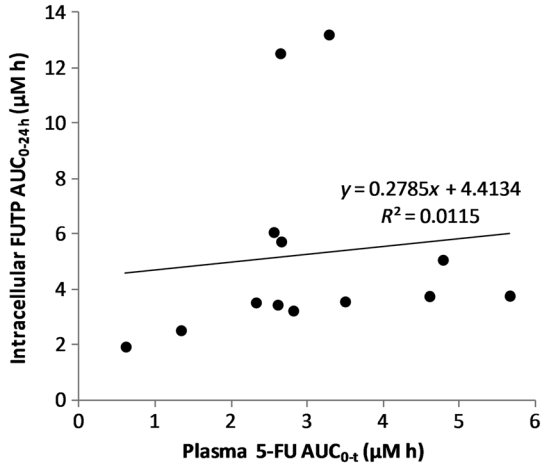

Figure 4

Relationship between the $AUC_{0\text{-}t}$ of 5-FU in plasma and the $AUC_{0\text{-}24h}$ of intracellular FUTP in PBMCs determined for group A on day 1 of the treatment

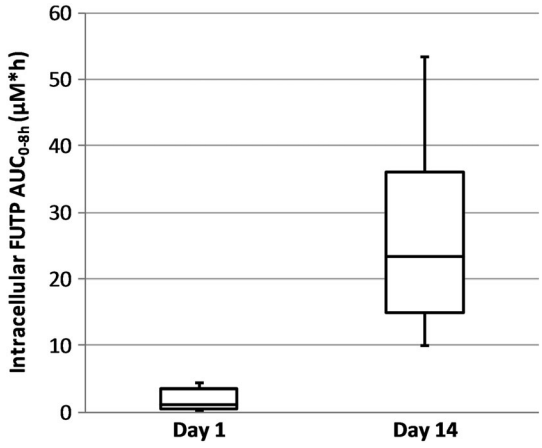

Figure 5

Box plot for the $AUC_{0\text{-}8h}$ of FUTP on day 1 and on day 14 of capecitabine treatment (twice daily). The box represents the median and the 25th and 75th percentiles of the data. The whiskers represent the range. The $AUC_{0\text{-}8h}$ for intracellular FUTP was significantly increased on day 14 of the treatment compared to day 1 (Mann–Whitney U = 20, $P = 0.008$, one-sided)

On the basis of this exploratory study, we now know that FUTP retains in cells for at least 24 h after a single capecitabine dose. The prolonged retention of FUTP in (tumour) tissue must be taken into consideration when designing new dose regimens.

It would be interesting to know whether also intracellular accumulation of FdUTP and FdUMP occurs during 14 days of treatment with capecitabine, especially given that the effects of these nucleotides are more cell cycle dependent than the effect of FUTP [21]. Prolonged intracellular exposure to FdUMP and FdUTP will affect more cells during their S-phases. Unfortunately, a more sensitive assay is required to measure the intracellular FdUTP and FdUMP levels.

Capecitabine was developed as an oral alternative to continuous intravenous 5-FU infusion [22]. It is known that prolonged exposure to 5-FU has a beneficial effect compared to brief exposure. In advanced colorectal cancer, continuous intravenous infusion resulted in significantly higher response rates and less toxicity compared with intravenous bolus injections [23]. Prolonged 5-FU exposure will probably lead to a *prolonged* intracellular exposure to 5-FU nucleotides, which is beneficial, especially as the effects of the 5-FU nucleotides are cell cycle dependent. We wondered if prolonged 5-FU plasma levels also lead to *higher* intracellular nucleotide concentrations than brief exposure to an equal amount of 5-FU. This would be the case if the transporters involved in the cellular uptake or the enzymes involved in the intracellular ribosylation or phosphorylation would become saturated above a certain 5-FU concentration. Remarkably, the FUTP concentrations which we reported in our analytical paper, measured 30 min after an intravenous 5-FU bolus (400 mg m^{-2} in 30 min) were in the same range as the FUTP concentrations measured after 14 days of capecitabine treatment twice daily (4.7–11 μM *vs.* 0.64–14 μM) [13]. This indicates that also brief, high 5-FU exposure can rapidly lead to high intracellular FUTP concentrations and the capacities of the cell membrane transporters and intracellular enzymes are probably not the limiting factors.

When interpreting the results of this intracellular PK study, we should keep in mind that the capecitabine dose that patients received during the study, 1000 mg QD for group A and 850 mg m^{-2} BID for group B, is lower than the typical dose that is used when capecitabine is given as a single agent: 1250 mg m^{-2} twice daily for 14 days followed by a 7-day rest period.

Furthermore, it should be realized that the FdUMP fraction which is bound to thymidylate synthase is not measured by the employed assay, because this fraction is precipitated during the extraction step with methanol [24, 25].

In addition, we have to keep in mind that the nucleotide concentrations measured in PBMCs may be different than the concentrations that would be found in tumour cells. PBMCs were used as a cell model for the intracellular activation, because these cells can be obtained more easily than tumour biopsies. This was particularly relevant as we wanted to follow the intracellular FUTP concentrations over time. Therefore samples had to be collected at multiple time points after capecitabine intake, which was not possible for tumour tissue.

However, by measuring the 5-FU nucleotide concentrations in PBMCs, no account is taken of the tumour-specific activation of capecitabine. The third activation step, the conversion of 5′-dFUR to 5-FU preferentially takes place within the tumour, because the responsible enzyme, thymidine phosphorylase, is more present in tumour tissue [1]. This means that the FUTP concentrations in tumour cells could be higher than those measured in PBMCs. On the other hand, it could be argued that PBMCs will probably be exposed to higher 5′-dFUR concentrations than solid tumour tissues, which in turn could lead to higher FUTP concentrations in PBMCs than in tumour cells.

Nevertheless, we believe that measurement of the active metabolites in PBMCs will provide useful information. By measuring in cells, the intracellular 'activation machinery' is at least to some extent represented. Therefore we hypothesize that these measurements in PBMCs will better reflect the active metabolite concentrations in other tissues (including tumour tissue) than the capecitabine and 5-FU plasma concentrations.

Further research is needed to determine whether there is indeed a correlation between the intracellular 5-FU nucleotide levels measured in PBMCs and clinical response or the occurrence of adverse reactions. Depending on the outcome of this study, PBMCs could serve as a surrogate matrix to give an impression of the cytotoxic metabolite concentrations in other tissues.

These intracellular measurements would be primarily useful to optimize dosing regimens for the total population. How long are the active metabolites present within the cell? What would be a suitable dose regimen (i.e. dose and dose interval) based on these results?

The use of intracellular measurements to optimize the capecitabine dose for individual patients (i.e. intracellular therapeutic drug monitoring; TDM) seems further away. The question is, first of all, whether there is a need for individual dose optimization. Our findings indicate that the intracellular FUTP concentrations show considerable interpatient variation, but is this variation associated with a different clinical outcome, and what about the other two active metabolites? If there is a need for individual dose optimization, a second question is whether intracellular TDM would be feasible. At present the isolation of PBMCs and measurement of the nucleotides is quite laborious, which makes deployment for TDM less practical.

Finally, intracellular measurement of the active metabolite levels might be useful as a predictive marker for treatment response, at least for early stage recognition of nonresponders as a result of a deficient intracellular metabolism.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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