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Short communication

Optimized blood sampling with cytidine deaminase inhibitor for improved analysis of capecitabine metabolites

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ABSTRACT

The 5FU prodrug capecitabine undergoes a 3-step enzymatic conversion, including the conversion of 5′DFRC into 5′DFUR by cytidine deaminase (CDA). The presence of CDA activity in blood led us to analyze the possible *ex vivo* conversion of 5′DFCR into 5′DFUR in blood samples. We thus examined the impact of the addition of a CDA inhibitor (tetrahydrouridine (THU) 1 μM final) in blood. Blood samples from 3 healthy volunteers were taken on tubes containing or not THU. Blood was spiked with 5′DFCR (20 μM final) (T0) and was maintained at room temperature for 2 h. Plasma concentrations of 5′DFRC and 5′DFUR were analyzed with an optimized HPLC assay. In the absence of THU, 5′DFUR was detectable as early as T0. The percent of 5′DFUR produced relative to 5′DFCR increased over time, up to 7.7 % at 2 h. In contrast, the presence of THU totally prevents the formation of 5′DFUR. The impact of THU for preventing the conversion of 5′DFCR was confirmed by the analysis of blood samples from 2 capecitabine-treated patients. Addition of THU in the sampling-tube before the introduction of blood is thus strongly recommended in order to guarantee accurate conditions for reliable measurement of capecitabine metabolites in plasma, and thus faithful pharmacokinetic data.

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1. Introduction

Capecitabine (Xeloda®) is an oral 5-fluorouracil (5FU) prodrug which is tending to replace 5FU in the treatment of a number of malignancies including breast cancer and gastrointestinal cancers [1]. Capecitabine (*N*4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine) was designed to generate 5FU preferentially in tumor tissue compared with healthy tissue [2,3]. Capecitabine is firstly hydrolyzed by carboxylesterase in the liver into 5′-deoxy-fluorocytidine (5'DFCR) (Fig. 1) [2,3]. 5′DFCR is then converted to 5′-deoxy-5fluorouridine (5′DFUR) by cytidine deaminase (CDA), which is highly active in the liver and tumor tissues and is also present in the blood [2,3]. Finally, 5′DFUR is converted to 5FU by thymidine phosphorylase (TP), which is present in tumor tissue, resulting in the release of 5FU preferentially in tumor tissue [2,3].

Numerous clinical pharmacokinetic studies have been published on capecitabine [4–7] and it has been reported that 5′DFUR concentrations are linked to treatment response [7]. A pharmacokinetic survey of patients treated by capecitabine may thus be of clinical relevance and particular attention should be paid to the plasma concentrations of 5′DFUR. The 5′DFUR/5′DFCR ratio varies among the reported studies ranging from 0.83 to 1.24 [8,9]. According to the time elapsed between blood sampling and sample processing, CDA can thus transform, *ex vivo*, 5′DFCR into 5′DFUR. Recommendations for gemcitabine pharmacokinetic analysis, for which CDA regulates the catabolic route [10], specify that CDA blood activity must be inhibited to ensure a faithful analysis. Numerous analytical methods for measuring capecitabine metabolites in plasma have been reported [4,11–14]. Among these latter, only one mentioned the use of a CDA inhibitor [14]. This study, however, did not examine the possible *ex vivo* conversion of 5′DFCR into 5′DFUR. The purpose of the present study was thus to examine more closely the impact of CDA activity on the relative stability of 5′DFCR in order to improve the analytical conditions of capecitabine and metabolite measurement in plasma.

2. Experimental

2.1. Analysis of the impact of CDA activity on 5′DFCR and 5′DFUR

Forty milliliter samples of blood were taken from three healthy volunteers who had previously given informed consent. Half

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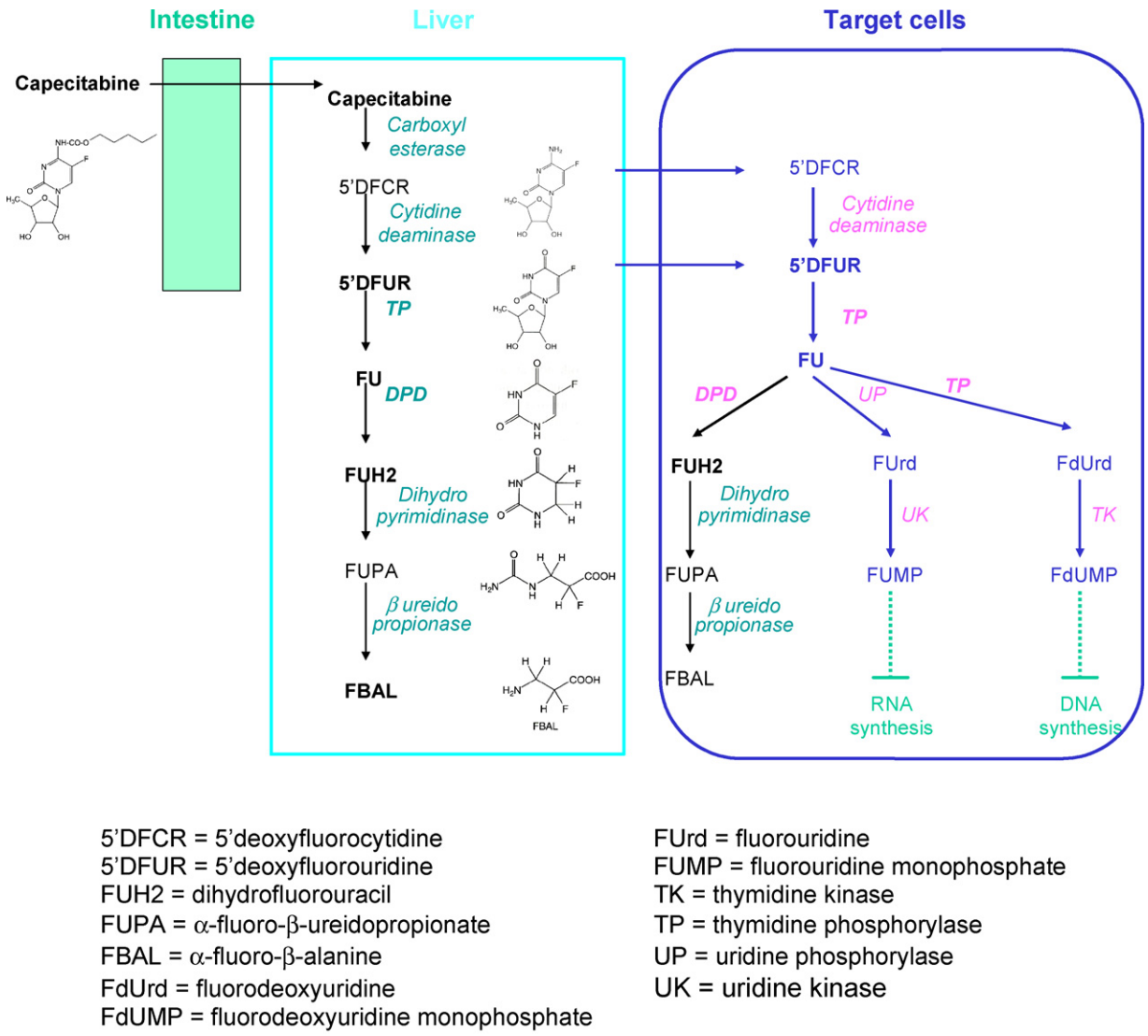

Fig. 1. Capecitabine metabolic pathways.

(20 ml) was taken in EDTA tubes containing tetrahydrouridine (THU, CDA inhibitor) at a final concentration of 1 μM, and half (20 ml) was taken in EDTA tubes without THU. The tubes were then immediately spiked with a 5 mM solution of 5′DFCR to reach a final concentration of 20 μM. This concentration was chosen since it matches the 5′DFCR C_{max} concentrations observed in the clinical setting with conventional dosing [4,13]. The enrichment time was defined as T0 and blood was maintained at room temperature (21 °C), reproducing conditions similar to those applied after patient blood sampling. Aliquots (3 ml of blood) were taken at T0, 15 min, 30 min, 1 h and 2 h. Each aliquot was immediately centrifuged at 4 °C and plasma was stored at −20 °C until HPLC analysis. Each sample was measured in duplicate.

In addition, plasma samples from 2 colorectal cancer patients receiving capecitabine (1600 mg/m^2/day in 2 daily intakes), who had previously given informed consent, were taken in 5 ml EDTA-tubes containing or not 1 μM THU (plasma from patients were handled as spiked plasma). Blood sampling was performed 2 and 3 h after the capecitabine morning intake for patients 1 and 2, respectively.

2.2. HPLC measurement of 5′DFCR and 5′DFUR

5′DFCR and 5′DFUR plasma concentrations were analyzed according to an optimized HPLC method described below. The internal standard was tegafur (Sigma, 4 μM final concentration). 5′DFCR and 5′DFUR were provided by Roche (Japan). The first step consisted in the precipitation of plasma proteins with acetonitrile (500 μl plasma + 4 ml acetonitrile) and evaporation to dryness of the top layer, under a stream of nitrogen, at 37 °C. The dry residue was then reconstituted with 500 μl of the mobile phase consisting of methanol/Titrisol® H_2SO_4 pH 4.0 (Merck)/water (220/25/870, v/v/v). Samples were then applied to a Bond-Elut® extraction cartridge (3.0 ml, Varian, Middelburg, Netherlands) conditioned with 3 ml methanol and 5 ml water. The cartridges were washed with 3 ml ammonium acetate (0.2 M) and elution was performed with 2 ml of methanol. Samples were evaporated to dryness at 37 °C and reconstituted in 250 μl of the HPLC mobile phase (20–40 μl injected). HPLC separation was performed on a Superiorex ODS column (4.6 mm × 250 mm, SHISEIDO) with a flow rate at 0.8 ml/min and UV detection at 267 nm. Retention times were 8.05, 10.1 and 12.4 min for 5′DFCR, 5′DFUR and tegafur, respectively (Figs. 2 and 3).

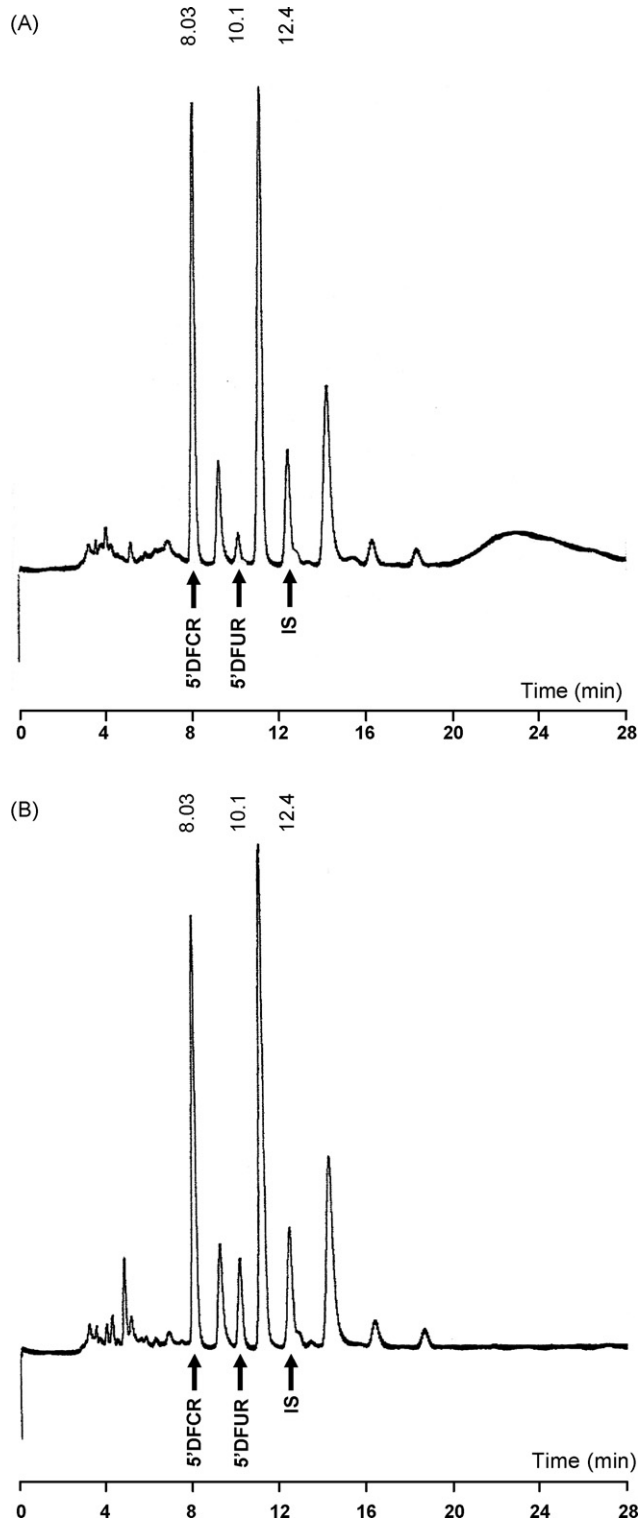

Fig. 2. HPLC profile of a plasma sample (volunteer A, 20 μl injected) obtained without THU, after blood enrichment at 20 μM of 5′DFCR. (A) Profile obtained at T0 (5′DFCR at 20.5 μM, 5′DFUR at 0.9 μM). (B) Profile obtained at T2h (5′DFCR at 19.9 μM, 5′DFUR at 1.8 μM). IS means internal standard (tegafur).

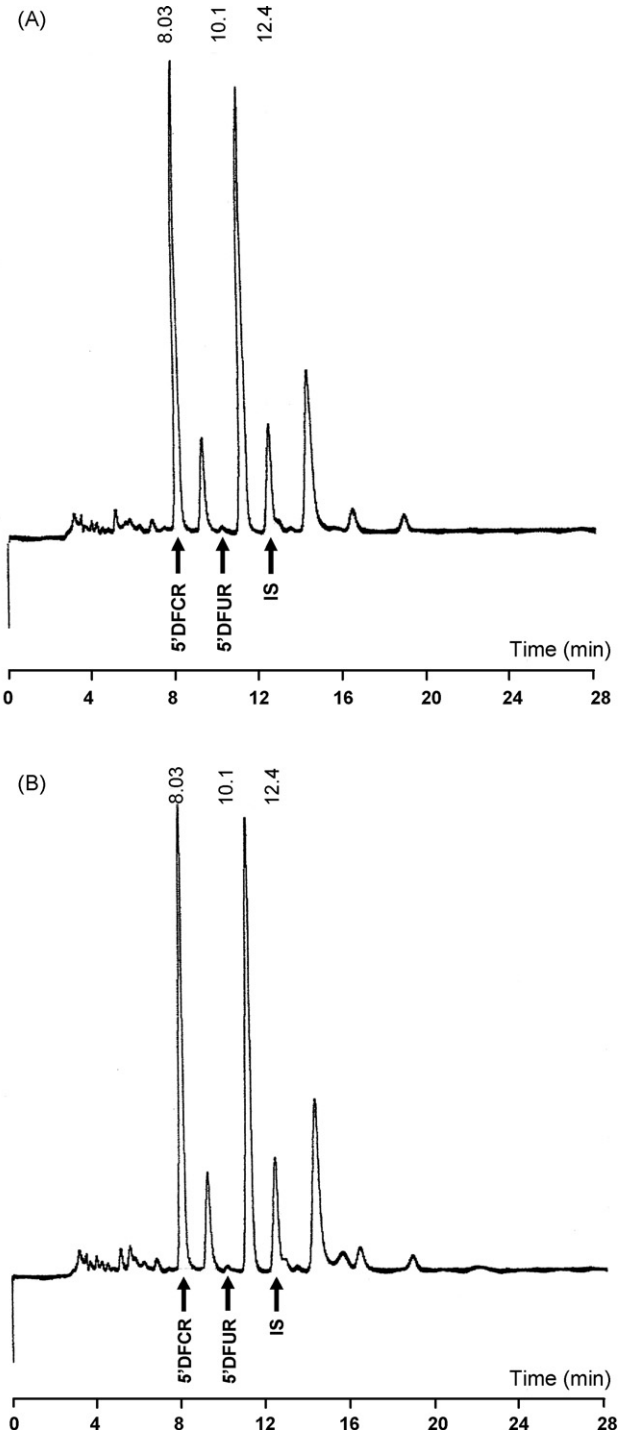

Fig. 3. HPLC profile of a plasma sample (volunteer A, 20 μl injected) obtained in the presence of 1 μM THU, after blood enrichment at 20 μM of 5′DFCR. (A) Profile obtained at T0 (5′DFCR at 21.9 μM, 5′DFUR below the limit of detection). (B) Profile obtained at T2h (5′DFCR at 20.0 μM, 5′DFUR below the limit of detection). IS means internal standard (tegafur).

Recovery rates were 85%, 83% and 80% for tegafur, 5′DFCR and 5′DFUR, respectively. The limit of quantification was 0.20 μM for both 5′DFCR and 5′DFUR. The linearity was satisfactory between 0.25 and 50 μM for both 5′DFCR and 5′DFUR. The coefficient of variation for inter-day reproducibility was <10% at both low (2.5 μM) and high (12.5 μM) concentrations of 5′DFCR and 5′DFUR.

2.3. HPLC measurement of 5FU and capecitabine

In blood samples obtained in capecitabine-treated patients, plasma concentrations of capecitabine and 5FU were analyzed by means of HPLC assay, as previously described [4].

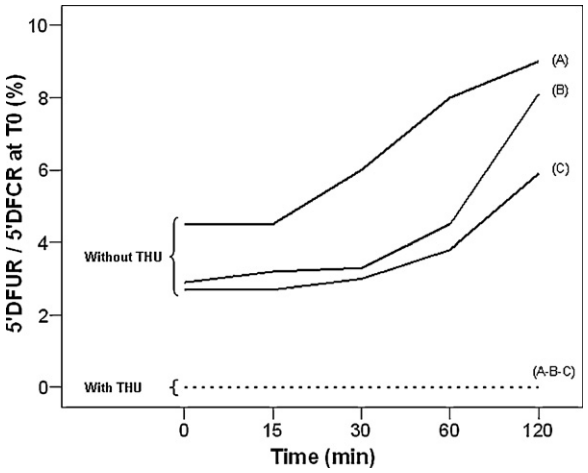

Fig. 4. Plot of the evolution of the percent of 5′DFUR produced (relative to 5′DFCR measured at T0) as a function of incubation time in total blood, with (- - -) or without (—) CDA inhibitor (1 μM final concentration in blood). The experiment was performed in triplicate, i.e. in blood taken from 3 healthy volunteers (A–C). The dotted lines corresponding to the 3 subjects are superimposable since 5′DFUR was never detected in the presence of THU.

3. Results and discussion

Of note, these experiments on blood samples spiked with 5′DFRC clearly show the appearance of a peak of 5′DFUR in the absence of THU. This peak of 5′DFUR is detectable as early as T0 (Fig. 2A), meaning that the production of 5′DFUR from 5′DFCR occurs immediately after the introduction of 5′DFCR into the blood sample. The kinetics of the production of 5′DFUR over the observation time (2 h) is illustrated in Fig. 4. The percent of 5′DFUR produced relative to 5′DFCR increased over time in the 3 different tested blood samples, ranging on average from 3.4% at T0 up to 7.7% at T2h. In contrast, the presence of 1 μM THU totally prevents the formation of 5′DFUR, as illustrated in Figs. 3A, B and 4. Table 1 illustrates the validation of this procedure on plasma samples from two capecitabine-treated patients. For both patients, the presence of THU was associated with higher 5′DFCR concentrations, along with lower 5′DFUR concentrations. In contrast and as anticipated, the presence of THU had no impact on capecitabine or 5FU measurement. Of note for patient 1, in the absence of THU the observed loss of 5′DFCR perfectly concords with the gain in 5′DFUR (3.6 μM).

Addition of THU (at a final concentration of 1 μM) in the sampling-tube before the introduction of blood is thus strongly recommended in order to guarantee accurate conditions for reliable measurement of capecitabine metabolites in plasma, and thus faithful pharmacokinetic data. In the absence of CDA inhibitor, the variability arising from the 5′DFCR conversion into 5′DFUR probably explains the discrepancies in the literature data concerning the relative proportion of 5′DFUR formed from 5′DFCR [8,9]. Reliable measurement of 5′DFUR is recommended, more especially as pharmacokinetic-pharmacodynamic relationships have been shown for 5′DFUR in the clinical setting for toxicity [15,16] and capecitabine responsiveness [7,16].

Table 1
Capecitabine and metabolites concentrations obtained from 2 patients under capecitabine therapy

		Concentrations measured with THU (μM)	Concentrations measured without THU (μM)
Patient 1[a]	Capecitabine	1.7	1.7
	5′DFCR	14.4	10.8
	5′DFUR	16.1	19.7
	5FU	0.8	0.8
Patient 2[b]	Capecitabine	0.6	0.6
	5′DFCR	10.0	9.1
	5′DFUR	4.9	6.5
	5FU	0.3	0.3

[a] Blood sample taken 2 h after oral administration of 800 mg/m^2 of capecitabine.
[b] Blood sample taken 3 h after oral administration of 800 mg/m^2 of capecitabine.

The metabolic pathway of another major anticancer agent, gemcitabine, is controlled by the CDA enzyme [17]. Analytical conditions for gemcitabine measurement in plasma specify that blood must be taken in tubes containing a CDA inhibitor (THU) [18]. We thus strongly recommend a procedure similar to that already applied to gemcitabine, for plasma analysis of capecitabine and metabolites in patients. It is hoped that the widespread use of such a validated sampling procedure will improve the reliability of capecitabine pharmacokinetic analysis.

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