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ORIGINAL ARTICLE

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Cytosolic and microsomal activation of doxifluridine and tegafur to produce 5-fluorouracil in human liver

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Abstract *Purpose*: The enzymatic formation of 5-fluorouracil (5-FU) from two fluoropyrimidine prodrugs, doxifluridine (5′-DFUR) and tegafur (FT), was compared in vitro in order to determine whether there are differences between the metabolic profiles of the two prodrugs. *Methods*: Conversion of the two fluoropyrimidine prodrugs to 5-FU was measured by high-performance liquid chromatography at a concentration of 500 μ*M* using the microsomal and cytosolic fractions of 12 human livers. The degree of correlation between the 5-FU-forming activities was determined using various cytochrome P450-dependent reactions. *Results*: Liver microsomes catalyzed 5-FU formation from 5′-DFUR at rates of 10.0–160.1 pmol/min per mg protein and correlated well with CYP2A6-dependent coumarin 7-hydroxylase activity. The rates of microsomal 5-FU formation from FT ranged from 44.9 to 808.3 pmol/min per mg protein and also correlated with coumarin 7-hydroxylase activity. The cytosol fractions catalyzed 5-FU formation from 5′-DFUR at rates of 3164.6 to 6026.6 pmol/min per mg protein, almost two orders of magnitude higher than the rates of cytosolic 5-FU formation from FT (46.8–219.0 pmol/min per mg protein). *Conclusions*: The cytosolic enzymes in livers appear to be important for 5-FU formation from 5′-DFUR. Both cytosolic and microsomal enzymes were involved almost equally in 5-FU formation from FT. The increased formation of 5-FU from 5′-DFUR might provide an answer to the question of why similar blood 5-FU levels were retained despite blood 5′-DFUR levels lower than blood FT levels.

Keywords 5-Fluorouracil · Doxifluridine · Tegafur · CYP2A6 · Thymidine phosphorylase

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Introduction

5′-Doxifluridine (5′-DFUR) and tegafur (FT) are antitumor fluoropyrimidine prodrugs [3, 11, 12]. Various enzymes have been shown to metabolize these prodrugs to the active metabolite, 5-fluorouracil (5-FU). 5-FU subsequently exerts its cytotoxic effects on tumor cells through thymidylate synthase inhibition [15] and incorporation of fluoro-UTP into RNA [16]. 5′-DFUR is converted to 5-FU by thymidine phosphorylase in tumor tissues, where the activity of this enzyme is elevated [5]. Recently, we and others have reported that cytochrome 450 (CYP)2A6 (CYP2A6) also metabolizes FT to 5-FU [8, 9, 13]. Komatsu et al. have reported that the formation of 5-FU from FT is catalyzed by both the microsomal and the cytosolic fractions of human liver [10]. Little is known, however, about the hepatic metabolism of 5′-DFUR, although it has been shown that tumor cells catalyze the activation of 5′-DFUR [4, 7].

In the present study, we investigated the hepatic metabolism of 5′-DFUR and FT in vitro. In addition, as it

has previously been demonstrated that similar levels of the active metabolite 5-FU are attained with blood 5′-DFUR levels lower than those of FT [1, 6], we examined the relationship between the in vitro metabolic activation of 5′-DFUR and FT and their reported pharmacokinetics.

Methods

Reagents

The chemicals used in the present study were of the highest grade available. Microsomes and cytosols from 12 human livers were purchased from Gentest (Woburn, Mass.). The Institutional Review Board of the National Institute of Health Sciences approved the use of these human materials. The manufacturer provided data on the cytochrome P450-dependent activities of these liver samples.

Determination of 5-FU formation by liver microsomes and cytosols using 5′-DFUR or FT as substrate

The formation of microsomal 5-FU was determined using either 0.5 m*M* 5′-DFUR or FT as substrate. The incubation mixture consisted of 100 m*M* potassium phosphate (pH 7.4), 400 μg/ml of microsomal fraction (Gentest, Woburn, Mass.), one of the prodrug substrates, and 0.33 m*M* NADPH. The reaction was initiated by the addition of NADPH, the mixture was incubated at 37°C for 30 min, and the reaction terminated by the extraction of the 5-FU produced with 750 μl ethyl acetate containing 12.5 nmol acetaminophen. The 5-FU produced was extracted once more with 500 μl ethyl acetate without acetaminophen and the organic phase was evaporated to dryness. The residue was dissolved in 10 m*M* potassium phosphate (pH 5.5) and the amount of 5-FU formed quantified by HPLC (LC-VP, Shimadzu, Tokyo). The HPLC conditions were as follows: C8 column (150×4.6 mm i.d., Senshu Pak PEGASIL); mobile phase 10 m*M* potassium phosphate buffer (pH 5.5) for 10 min, then a linear gradient to 10% methanol over 10 min, 10% methanol for 10 min, and then a decrease in methanol concentration to 0% over 10 min with a linear gradient. 5-FU was detected at 266 nm.

The formation of cytosolic 5-FU was measured in the presence of 10 μ*M* gimestat (CDHP), an inhibitor of dihydropyrimidine dehydrogenase. The incubation mixture consisted of 100 m*M* potassium phosphate (pH 7.4), 800 μg/ml of cytosolic fraction, one of the prodrug substrates (0.5 m*M*) and 10 μ*M* CDHP. The mixture was incubated at 37°C for 10 min. The 5-FU formed was detected in the same way as described for the microsomal incubation. The activity was calculated by subtracting the amount of 5-FU produced in the absence of NADPH (in the case of microsomes) from that in the presence of NADPH. The activity was calculated by dividing the amount of 5-FU formed enzymatically by the incubation time and amount of protein used.

Measurement of thymidine phosphorylase activity

Thymidine phosphorylase activity was measured using thymidine as substrate. The incubation mixture (250 μl) consisted of 100 m*M* potassium phosphate (pH 7.4), 800 μg/ml of the cytosolic fraction, thymidine (0.5 m*M*) and 50 μ*M* CDHP. The mixture was incubated at 37°C for 10 min. The thymine formed was detected as described above for microsomal incubation. We used boiled cytosols for the determination of spontaneously produced amounts, which were subtracted from the amounts in the presence of intact cytosols. The activity was calculated by dividing the amount of 5-FU formed enzymatically by the incubation time and amount of protein used.

Statistical analysis

Analysis of the degree of correlation between the various enzymatic activities was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, Calif.). Correlations were analyzed by Pearson's test.

Pharmacokinetic analysis of reported data for 5′-DFUR and FT

The average blood concentration ($n = 3$–5) after oral administration of 5′-DFUR (800 mg/patient) and FT (1 g/patient) obtained from two previous reports [1, 6] was fitted to a one-compartment model, and absorption (k_a), formation (k_f) and elimination (k_e) rate constants were estimated using WinNonlin (Pharsight Corporation, Mountain View, Calif.).

Results

Microsomal production of 5-FU from 5′-DFUR and FT, and its correlation with coumarin 7-hydroxylase activity

Microsomal 5-FU formation from 5′–DFUR and FT was examined using microsomes from 12 livers. The rates of 5-FU formation were in the range 10.0–160.1 pmol/min per mg protein using 5′–DFUR as substrate and 44.9–808.3 pmol/min per mg protein using FT (Table 1). For both substrates, 5-FU formation correlated well with coumarin 7-hydroxylase activity, which is known to be catalyzed by CYP2A6 (Figs. 1 and 2). On the other hand, 5-FU formation from both 5′-DFUR and FT showed no appreciable correlation with phenacetin *O*-deethylase activity (mainly catalyzed by CYP1A2) or testosterone 6*β*-hydroxylase activity (catalyzed by CYP3A4) (Figs. 1 and 2).

Cytosolic 5-FU formation from 5′-DFUR and FT, and its correlation with thymidine phosphorylase activity

The production of 5-FU from 5′-DFUR and FT by liver cytosolic fractions was determined. The rates of cyto-

Table 1 Formation of 5-FU from 5′-DFUR and FT, and cytochrome P450-dependent activities measured in microsomal fractions isolated from 12 human livers (*POD* phenacetin *O*-deethylase, *CHD* coumarin 7-hydroxylase, *T6β* testosterone 6*β*-hydroxylase)

Sample	5-FU formation (pmol/min/mg protein)		Enzyme activities (pmol/min/mg protein)		
	5′-DFUR	FT	POD	CHD	T6*β*
HG03	160.1	430.0	112.0	2358.0	5854.0
HG06	14.7	140.3	980.0	490.0	2170.0
HG23	72.6	539.5	740.0	650.0	3150.0
HG30	44.9	313.0	2380.0	940.0	10610.0
HG42	86.3	644.7	550.0	1500.0	14530.0
HG43	40.1	633.6	356.0	471.0	3408.0
HG56	114.2	525.8	2009.0	1333.0	5028.0
HG66	37.3	606.8	264.0	1571.0	5802.0
HG70	12.3	808.3	496.0	2047.0	8960.0
HG89	57.2	117.7	1197.0	514.0	11732.0
HG93	69.5	200.7	501.0	329.0	2090.0
HG112	10.0	44.9	244.0	426.0	17519.0

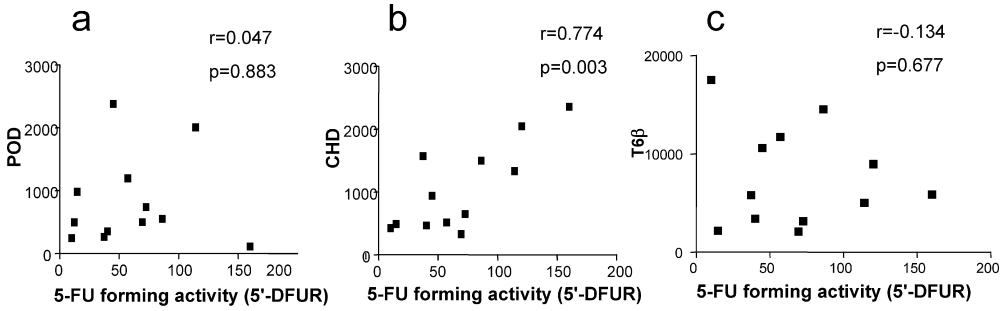


Fig. 1a–c Correlation between 5-FU formation from 5′-DFUR and (**a**) phenacetin *O*-deethylase (*POD*), (**b**) coumarin 7-hydroxylase (*CHD*) and (**c**) testosterone 6*β*-hydroxylase (*T6β*) activities (in picomoles per minute per milligram protein) in human liver microsomes from 12 individuals. The formation of 5-FU from 5′-DFUR was determined by HPLC as described in Methods. The activities of POD, CHD, and T6*β* were determined by the company (Gentest, Woburn, Mass.) that supplied the human liver microsomes. *P* values <0.05 were assumed to indicate statistical significance

solic 5-FU formation from 5′-DFUR and FT were in the ranges 3164.6–6026.6 and 46.8–219.0 pmol/min per mg protein, respectively (Table 2). The rates of formation of 5-FU from both 5′-DFUR and FT were significantly correlated with thymidine phosphorylase activity (Fig. 3).

Table 2 5-FU formation and thymidine phosphorylase activity by cytosolic fractions isolated from livers of 12 human individuals

Sample	5-FU formation (pmol/min/mg protein)		Thymidine phosphorylase activity (pmol/min/mg protein)
	5′-DFUR	FT	
HG03	5161.3	90.3	9937.0
HG06	3164.6	59.6	5806.0
HG23	3887.0	97.1	7840.0
HG30	4437.6	219.0	10244.0
HG42	3573.8	57.7	7810.0
HG43	3756.2	57.2	6476.0
HG56	4278.6	81.1	7816.0
HG66	6026.6	156.0	11258.0
HG70	3781.4	103.0	7957.0
HG89	3377.6	46.8	5523.0
HG93	4879.0	66.1	8802.0
HG112	4438.8	74.2	7918.0

Pharmacokinetic parameters estimated from reported blood concentrations of 5′-DFUR, FT and 5-FU after oral administration

We attempted to draw concentration-time profiles for 5′-DFUR, FT and 5-FU by fitting reported blood concentrations of 5′-DFUR, FT and 5-FU into a one-compartment model. The absorption and elimination rate constants, k_a and k_e, were estimated for 5′-DFUR and FT, and k_f and k_e estimated for 5-FU, using WinNonlin Professional software. The results are summarized in Table 3. Both the elimination rate constant for FT and the formation constant for 5-FU from FT were much lower than the values obtained from 5′-DFUR.

Discussion

The fluoropyrimidine prodrugs 5′-DFUR and FT have been widely used in the treatment of stomach, colon and breast cancers. Microsomal and cytosolic enzymes are involved in the bioactivation of these two drugs, which results in the production of the active metabolite, 5-FU [10]. In the present study, the in vitro metabolism of these prodrugs to 5-FU was examined using microsomal and cytosolic fractions of 12 human livers. The pro-

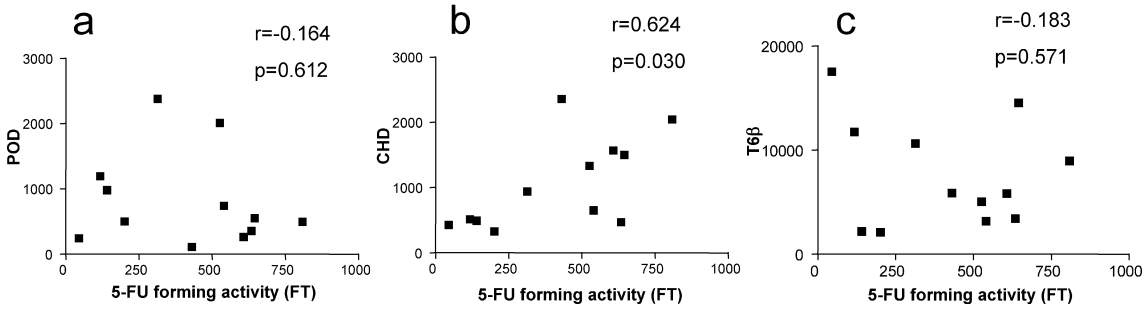


Fig. 2a–c Correlation between the formation of 5-FU from FT and (**a**) phenacetin *O*-deethylase (*POD*), (**b**) coumarin 7-hydroxylase (*CHD*) and (**c**) testosterone 6*β*-hydroxylase (*T6β*) activities (in picomoles per minute per milligram protein) in human liver microsomes from 12 individuals. The formation of 5-FU from FT was determined by HPLC as described in Methods. The activities of POD, CHD and T6*β* were determined by the company (Gentest, Woburn, Mass.) that supplied the human liver microsomes. *P* values <0.05 were assumed to indicate statistical significance

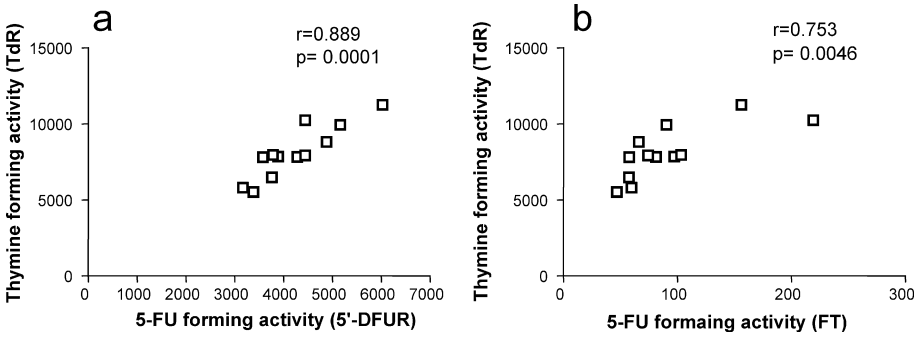


Fig. 3a, b Correlation between the production of 5-FU from (**a**) 5′-DFUR and (**b**) FT and thymidine phosphorylase activities (in picomoles per minute per milligram protein) in 12 human liver cytosolic fractions. 5-FU formation from 5′-DFUR and FT was determined by HPLC as described in Methods. Thymidine phosphorylase activity was also measured by HPLC as described in Methods. *P* values <0.05 were assumed to indicate statistical significance

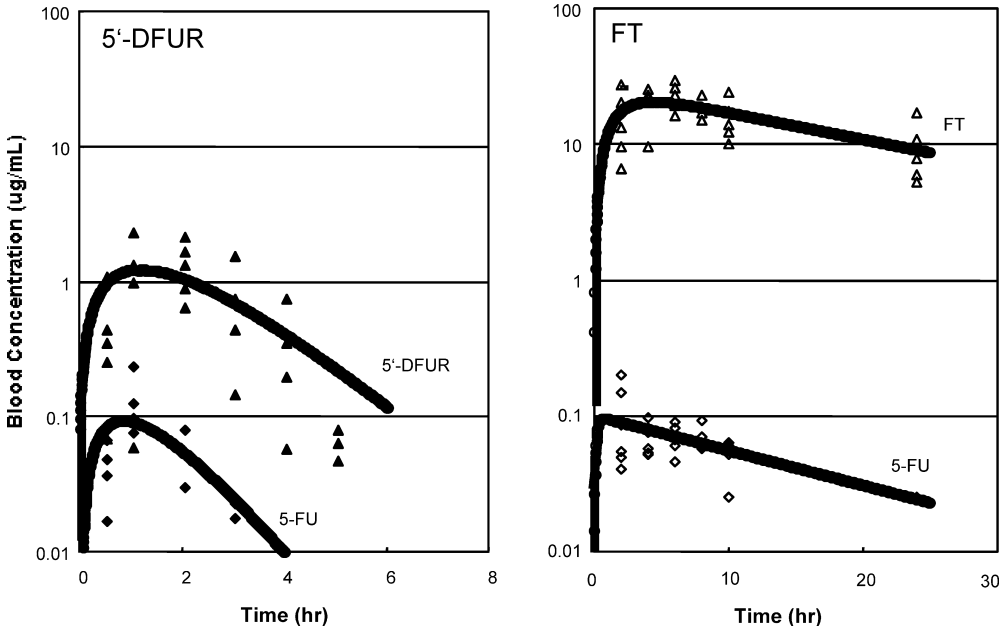
Table 3 Absorption, formation and elimination rate constants for orally administered 5′-DFUR, FT and 5-FU as determined by pharmacokinetic analysis of reported blood concentrations after oral administration of the prodrugs

	k_a or k_f (min^{-1})	k_e (min^{-1})
5′-DFUR	0.82	0.83
FT	0.66	0.045
5-FU (from 5′-DFUR)	1.23	1.20
5-FU (from FT)	0.059	6.19

duction of 5-FU from 5′-DFUR and FT by the microsomal fractions varied by 16-fold and 18-fold, respectively. 5-FU formation by cytosolic fractions was less variable, with 1.9-fold and 4.7-fold variations for 5′-DFUR and FT, respectively. The thymidine phosphorylase activities measured in samples from all 12 livers were similar. The cytosol production of 5-FU from 5′-DFUR was about two orders of magnitude higher than that obtained from FT, whereas the microsomal production of 5-FU from 5′-DFUR was about one order of magnitude lower than that obtained from FT. Production of 5-FU from both 5′-DFUR and FT correlated with CYP2A6-dependent coumarin 7-hydroxylase activity. The results of FT metabolism reported here are consistent with those reported by Komatsu et al. [9].

A correlation between the microsomal production of 5-FU from 5′-DFUR and CYP2A6-dependent enzyme activity has not been previously described. The contribution of this microsomal activity, however, to overall 5-FU production was relatively small in relation to the total hepatic activity as the cytosolic fraction rapidly metabolized 5′-DFUR to 5-FU (Table 2). Of more importance, however, is the large difference in the rate of production of 5-FU from 5′-DFUR and from FT by the liver cytosol. These results may have been due to

Fig. 4a, b Simulated kinetic profiles of blood levels of 5-FU, 5′-DFUR and FT after oral administration of (**a**) 5′-DFUR and (**b**) FT. Blood level simulations were performed as described in Methods, based on the reported data of drug concentrations after oral administration of 800 mg 5′-DFUR and 1 g FT [1, 5]. (**a** *triangles* 5′-DFUR, *diamonds* 5-FU; **b** *triangles* FT, *diamonds* 5-FU)



differences in the hydrophobic properties of these two prodrugs. As judged from their retention times in reverse-phase HPLC and their chemical structures, 5′-DFUR appears to be more water-soluble than FT. Recently, different alleles including defective ones of the CYP2A6 gene have been reported. Therefore, for those who carry the defective CYP2A6 alleles, thymidine phosphorylase is extremely important in the metabolic activation of FT, the 5-FU prodrug in liver. 5-FU formation activities in liver were similar between microsomal and cytosolic fractions, which suggests equally important roles for thymidylate synthase and CYP2A6. The Km value of thymidine phosphorylase for the conversion of FT into 5-FU has been reported to be higher than that of CYP2A6 [10]. On the other hand, thymidine phosphorylase is expressed in a wider variety of tissues than CYP2A6. Thymidine phosphorylase seems to be important in tissues other than liver.

Whole liver cell production of 5-FU from 5′-DFUR is approximately ten times higher than that from FT. This is due to the fivefold greater amount of protein required to form a comparable amount of 5-FU within the cytosolic fraction when compared to the microsomal fraction. As previously mentioned, Abe et al. and Hara et al. have reported that blood levels following oral administration of FT are approximately 20-fold higher than those following oral administration of 5′-DFUR, despite similar 5-FU levels [1, 6]. We performed a pharmacokinetic analysis of their data and the reported individual blood concentrations, and calculated average blood concentrations of 5′-DFUR, FT, and 5-FU were used to estimate the parameter values plotted on Fig. 4. The elimination rate constant for FT, and the formation rate constant for 5-FU from FT were much lower than the elimination rate constant for 5′-DFUR, and the formation rate constant for 5-FU from 5′-DFUR, as summarized in Table 3. These data are in accord with the results obtained from our in vitro study. The rapid hepatic conversion of 5′-DFUR to 5-FU could explain the similar levels of blood 5-FU and faster elimination of 5′-DFUR. A difference in the renal clearance (5′-DFUR greater than FT) [14] may also contribute to the reduced blood levels of 5′-DFUR.

In the present study, we investigated the in vitro metabolism of 5′-DFUR and FT to the active metabolite, 5-FU, using cytosolic and microsomal fractions from human livers. The pathways and the enzymes involved in 5-FU formation were quite different for the two prodrugs. While our studies would suggest that there are relatively small individual differences in the cytosolic level of thymidine phosphorylase, large individual differences in the microsomal CYP2A6 enzyme activity were observed. Therefore, dosage regimen and therapeutic drug monitoring may be required when using these prodrugs [2].

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