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Design and Synthesis of the Tumor-Activated Prodrug of Dihydropyrimidine Dehydrogenase (DPD) Inhibitor, RO0094889 for Combination Therapy with Capecitabine

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Abstract—A series of tumor-activated prodrugs of the inhibitors of dihydropyrimidine dehydrogenase (DPD), an enzyme catabolizing 5-fluorouracil (5-FU: **4g**), has been designed and synthesized. RO0094889 (**11c**) is a prodrug of 5-vinyluracil (**4c**), a known DPD inhibitor, and was designed to generate **4c** selectively in tumor tissues by sequential conversion of **11c** by three enzymes: esterase, cytidine deaminase and thymidine phosphorylase, the latter two of which are known to be highly expressed in various tumor tissues. When capecitabine (**1**), a tumor-activated prodrug of 5-FU, was co-administered orally with **11c**, 5-FU in tumor tissues was significantly increased with only a slight increase of 5-FU in plasma as compared with oral capecitabine alone.

Introduction

Previously, we designed an oral fluoropyrimidine, capecitabine (**1**),[1,2] which generates 5-fluorouracil (5-FU: **4g**) selectively in tumors through its sequential metabolism by three enzymes highly expressed in the liver and tumors: carboxylesterase, cytidine deaminase (Cyd deaminase) and thymidine phosphorylase (dThdPase). The susceptibility of human cancer xenografts to capecitabine correlated with the ratio of two enzymes in tumors, dThdPase and dihydropyrimidine dehydrogenase (DPD),[3] which respectively generate 5-FU from the intermediate 5′-deoxy-5-fluorouridine (**3**: 5′-DFUR) and catabolize 5-FU to an inactive 5,6-dihydro-5-fluorouracil (FUH_2) (Fig. 1).

DPD exists in various types of human cancers.[4] Therefore, modulators of either dThdPase or DPD in tumors would optimize capecitabine therapy. To enhance the efficacy of capecitabine, but not its toxicity, we designed a series of 5′-deoxy-5-(substituted)-cytidine derivatives as prodrugs of known DPD inhibitors so as to generate an active DPD inhibitor, 5-(substituted)-uracil derivative[5] selectively in tumors, similar to the design of capecitabine.[2] These cytidine derivatives were screened for sequential activation by carboxylesterase, Cyd deaminase and dThdPase in vitro (Fig. 2). We identified RO0094889 (**11c**) as a tumor-activated prodrug of 5-vinyluracil (5-VU: **4c**). When both capecitabine and **11c** were given orally, a much higher concentration of 5-FU in tumor was attained without an increase in plasma or other normal tissues as compared with oral capecitabine alone.

In this paper, we describe the discovery of RO0094889 as well as its potentiation of the antitumor activity of capecitabine in vivo.

Chemistry

After evaluation of known DPD inhibitors,[5] we selected the following six 5-substituted-uracil derivatives as parent drugs with IC_{50} values of less than 1 μM: **4a** (0.014

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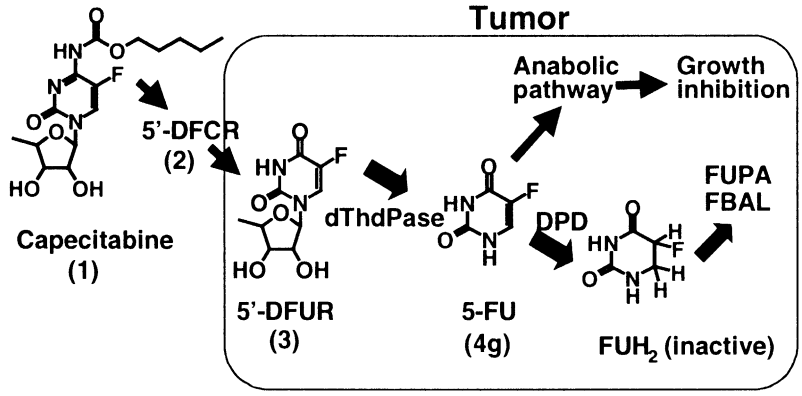


Figure 1.

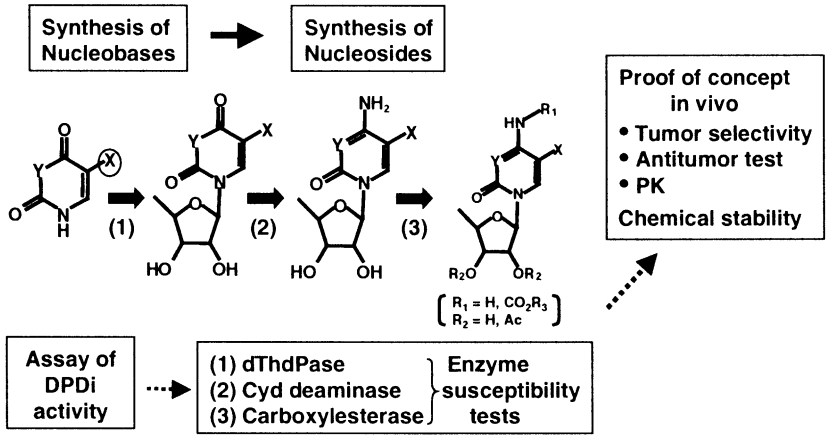


Figure 2.

μM),[6] **4b** (0.22 μM),[7] **4c** (0.089 μM), **4d** (0.20 μM), **4e** (0.097 μM) and **4f** (0.043 μM). These compounds were either purchased or synthesized according to the procedures in the literature.[8] The synthesis of the corresponding 5′-deoxyuridine, 5′-deoxycytidine and N^4-alkoxycarbonyl-5′-deoxycytidine derivatives are outlined in Figures 3 and 4. The 5′-deoxyuridine derivatives, **6a**, **6b** and **6e**, were synthesized by glycosidation reaction of 5-substituted uracil derivatives and 1,2,3-tri-*O*-acetyl-5-deoxyribose with $SnCl_4$, followed by hydrolysis of the acetyl group with NaOH in aqueous methanol. The vinyl derivative (**6c**) was synthesized by the Stille coupling reaction of **5b** with tri-butylvinylstanane, followed by hydrolysis of the acetyl group under the same conditions. The cyano derivative (**6d**) was synthesized by substitution reaction of the 5-iodo derivative (**7b**) with sodium cyanide, followed by removal of the TBS group with tetrabutylammonium fluoride (TBAF). The (*Z*)-styryl derivative (**6f**) was synthesized by palladium-catalyzed coupling reaction of **7b** with phenylacetylene, followed by hydrogenation with Lindlar catalyst and then removal of the TBS group with TBAF. The 5′-deoxycytidine derivatives (**9a**) and (**9e**) were synthesized by treatment of the TBS derivatives (**7a** and **7e** respectively) with $POCl_3$ in pyridine/acetonitrile in the presence of dimethylaminopyridine, followed by aminolysis with aqueous ammonia or liquid ammonia and then removal of the TBS group with HF-NEt_3 or HF-pyridine.

The 5′-deoxycytidine derivative (**9b**) was synthesized from 5-iodocytosine (**10**) under similar reaction conditions as the 5′-deoxyuridine derivative (**5b**) as mentioned above, followed by hydrolysis of the acetyl group with NaOH in aqueous methanol. The cyano derivative (**9d**) was synthesized from the 5-iodo derivative (**8b**) under the same reaction conditions as the 5′-deoxyuridine derivative (**6d**). 2′,3′-Di-*O*-acetyl-5′-deoxy-5-vinylcytidine (**11c**: RO0094889) was synthesized from **11b** under the same reaction conditions as the 5′-deoxyuridine derivatives (**6c**) as mentioned above. The 5′-deoxycytidine derivative (**9c**) was prepared by hydrolysis of **11c** with NaOH in aqueous methanol. The N^4-pentyloxycarbonyl-5′-deoxycytidine derivatives, **12a**, **12b**, **12c** (RO0094226), **12d** and **12e**, were prepared by acylation of the intermediates (**8a–e** and **11c**) with *n*-pentyl chloroformate in pyridine/CH_2Cl_2, followed by removal of the *O*-protecting group with TBAF (for the TBS group) or K_2CO_3 in aqueous methanol (for the Ac group).

Results and Discussion

The 5-substituted-5′-deoxycytidine derivatives synthesized above were screened for sequential activation by carboxylesterase/esterase, Cyd deaminase and dThdPase in vitro (Fig. 2). We selected the compounds whose susceptibility to those enzymes were more than 0.1 relative to that of capecitabine (1), 5′-DFCR (2) and

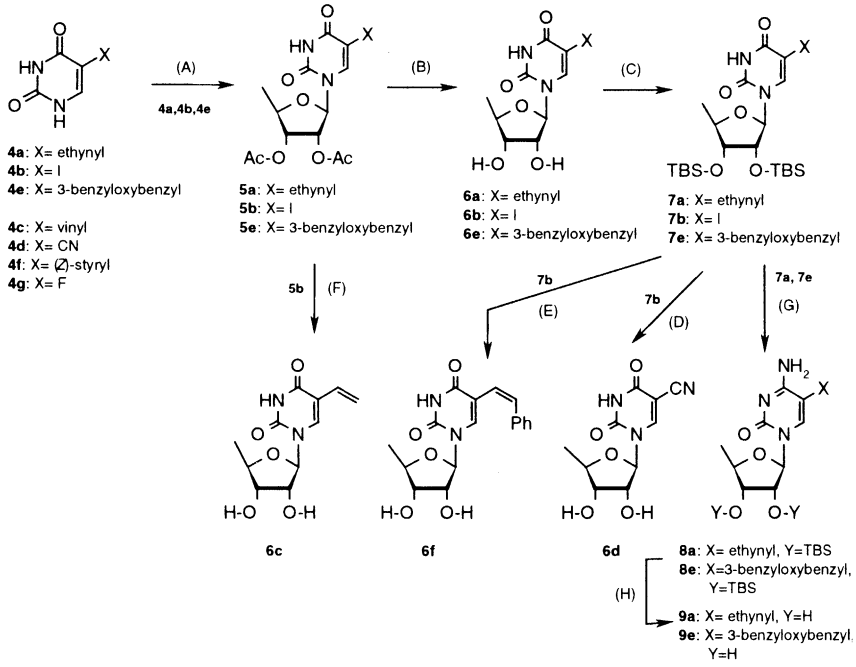


Figure 3. Reagents: (A) Synthesis of **5a** (starting from **4a**): (i) HMDS, $(NH_4)_2SO_4$, reflux, 5 h; (ii) 1,2,3-tri-*O*-acetyl-5-deoxyribose, $SnCl_4$, CH_3CN, CH_3NO_2, 0 °C, 2 h (64%, two steps); Synthesis of **5b** (starting from **4b**): (i) HMDS, $(NH_4)_2SO_4$, benzene, reflux, 2 h; (ii) 1,2,3-tri-*O*-acetyl-5-deoxyribose, $SnCl_4$, CH_3CN, CH_3NO_2, 0 °C, 2 h (quant, two steps); Synthesis of **5e** (starting from **4e**): (i) HMDS, $(NH_4)_2SO_4$, reflux, 5 h; (ii) 1,2,3-tri-*O*-acetyl-5-deoxyribose, $SnCl_4$, CH_3CN, CH_3NO_2, 0 °C, 3 h (89%, two steps); (B) Synthesis of **6a**, **6b** and **6e** (starting from the corresponding **5**): NaOH, MeOH, H_2O, 0 °C, 0.5 h; (C) Synthesis of **7a** (starting from **6a**): TBSCl, imidazole, DMF, rt, 23 h (76% from **5a**); Synthesis of **7b** (starting from **6b**): TBSCl, imidazole, DMF, 60 °C, 23 h (94% from **5b**); Synthesis of **7e** (starting from **6e**): TBSCl, imidazole, DMF, rt, 23 h (quant. from **5e**); (D) Synthesis of **6d** (starting from **7b**): (i) NaCN, DMF, rt, 17 h; (ii) TBAF, THF, rt, 3 h (63% two steps); (E) Synthesis of **6f** (starting from **7b**): (i) phenylacetylene, $PdCl_2(PPh_3)_2$, CuI, NEt_3, CH_2Cl_2, reflux, 6 h; (ii) H_2, Lindlar cat., quinoline, hexane, rt, 36 h; (iii) TBAF, THF, rt, 1 h (12% three steps); (F) Synthesis of **6c** (starting from **5b**): (i) vinyltributylstannane, $Pd_2(dba)_3$, tri-2-furylphosphine, DMF, rt, 96 h; (ii) NaOH, MeOH, H_2O, rt, 0.5 h (49% two steps); (G) Synthesis of **8a** (starting from **7a**): $POCl_3$, DMAP, pyridine, CH_3CN, then NH_4OH, 0 °C to rt, 2 h (99%); Synthesis of **8e** (starting from **7e**): $POCl_3$, DMAP, pyridine, CH_3CN, then liq.NH_3, 0 °C to rt, 2 h (75%); (H) Synthesis of **9a** (starting from **8a**): HF-NEt_3, THF, 50 °C, 14 h (80%); Synthesis of **9e** (starting from **8e**): HF-pyridine, THF, rt, 1 h (36%).

5′-DFUR (3), respectively, in order to minimize the release of an active DPD inhibitor in the blood stream and to achieve the delivery of the prodrug to tumor tissues. The enzyme conversion assays were carried out by the same method described in our previous paper.[9]

Table 1 shows the susceptibility of 5′-deoxyuridine derivatives, **6a–6f**, to dThdPase with a value relative to that of 5′-DFUR (**3**). 5-Ethynyl- (**6a**) and 5-iodo- (**6b**) uridine derivatives were 5, and 2.3 times more susceptible to dThdPase than 5′-DFUR, respectively. 5-Vinyl derivative (**6c**) was 5 times less susceptible than 5′-DFUR. 5-Cyano- (**6d**), 5-(3-benzyloxybenzyl)- (**6e**) and 5-(*Z*)-styryl- (**6f**) uridine derivatives were not susceptible to dThdPase. Thus, **6a**, **6b** and **6c** were selected and converted to the corresponding cytidine derivatives (**9a–c**). The compounds (**9a–c**) were then evaluated for the susceptibility to Cyd deaminase.

Table 2 shows the susceptibility of 5′-deoxycytidine derivatives (**9a–c**) to Cyd deaminase, with a value relative to that of 5′-DFCR (**2**). Among them, the susceptibility of 5-iodo derivative (**9b**) and 5-vinyl derivative (**9c**) were more than 0.1 relative to that of 5′-DFCR (2). The 5-ethynyl derivative (**9a**) was the least susceptible among them. Thus, **9b** and **9c** were selected and converted to the corresponding N^4-alkoxycarbonyl-5′-deoxycytidine derivatives (**12b** and **12c**), which were evaluated for susceptibility to carboxylesterase from human liver and intestine.

Table 3a shows the susceptibility of N^4-alkoxycarbonyl-5′-deoxycytidine derivatives (**12b** and **12c**) to carboxylesterase, in which the conversion rates are expressed in ng/mg protein/h. Between these two compounds, 5-vinyl derivative (**12c**: RO0094226) showed a higher selectivity to human liver carboxylesterase over intestinal carboxylesterase (>22 times). The selectivity ratio of **12b** was only 5. The potentiation effect of **12b** on the antitumor activity of capecitabine in human colon cancer xenograft (HCT 116) was inferior to that of **12c** (data not shown). Thus, we selected 5-vinyl derivative (**12c**) for further evaluation of tumor selectivity and potentiation of the antitumor activity of capecitabine in other xenograft models. However, it was found that **12c** was chemically unstable, and easily underwent dimerization through Diels–Alder cyclo-addition reaction between the vinyl group and the diene system composed of the 5,6-double bond and the vinyl side chain even at room temperature.[10] This enabled us to search for another chemically stable prodrug.

We examined the possibility of 2′,3′-di-*O*-acetyl-5′-deoxy-5-vinylcytidine (**11c**: RO0094889) as an alternative prodrug. Interestingly, the crystalline form of **11c** was chemically stable and its stability was better than the corresponding deacetyl derivative (**9c**).[10]

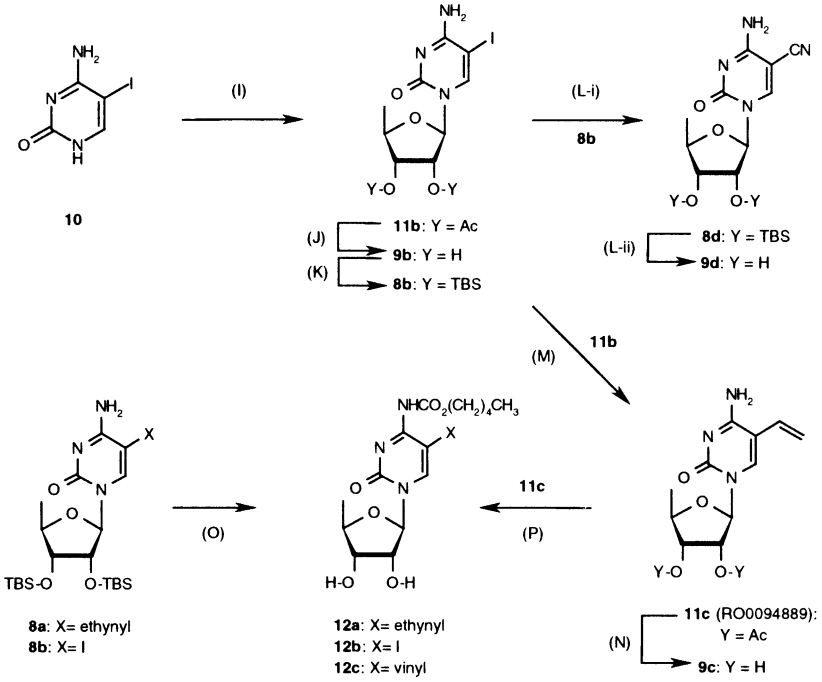
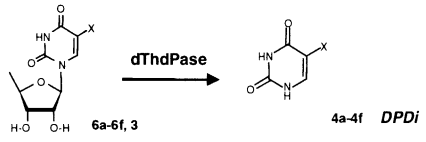
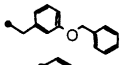


Figure 4. (I) Synthesis of **11b** (starting from **10**): (i) HMDS, toluene, reflux, 3 h; (ii) 1,2,3-tri-*O*-acetyl-5-deoxyribose, $TiCl_4$, CH_2Cl_2, 0 °C, 2 h (78%, two steps); (J) Synthesis of **9b** (starting from **11b**): NaOH, MeOH, 0 °C, 0.5 h (quant); (K) Synthesis of **8b** (starting from **9b**): TBSCl, imidazole, DMF, 60 °C, 3 h (quant); (L) Synthesis of **9d** (starting from **8b**): (i) NaCN, DMF, rt, 24 h; (ii) TBAF, THF, rt, 0.5 h (44% two steps); (M) Synthesis of **11c** (starting from **11b**): vinyltributylstannane, $Pd_2(dba)_3$, tri-2-furylphosphine, DMF, rt, 96 h (86%); (N) Synthesis of **9c** (starting from **11c**): NaOH, MeOH, H_2O, 0 °C, 0.5 h (98%); (O) Synthesis of **12a** (starting from **8a**): (i) *n*-pentyl chloroformate, pyridine, CH_2Cl_2, rt, 2 h; (ii) TBAF, THF, rt, 2 h (58%, two steps); Synthesis of **12b** (starting from **8b**): (i) *n*-pentyl chloroformate, pyridine, CH_2Cl_2, rt, 1 h; (ii) TBAF, THF, rt, 2 h (80%, two steps); Synthesis of **12d** (starting from **8d**): (i) *n*-pentyl chloroformate, pyridine, CH_2Cl_2, rt, 2 h; (ii) TBAF, THF, rt, 3 h (91%, two steps); Synthesis of **12e** (starting from **8e**): (i) *n*-pentyl chloroformate, pyridine, DMAP, CH_2Cl_2, rt, 2 h; (ii) TBAF, THF, rt, 2 h (45%, two steps); (P) Synthesis of **12c** (starting from **11c**): (i) *n*-pentyl chloroformate, pyridine, CH_2Cl_2, rt, 2 h; (ii) K_2CO_3, MeOH, H_2O, rt, 0.5 h (85%, two steps).

Table 1. Susceptibility of 5-substituted uridine derivatives to dThdPase

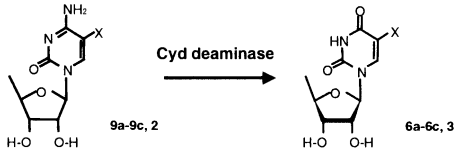


Compd.	X	Relative susceptibility[a] (analogues/5′-DFUR)
6a		5.0
6b	I	2.3
6c		0.20
6d		<0.01
6e		<0.01
6f		<0.01
5′-DFUR (**3**)	F	1

[a]Concn of the substrate: 1.0 mM.

As shown in Table 3b, compound **11c** was also stable in aqueous solution at pH 2.0–7.4 for 2 h (37 °C). The diacetate group of **11c** could be rapidly hydrolyzed by the esterase mainly in the liver after oral absorption since **11c** is 4.6 times more susceptible to human liver enzyme than intestinal enzyme (crude enzyme extract): the susceptibility to human liver and intestinal enzymes were 130 and 28 nmol/mg protein/h, respectively.

Table 2. Susceptibility of 5-substituted cytidine derivatives to Cyd deaminase



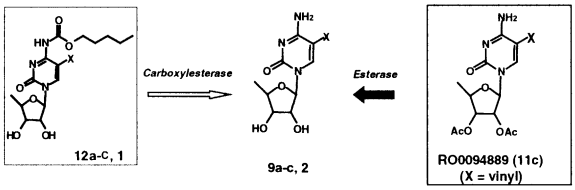
Compd.	X	Relative susceptibility[a] (analogues/5′-DFCR)
9a		0.034
9b	I	0.33
9c		0.14
5′-DFCR (**2**)	F	**1**

[a]Concn of the substrate: 2.0 mM.

The tumor-selective delivery of active DPD inhibitor, 5-VU, by oral RO0094889 in vivo, and tumor-selective increase of 5-FU concentration by its co-administration with capecitabine

The tumor selective delivery of the active DPD inhibitor, 5-vinyluracil was demonstrated by the following experiment in vivo (Fig. 5). RO0094889 (33.7 mg/kg)

Table 3. Susceptibility of N^4-alkoxycarbonyl-5-substituted-5′-deoxycytidine and 2′,3′-*O*-diacetyl-5-substituted-5′-deoxycytidine derivatives to human carboxylesterase and esterase, respectively



(a) (b)

Compd.	X	Susceptibility[a] to carboxylesterase (nmol/mg protein/h)			RO0094889 (**11c**)
		Liver (L)	Intestine (I)	Ratio L/I	● Susceptibility to esterase (nmolmg protein/h)
12b	I	90	18	5	Human liver (130)
12c: RO0094226		110	<5.0	>22	intestine (28)
Capecitabine (1)	F	79	5.3	15	● Stability in serum 61% (remaining % after 1 h)
					● Stability at pH 2.0–7.4 100% (remaining % after 2 h)

[a]Concn of the substrate: 2.5 mM.

and capecitabine (135 mg/kg) given orally to a HT-3 human cervical cancer xenograft generated high 5-VU levels in tumors (2.2 nmol/g) and the local administration site intestine (2.0 nmol/g), but only low levels in other normal tissues studied (<0.6 nmol/g). In contrast, 5-VU itself was not tumor-selective and gave 5-VU at much higher concentrations in the stomach and intestine than in tumors. Since the uridine phosphorylase[11] activity in mouse intestine is known to be 17 times higher than dThdPase activity in human, the exposure of 5-VU at intestine in human is expected to be lower.

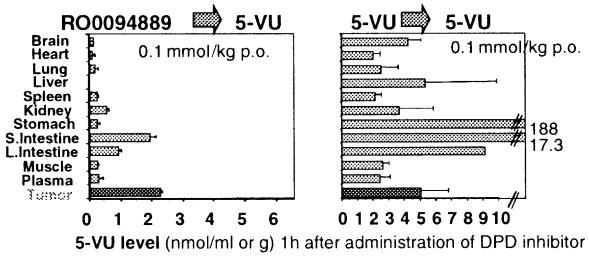


Figure 5. Tumor selective delivery of active DPDi, 5-VU, by RO0094889 (po): (HT-3 human cervical cancer xenograft).

As a result of this tumor-selective delivery of active DPD inhibitor by RO0094889, a significant increase of 5-FU concentration in tumor tissues can be achieved when both capecitabine (**1**) and RO0094889 are given orally (Table 4). Capecitabine (135 mg/kg) alone or in combination with either 5-VU (1.38 mg/kg) or RO0094889 (33.7 mg/kg) was given orally to mice bearing HT-3 human cervical cancer. At various time points, tumor and plasma levels of 5-FU were determined by LC/MS/MS. As seen in Table 4, the combination of RO0094889 and capecitabine significantly increased the Cmax of 5-FU in tumor with only a slight

Table 4. 5-FU levels in tumor and plasma after treatment with capecitabine + 5-vinyluracil versus capecitabine + RO0094889 (HT-3 human cervical cancer xenograft)

Compd	5-FU Cmax (nmol/mL or g)		Ratio T/P
	Tumor (T)	Plasma (P)	
Capecitabine	3.5	0.62	5.6
Capecitabine + 5-vinyluracil	12	3.2	3.8
Capecitabine + RO0094889	*13*	*1.0*	*13*

Capecitabine (135 mg/kg) alone or in combination with either 5-VU (1.38 mg/kg) or RO0094889 (33.7 mg/kg), was given orally. At various time points, tumor and plasma levels of 5-FU were determined by LC/MS/MS.

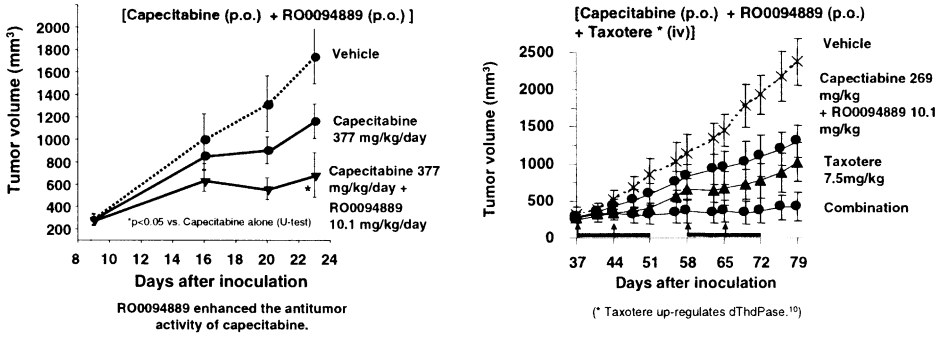


Figure 6. Antitumor activity of capecitabine with RO0094889: (a) NCl-H460 human NSCLC xenograft; (b) Calu-3 human NSCLC xenograft.

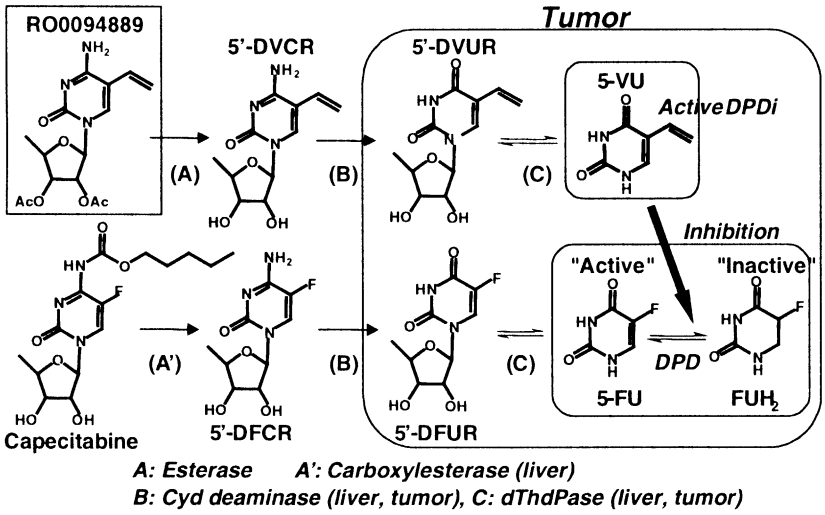


Figure 7. Sequential bioconversion of RO0094889 to 5-VU.

increase of 5-FU in plasma, as compared with administration of capecitabine alone. This selectivity was not observed in the combination of capecitabine with 5-VU.

Enhancement of antitumor activity of capecitabine in combination with RO0094889 in human non-small cell lung carcinoma (NSCLC) xenografts

In the NCI-H460 human NSCLC xenograft, a combination of capecitabine (377 mg/kg) and RO0094889 (10.1 mg/kg) given orally five times a week for 2 weeks resulted in a significant increase of tumor growth inhibition as compared with that of the same dose of capecitabine alone (Fig. 6a).

As mentioned before, antitumor activity of capecitabine is influenced by the ratio of dThdPase and DPD. Therefore, the combination of both dThdPase up-regulator and DPD inhibitor is expected to maximize the antitumor activity of capecitabine as a result of a maximum increase of 5-FU concentration in tumor tissues. Ishitsuka et al. reported taxotere up-regulates dThdPase in tumors.[12] Thus, the following triple combination was investigated (Fig. 6b). In the Calu-3 human NSCLC xenograft, a triple combination of capecitabine (269 mg/kg/day, po), RO0094889 (10.1 mg/kg/day, po) plus taxotere (7.5 mg/kg/weekly, iv) showed more than additive tumor growth inhibition as compared with that of the same dose of capecitabine plus RO0094889 (Fig. 6b).

In conclusion, RO0094889 generates the DPD inhibitor 5-VU preferentially in tumors by the same enzymes as in capecitabine activation. The combination of RO0094889 and capecitabine is therefore expected to give the active compounds selectively in tumor tissues and show better efficacy against tumors that express high DPD levels,[13] such as NSCLC,[14] than capecitabine alone (Fig. 7).

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