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Discovery of an Orally Active and Liver-Targeted Prodrug of 5-Fluoro-2′-Deoxyuridine for the Treatment of Hepatocellular Carcinoma

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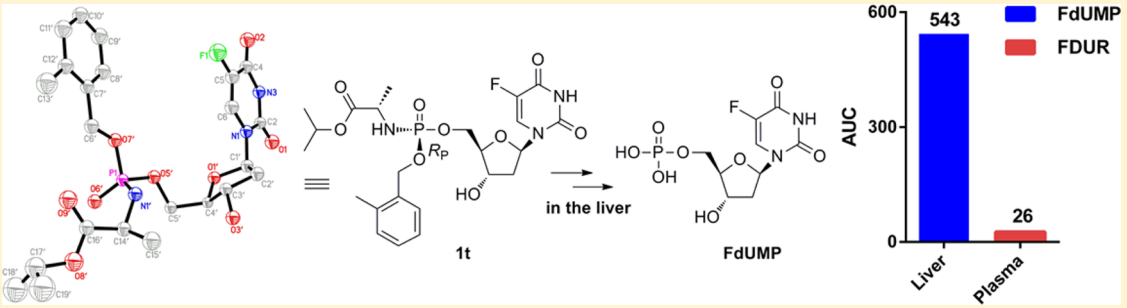
Supporting Information



ABSTRACT: We report a series of novel O-(substituted benzyl) phosphoramidate prodrugs of 5-fluoro-2′-deoxyuridine for the treatment of hepatocellular carcinoma. Through structure optimization, the *o*-methylbenzyl analog (**1t**) was identified as an orally bioavailable and liver-targeted lead compound. This lead prodrug is well-tolerated at a dose up to 3 g/kg in Kuming mice via oral administration. An efficacy study demonstrated that it possesses good inhibitory effect (61.67% and 72.50%, respectively) on tumor growth in a mouse xenograft model. A metabolism study in Sprague−Dawley rats suggested that **1t** can release the desired 5′-monophosphate in the liver with high liver-targeting index.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most lethal cancers and the third most common cause of cancer-related deaths globally with 626,000 new patients per year.[1,2] The current therapeutic options for HCC include resection, transplantation, radiofrequency ablation, chemoembolization, and sorafenib (4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]-carbamoylamino] phenoxy]-*N*-methyl-pyridine-2-carboxamide).[3] However, most HCC cases are diagnosed in their advanced stages, which strictly limits the treatment options.[4] As a multikinase inhibitor, sorafenib is the only systemic therapy for advanced HCC, but its actual benefit is still relatively small.[1] The difficulties associated with chemotherapy in HCC may derive from drug resistance mechanisms, such as rapid drug metabolism via primary and/or secondary metabolism pathways, removal of drugs from tumor cells by drug transport systems, and inactivation of oncolytic drugs by intracellular agents such as glutathione. These mechanisms limit the uptake and retention of the drug molecules in tumor cells and consequently decrease their overall therapeutic benefit.[5]

A major reason for the inefficacy of nucleoside antitumor drugs is that they cannot be efficiently phosphorylated by specific nucleoside kinases.[5,6] On the other hand, nucleoside phosphates, or nucleotides, cannot themselves be used as viable drugs due to their poor chemical stability along with high polarity that vitiates their cellular uptake.[7,8] In order to improve the potency of nucleosides, numerous prodrugs have been designed by masking the monophosphates thus bypassing the first-step phosphorylation, which is rate-limiting. Previously, phosphate and phosphonate prodrugs containing cyclic 1,3-propanyl esters have been reported. These can be activated via cytochrome P450-catalyzed oxidative cleavage reaction in the liver to form higher concentrations of the biologically active nucleoside triphosphate.[6,9−11] However, prodrugs of this type will also produce a potentially toxic byproduct, aryl vinyl ketone, which may cause cytotoxicity and genetic toxicity.[12,13] Aryloxy phosphoramidates, another type of prodrug, can partially avoid the dependence of the current drugs on active transport, saturate efflux transportion and nucleoside kinase-mediated activation,[14,15] and they are also resistant to metabolic deactivation.[15] But once the carboxyl ester is hydrolyzed by esterases in blood and nontargeted tissues, the phenol group in the phosphate moiety is cleaved spontaneously by an

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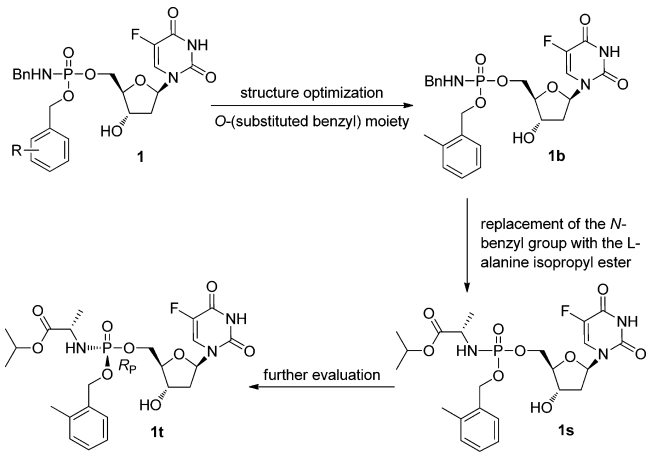
intramolecular nucleophilic attack,[16,17] resulting in an alanyl phosphate metabolite, which is a very polar diacid, and will be poorly transported across cell membranes to the targeted tissues. In addition, studies have shown that although teratogenic effects have not been associated with exposure to phenol, high doses of phenol are fetotoxic.[18]

This report describes the development of novel phosphoramidate prodrugs of 5-fluoro-2′-deoxyuridine (FDUR) for the treatment of HCC by oral administration. FDUR itself is of particular value in the clinical treatment of liver cancer,[19−21] but like most nucleosides, it is inactive and requires phosphorylation to the primary bioactive entity, the 5′-monophosphate (FdUMP).[15] FdUMP acts as a potent suicide-type inhibitor of thymidylate synthase, a key enzyme in DNA synthesis, which prompts a potent toxic event in the cell.[22] In order to reach the therapeutic goal and minimize systemic toxicities, FDUR in clinical practice was administered via hepatic arterial infusion (HAI) to provide direct drug delivery into the tumor feeding vessels.[20,21] Unfortunately, many liver cancer patients cannot tolerate a catheter inserted into the hepatic artery and a pump implanted under the skin.

In this paper, we report the design and synthesis of a series of novel phosphoramidate prodrugs of FDUR bearing O-(substituted benzyl) moieties (Scheme 1). Through structure optimization, the *O*-(*o*-methylbenzyl) analog (**1t**) was identified as an orally bioavailable lead compound which targets the liver.[23] Metabolism studies in Sprague−Dawley rats demonstrated that this phosphoramidate prodrug can release the desired FdUMP in the liver with a high liver-targeting index and thus minimize extra hepatic exposure. The acute toxicity and efficacy of compound **1t** were also assessed in Kunming (KM) mice and in a mouse xenograft model.

Scheme 1. Lead Optimization of the O-(Substituted Benzyl) Phosphoramidate Prodrugs of FDUR



2. CHEMISTRY

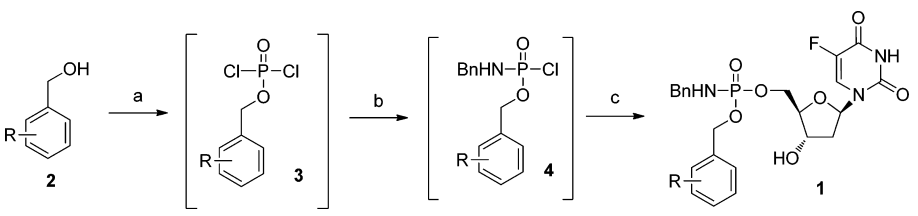
Prodrug **1** was synthesized as outlined in Scheme 2. Treatment of a benzyl alcohol (**2**) with phosphoryl oxychloride ($POCl_3$) in the presence of triethylamine gave the phosphorodichloride (**3**), which is further treated with benzylamine to provide the chlorophosphoramidate (**4**). Finally, the reaction of **4** with FDUR (**5**) in the presence of *N*-methylimidazole (NMI) afforded the desired prodrug **1a−r**. The analog **1s** was synthesized by using L-alanine isopropyl ester hydrochloride in place of benzylamine (Scheme 3).

To synthesize the R_P-enantiomer (**1t**) of the racemic prodrug **1s**, (S_P)-*O*-perfluorophenyl phosphoramidate **7** was prepared by the procedure used for the synthesis of **1s**. Treatment of phosphorochloridate **4s** with pentafluorophenol gave diastereomers **6** with the S_P-enantiomer as the major isomer. The enantiomer **7** can be obtained through recrystallization from a mixture of diisopropyl ether and petroleum ether (Scheme 3). Selective protection of the 5′- and 3′-hydroxy groups in the nucleoside **5** with DMTrCl and TBSCl, respectively, gave intermediate **9**, which was treated with TFA to provide the 3′-TBS protected nucleoside (**10**). Reaction of compound **10** with intermediate **7** in the presence of *t*-butyl magnesium chloride, followed by recrystallization in a mixture of ethanol and water afforded prodrug **1t** (Scheme 4) as the pure R_P-enantiomer (confirmed by X-ray, see the Supporting Information).

3. RESULTS AND DISCUSSION

3.1. In Vitro Evaluation and Lead Optimization. First, we performed the optimization of the *O*-benzyl moiety in the newly designed prodrug structure. In view of the poor stability of the amino acid ester moiety in rodents,[24] to blood circulating esterases, we initially synthesized the prodrugs (**1a−r**) containing a benzylamine group for optimization. These *N*-benzyl phosphoramidate prodrugs are all quite stable in rat plasma ($t_{1/2}$ > 24 h), the single exception being the *O*-(*o*-methoxy)benzyl analog (**1e**) for which $t_{1/2}$ = 3.4 ± 0.5 h. The activation rate in vitro of the prodrug analogs (**1a−r**) in rat liver S9 were determined by measurement of the formation of FdUMP, the primary bioactive entity, upon incubation for 0, 10, 20, 40, or 60 min with 50 μM of the tested compounds. As shown in Table 1, the unsubstituted *O*-benzyl analog (**1a**) showed a moderate activation rate for the formation of FdUMP. In most cases, incorporation of substituents in the benzene ring of the *O*-benzyl group resulted in higher rate of activation. Generally, compounds bearing electron-donating groups showed better activation rates than those with electron-withdrawing groups, and ortho-substituted analogs possess slightly better activation rates than the meta- and para-

Scheme 2. Synthesis of O-(Substituted Benzyl) Phosphoramidate Prodrugs (1)[a−c]



[a]$POCl_3$, Et_3N, CH_2Cl_2, −78 °C. [b]$BnNH_2$, Et_3N, DCM, −78 °C to rt. [c]FDUR (5), NMI, CH_2Cl_2, rt, overnight.

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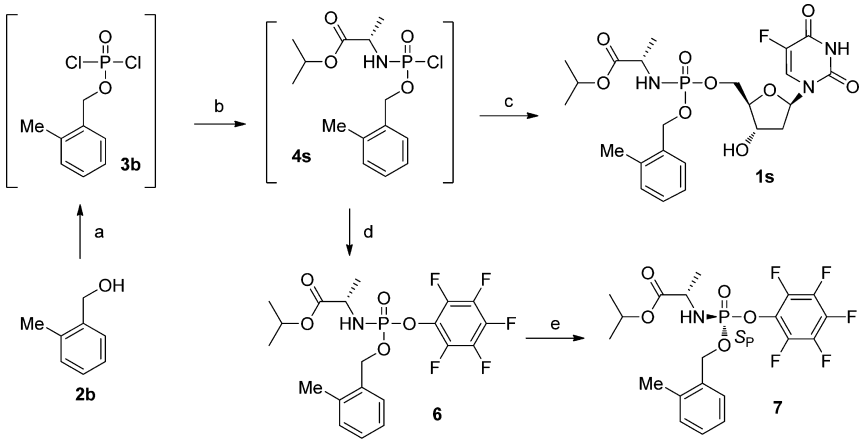
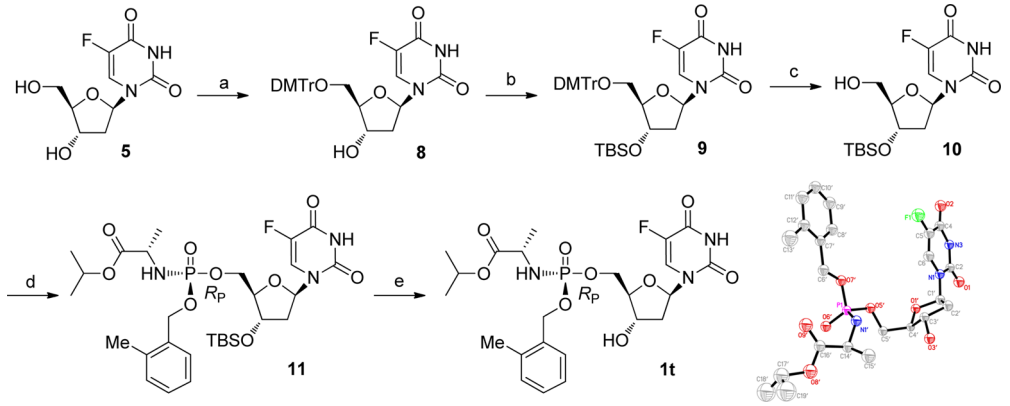
Scheme 3. Synthesis of *O*-(*o*-Methylbenzyl) Phosphoramidate Prodrug (1s) and (S_P)-*O*-Perfluorophenyl Phosphoramidate (7)[a−e]



[a]$POCl_3$, Et_3N, CH_2Cl_2, −78 °C. [b]L-alanine isopropyl ester hydrochloride, Et_3N, DCM, −78 °C to rt. [c]**5**, Et_3N, CH_2Cl_2, rt. [d]pentafluorophenol, Et_3N, CH_2Cl_2, rt. [e]recrystallization in a mixture of diisopropyl ether and petroleum ether.

Scheme 4. Synthesis of the (S_P)-*O*-(*o*-Methylbenzyl) Phosphoramidate Prodrug 1t[a−e]



[a]DMTrCl, Py., 0 °C. [b]TBDMSCl, imidazole, DCM, rt. [c]TFA, DCM, rt. [d]**7**, *t*-BuMgCl/THF, 0 °C to rt. [e]TBAF/THF, rt.

substituted analogs. Cytostatic evaluation in vitro of these prodrugs with the SMMC7721 cell line identified the *o*-methylbenzyl (**1b**) and *o*-methoxybenzyl analogs (**1e**) with potent inhibitory activities. Due to the lower stability of the *o*-methoxybenzyl group (**1e**) in plasma, the *o*-methylbenzyl substituent (**1b**) was selected as the optimal structure design.

Replacement of the *N*-benzyl group in compound **1b** with the L-alanine isopropyl ester moiety led to the phosphoramidate **1s**. Although replacement of the *N*-benzyl group with the L-alanine isopropyl ester moiety decreased the in vitro activity, study of the tissue distribution indicated that the amino acid ester analog possesses a much higher liver-targeting index (see Section 3.4). Consistent with the previous data,[24] both the racemic prodrug **1s** and its R_P-enantiomer **1t** have a short half-life (<5 min) in rat plasma but good stability in human and dog plasma ($t_{1/2}$ > 24 h, Table 2). Further evaluation demonstrated that prodrug **1t** exhibited equally good inhibitory activity with the liver cancer cell line (SMMC7721) and slightly lower toxicity on a normal liver cell line (LO2) than its racemic form **1s**. Besides, considering its superiority in synthesis and purification (see Section 5.1), we focused our further development efforts on this R_P-phosphoramidate prodrug (**1t**).

3.2. Assessment of Acute Toxicity in KM Mice. Prior to the investigation of the in vivo anti-HCC efficacy, an acute toxicity study of the prodrug (**1t**) was conducted in KM mice. A single oral dose of the test compound at 2 and 3 g/kg was administered to the mice, and this was followed by observation for 15 days. During the study period, all mice remained alive, and no significant side effects were observed at either dose. Only negligible weight loss occurred at 1−5 days post-treatment (Figure 1). All the animals were sacrificed on day 15, and no damage to the heart, liver, spleen, lung or kidney was observed. These results demonstrated that orally administered prodrug **1t** was well-tolerated in KM mice at dosage of up to 3 g/kg in the course of treatment and during the post-treatment period.

3.3. Assessment of Efficacy in KM Mice. For the assessment of the antitumor activity in vivo, a mouse xenograft model established with H22 cells in KM mice was employed. The tumor-bearing mice were dosed daily by oral gavage with prodrug **1t** at 65, 130, or 260 mg/kg or sorafenib as the positive control (60 mg/kg) for 8 days (for detailed dose optimization see Section 5.7). The tumor weight was measured on day 8 and is given in Table 3. Compared to the control groups, the tested compound (**1t**) exhibited good inhibitory effect on the tumor growth. In particular, prodrug **1t** at the dose of 130 or 260 mg/kg significantly reduced tumor size with inhibitory rates of 61.67% and 72.50%, respectively, which are equally or more

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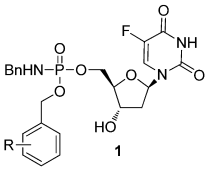
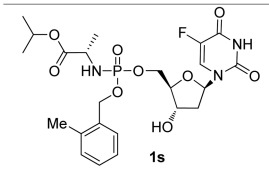
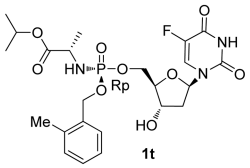
Table 1. Stability, Activation, and Cytostatic Activity of Prodrugs 1a–r[a]

compound			rat plasma $t_{1/2}$ (h)	rate of activation in rat liver S9 (nmol/min/mg)	SMMC7721 cell line IC_{50} (μM)
		R =			
	**1a**	H	> 24	17.4 ± 0.4	365 ± 51
	1b	*o*-Me	> 24	58.4 ± 4.6	18.6 ± 2.4
	1c	*m*-Me	> 24	49.4 ± 3.0	139 ± 10
	1d	*p*-Me	> 24	53.5 ± 2.6	31.7 ± 1.9
	1e	*o*-OMe	3.4 ± 0.5	238.9 ± 8.5	6.8 ± 0.7
	1f	*m*-OMe	> 24	118.7 ± 19.5	386 ± 4
	1g	*o*-F	> 24	34.7 ± 0.4	155 ± 10
	1h	*m*-F	> 24	12.5 ± 1.3	241 ± 32
	1i	*p*-F	> 24	26.3 ± 1.8	150 ± 7
	1j	*o*-Cl	> 24	29.2 ± 2.8	132 ± 11
	1k	*m*-Cl	> 24	22.6 ± 1.9	206 ± 15
	1l	*p*-Cl	> 24	25.6 ± 2.0	133 ± 6
	1m	*o*-Br	> 24	49.6 ± 2.5	151 ± 4
	1n	*m*-Br	> 24	32.8 ± 0.2	225 ± 3
	1o	*p*-Br	> 24	39.1 ± 2.7	107 ± 5
	1p	*o*-NO_2	> 24	44.7 ± 0.7	> 500
	1q	*m*-NO_2	> 24	14.9 ± 0.2	ND
	1r	*p*-NO_2	> 24	40.0 ± 1.2	> 1000

[a]Data are the means (±SD) of triplicate samples.

Table 2. Stability and Cytostatic Activity of Racemic Prodrug (1s) and Its R_P-Enantiomer (1t)[a]

compound	rat plasma $t_{1/2}$ (min)	human plasma $t_{1/2}$ (h)	dog plasma $t_{1/2}$ (h)	SMMC7721 IC_{50} (μM)	LO2 CC_{50} (μM)
 **1s**	< 5	> 24	> 24	276 ± 16	333 ± 34
 **1t**	< 5	> 24	> 24	250 ± 38	412 ± 28

[a]Data are the means (±SD) of two independent experiments, with each one performed in triplicates.

effective compared to sorafenib at the dose of 60 mg/kg (effective dose).[25] Over the course of this 8 day study, treatment of **1t** did not cause significant toxicity in any of the three dosed groups (Table 3).

3.4. Assessment of Tissue Distribution in Sprague–Dawley Rats. Tissue distribution study of prodrug **1t** and its *N*-benzyl analog **1b** was conducted in Sprague–Dawley rats to assess their liver-targeting indexes, compared to those of FDUR. Intragastric gavage (i.g.) administration of 52.17 mg/kg (0.096 mmol/kg) of **1t** and 49.87 mg/kg (0.096 mmol/kg) of **1b** produced 543 ng/g·h and <155 ng/g·h of FdUMP in the liver, respectively, while equimolar doses of FDUR (23.62 mg/kg) resulted in a very low level of the monophosphate (Table 4, Figure 2). Conversely, only 26 ng/mL·h and 41 ng/mL·h of

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J. Med. Chem. XXXX, XXX, XXX–XXX

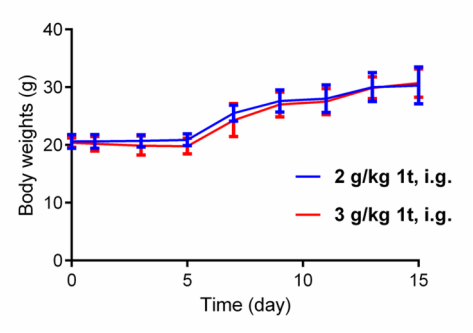

Figure 1. Mean body weight change of KM mice from day 1 to 15 after oral administration of prodrug **1t** at a single dose of 2 and 3 g/kg.

FDUR were determined in the plasma of **1t**- and **1b**-treated rats, respectively, whereas 308 ng/mL·h was observed in that of FDUR-treated animals. These results translate to a liver-targeting index ($AUC_{(inf)}$ FdUMP (liver)/$AUC_{(inf)}$ FDUR (plasma)) of 20.9 for **1t**, <3.8 for **1b**, and <0.11 for FDUR. Overall, the increase in the targeting index for **1t** over FDUR is therefore >190-fold.

In addition, much higher levels of the primary metabolite **A** (see Scheme 5) were observed in the liver than in the plasma of **1t**-dosed rats (Table 4). The $AUC_{(inf)}$ of **A** in the liver was 5509 ng/g·h, while the value in the plasma was only 316 ng/mL·h (a ratio of ~17.4:1). Formation of FDUR was also a liver-selective distribution, with $AUC_{(inf)}$ of 1541 ng/g·h in the liver but only 26 ng/mL·h in the plasma (a ratio of ~59.3:1). Moreover, no detectable amounts of FdUMP and 5-fluorouracil (5-FU) were observed in the plasma upon administration of **1t**, while a high level of 5-FU was formed in the plasma of FDUR-dosed aniamals. The liver-targeting release of the bioactive FdUMP and related metabolites can significantly reduce their exposure in nontargeted tissues, therefore diminishing the overall toxicity in vivo.

3.5. Prodrug Activation Mechanism. A putative mechanism for the activation of **1t** and formation of the bioactive monophosphate (FdUMP) in the liver is proposed and is shown in Scheme 5. The metabolism of this prodrug starts with (a) the hydrolysis of the carboxylic ester moiety by a carboxyesterase-type enzyme to give a stable intermediate **A**.[17] Then, (b) a plausible metabolite B[26] might be formed via a cytochrome P450-catayzed oxidative hydroxylation at the benzylic position.[6,9] Subsequently, (c) the benzyl moiety is cleaved via hydrolysis to produce a polar diacid intermediate **C** and 2-methylbenzaldehyde which will be further oxidized by aldehyde dehydrogenase to a nontoxic metabolite, 2-methyl-benzoic acid.[27] Finally, (d) cleavage of the P−N bond of **C** mediated by the histidine triad nucleotide-binding protein 1 (Hint1)[17] affords the desired monophosphate, FdUMP.

To prove the above putative mechanism, we carried out a metabolism study of prodrug **1t** in the presence of rat liver S9. Consistent with the previous results in rat plasma (see Table 2), most of the test compound was hydrolyzed to intermediate **A** within 20 min (Figure 3), and subsequently, the mono-phosphate FdUMP is formed. Then the level of this bioactive entity increased, and the concentration of compound **A** decreased both in a time-dependent manner (Figure 3). However, only a high concentration of **A** and no FdUMP were determined in the negative group without NADPH, indicating that P450-catalyzed oxidation reactions were involved in this debenzylation step. In addition, HPLC-MS/MS analysis of the liver and plasma samples indicated that the metabolite 2-methylbenzoic acid was further conjugated with glycine to form nontoxic *o*-methylhippuric acid[27] (m/z 192 → 148, also confirmed by running a standard sample). Overall, during this metabolism process (Scheme 5), only 2-methylbenzaldehyde possesses little acute toxicity,[28] most of which will be eventually excreted as *o*-methylhippuric acid in the urine.[27,8]

4. CONCLUSIONS

We have designed and synthesized a series of novel O-(substituted benzyl) phosphoramidate prodrugs of FDUR for the treatment of hepatocellular carcinoma. Evaluation in vitro allowed identification of the *o*-methylbenzyl analog (**1t**) which is orally bioavailable and liver targeted as a lead compound. Prodrug **1t** is well-tolerated upon oral administration at a dose up to 3 g/kg in KM mice. During an 8 day efficacy study, this lead prodrug (130 and 260 mg/kg/d) exhibited a good inhibitory effect on tumor growth (61.67% and 72.50%, respectively) in a mouse xenograft model. More importantly, a metabolism study in Sprague−Dawley rats demonstrated that this phosphoramidate prodrug can release the desired FdUMP in the liver with a high liver-targeting index. Further application of this prodrug strategy to discover more prodrugs of nucleosides for the treatment of the corresponding diseases is currently ongoing in our laboratory.

5. EXPERIMENTAL SECTION

5.1. Chemistry. ^{1}H NMR spectra were recorded on a 400 MHz spectrometer, and ^{13}C NMR spectra were recorded on a 100 MHz spectrometer. Chemical shift values are given in ppm and referred to an internal standard of tetramethylsilane. The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; dd, doublet of doublets; and dt, doublet of triplets. The coupling constants (J) are reported in Hertz (Hz). Optical rotations were measured on a PerkinElmer Polarimeter 341 in a 10 cm cuvette at 20 °C at 589 nm. The concentration (c) is given in g/100 mL. Flash

Table 3. Effect of Tumor Growth in H22 Tumor-Bearing Mice after Treatment for 8 Days[a]

groups	dosage (mg/kg/d)	body weight (g)		tumor weight (g)	inhibition rate (%)
		pretreatment	post-treatment		
control	–	22.1 ± 1.0	29.4 ± 2.4	1.20 ± 0.40	–
1t	65	21.7 ± 1.2	25.9 ± 3.6	0.67 ± 0.32[b]	44.17
1t	130	22.3 ± 1.1	23.8 ± 3.1	0.46 ± 0.24[b]	61.67
1t	260	22.3 ± 1.4	22.3 ± 2.1	0.33 ± 0.17[b,c]	72.50
sorafenib	60	22.1 ± 0.8	26.4 ± 1.8	0.59 ± 0.20[b]	50.83

[a]Data shown are means ± SD of tumor weights and mouse body weights for each group of mice (n = 10). [b]P < 0.01 versus control group. [c]P < 0.05 versus sorafenib group.

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J. Med. Chem. XXXX, XXX, XXX−XXX

Table 4. Comparative Tissue Distribution in Rat after Oral Administration of FDUR, 1b, and 1t

		plasma		liver	
compds	detected compound	C_{max} (ng/mL)	$AUC_{(inf)}$ [a] (ng/mL·h)	C_{max} (ng/g)	$AUC_{(inf)}$ (ng/g·h)
FDUR	FDUR	180	308	ND[b]	ND
	5-FU	169	209	60	232
	FdUMP	ND	ND	9	<34[c]
1b	FDUR	40	41	50	838
	5-FU	14	ND	60	ND
	FdUMP	ND	ND	55	<155[c]
1t	metabolite **A**	234	316	3673	5509
	FDUR	17	26	747	1541
	5-FU	ND	ND	139	257
	FdUMP	ND	ND	340	543

[a]$AUC_{(inf)}$, area under the curve from time zero to infinity. [b]ND, not detected. [c]Lower limit of quantitation and the $AUC_{(inf)}$ were calculated on the basis of the limit of quantification.

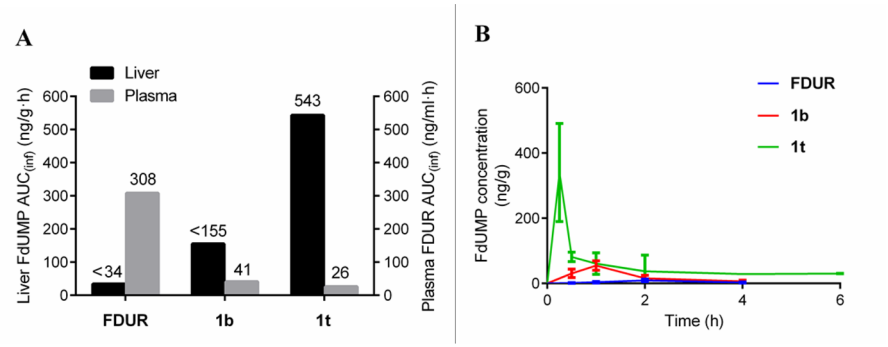
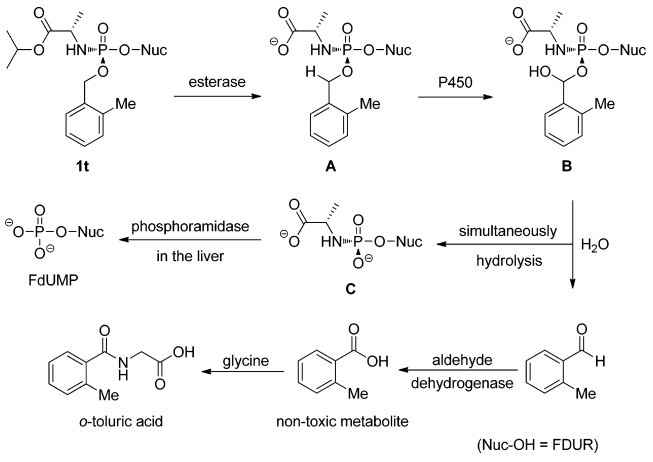


Figure 2. (A) Liver FdUMP $AUC_{(inf)}$ and plasma FDUR $AUC_{(inf)}$ after treating rats with equimolar FDUR (23.62 mg/kg, i.g.), **1b** (49.87 mg/kg, i.g.), and **1t** (52.17 mg/kg, i.g.). (B) Time course of liver FdUMP levels after i.g. administration of equimolar FDUR (23.62 mg/kg), **1b** (49.87 mg/kg), and **1t** (52.17 mg/kg).

Scheme 5. Proposed Mechanism for the Activation of Prodrug 1t



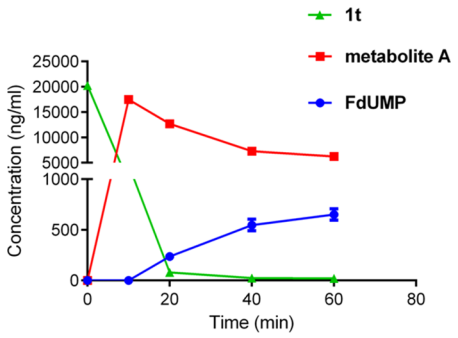


Figure 3. Concentration−time curve of prodrug **1t**, its metabolite **A**, and FdUMP during incubation of **1t** (20 μg/mL) with rat liver S9 (10 mg/mL).

column chromatography was performed over 200−300 mesh silica gel. High-resolution mass spectra (HRMS-ESI) were obtained on a Q-TOF mass spectrometer. All the compounds tested possess a purity of at least 95%. Analytical HPLC was run on a Thermo Fisher ACCELA HPLC system equipped with a Phenomenex Luna C8 column (150 mm × 4.6 mm, 5 μm) and UV detection at 267 nm. The mobile phase consisted of MeOH:H_2O = 68:32 (v/v). The flow rate was set at 0.80 mL/min, and the column temperature was maintained at 30 °C.

5.1.1. General Procedure for the Synthesis of Prodrug ***1a−r.*** At−78 °C, to a solution of phosphoryl chloride (307 mg, 2 mmol) in anhydrous CH_2Cl_2 (6 mL) was added a solution of substituted benzyl alcohol (2 mmol) and triethylamine (202 mg, 2 mmol) in anhydrous CH_2Cl_2 (2 mL) dropwise. After having been stirred at the same temperature for 3 h, the reaction mixture was treated slowly with a mixture of benzylamine (214 mg, 2 mmol) and triethylamine (202 mg, 2 mmol) in anhydrous CH_2Cl_2 (2 mL). It was stirred at −78 °C for another 1 h and then allowed to warm up to room temperature over 1 h. After being chilled to 0 °C, the above mixture was further treated with a solution of FDUR (98 mg, 0.4 mmol) and NMI (164 mg, 2 mmol) in anhydrous CH_2Cl_2 (1 mL) and stirred overnight at 0 °C. The reaction was quenched with H_2O (10 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layer was washed with 0.5 M dilute HCl (20 mL) and brine (20 mL) in sequence. After drying over anhydrous Na_2SO_4, it was concentrated and then purified through silica gel column chromatography to afford the desired products **1a−r**.

5.1.1.1. ((2R,3S,5R)-5-(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl 2-methylbenzyl

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J. Med. Chem. XXXX, XXX, XXX−XXX

*benzylphosphoramidate (**1b**).* White solid; yield, 33%; ^{1}H NMR (400 MHz, CD_3OD): δ 7.82−7.80 (m, 1H), 7.34−7.15 (m, 9H), 6.22−6.19 (m, 1H), 5.11−4.98 (m, 2H), 4.35−4.31 (m, 1H), 4.20−4.02 (m, 5H), 2.34 (s, 3H), 2.29−2.21 (m, 1H), 2.07−1.97 (m, 1H); HRMS (m/z) $[M + Na]^+$ calcd for $C_{24}H_{27}FN_3O_7PNa$ 542.1463, found 542.1439.

*5.1.2. Synthesis of Prodrug **1s**.* At−78 °C, to a solution of phosphoryl chloride (307 mg, 2 mmol) in anhydrous CH_2Cl_2 (6 mL) was added a solution of 2-methylbenzyl alcohol (245 mg, 2 mmol) and triethylamine (202 mg, 2 mmol) in anhydrous CH_2Cl_2 (2 mL) dropwise. After being stirred at the same temperature for 3 h, the reaction mixture was treated with amino acid ester hydrochloride (2 mmol) in one portion, followed by the addition of triethylamine (405 mg, 4 mmol) dropwise. It was stirred at −78 °C for another 1 h and then allowed to warm up to room temperature over 1 h. After chilling to 0 °C, the above mixture was further treated with a solution of FDUR (98 mg, 0.4 mmol) and NMI (164 mg, 2 mmol) in anhydrous CH_2Cl_2 (1 mL) and stirred overnight at 0 °C until TLC indicated that the FDUR was completely consumed. The reaction was quenched with H_2O (10 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layer was washed with 0.5 M dilute HCl (20 mL) and brine (20 mL) in sequence. After dried over anhydrous Na_2SO_4, it was concentrated and then purified through silica gel column chromatography to afford the desired product **1s** as colorless semisolid. Yield, 24%; ^{1}H NMR (400 MHz, CD_3OD): δ 7.85−7.83 (m, 1H), 7.37−7.32 (m, 1H), 7.24−7.15 (m, 3H), 6.25−6.20 (m, 1H), 5.13−5.07 (m, 2H), 4.99−4.92 (m, 1H), 4.42−4.35 (m, 1H), 4.24−4.16 (m, 2H), 4.06−4.03 (m, 1H), 3.85−3.77 (m, 1H), 2.37−2.35 (m, 3H), 2.31−2.24 (m, 1H), 2.17−2.10 (m, 1H), 1.36−1.32 (m, 3H), 1.23−1.19 (m, 6H); HRMS (m/z) $[M + Na]^+$ calcd for $C_{23}H_{31}FN_3O_9PNa$ 566.1674, found 566.1676.

*5.1.3. Preparation of (S_P)-O-Perfluorophenyl Phosphoramidate **7**.* At−78 °C, to a solution of phosphoryl chloride ($POCl_3$, 13.9 g, 0.091 mol) in anhydrous CH_2Cl_2 (150 mL) was added dropwise a solution of 2-methylbenzyl alcohol (**2b**, 11.1 g, 0.091 mol) and triethylamine (12.6 mL, 0.091 mol) in anhydrous CH_2Cl_2 (18 mL). After being stirred at the same temperature for 3 h, the reaction mixture was treated with L-alanine isopropyl ester hydrochloride (15.2 g, 0.091 mol) in one portion, followed 15 min later by the addition of a solution of triethylamine (26.4 mL, 0.190 mol) in anhydrous CH_2Cl_2 (17 mL) dropwise. It was stirred at −78 °C for another 1 h and then allowed to warm up to room temperature over 1 h. The above mixture was further treated with a premixed solution of pentafluorophenol (10.0 g, 0.054 mol) and triethylamine (15.1 mL, 0.109 mol) in anhydrous CH_2Cl_2 (15 mL) and stirred overnight at room temperature. The solid was collected by filtration, and the filtrate was concentrated. The resulting residue and collected solid were combined and partitioned between EtOAc (100 mL) and H_2O (60 mL). The organic layer was separated, and the aqueous layer was extracted with EtOAc (2 × 30 mL). The combined organic layer was washed with brine (80 mL), dried over anhydrous Na_2SO_4, and then concentrated. The given residue was treated with a mixture of diisopropyl ether (60 mL) and petroleum ether (120 mL) and heated to reflux. After the entire solid was dissolved, it was cooled and kept at room temperature for 2 days. Pure product **7** (11.7 g, S_P:R_P > 10:1) was obtained as white solid by filtration. The filtrate was concentrated and purified through silica gel chromatography (EtOAc/PE = 1:7−1:5) to afforded product **7** as a mixture of S_P:R_P isomers (~1.3:1), which was further recrystallized in a mixture of diisopropyl ether (30 mL) and petroleum ether (60 mL) to provide pure product **7** (4.1 g, S_P:R_P > 10:1) as white solid. The overall yield was 60%.

*5.1.4. Preparation of 3′-TBS Protected Nucleoside **10**.* At 0 °C, a solution of compound **5** (10.0 g, 0.041 mol) in anhydrous pyridine (100 mL) was treated with DMTrCl (27.5 g, 0.081 mol), stirred for 5 h until TLC indicated that the reaction was complete, and then quenched with H_2O (5 mL). The reaction mixture was concentrated, treated with H_2O (150 mL), and extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with brine (100 mL), dried over anhydrous Na_2SO_4, and concentrated to afford intermediate **8** as thick oil. A solution of compound **8** obtained above in anhydrous CH_2Cl_2 (100 mL) was treated with imidazole (11.0 g, 0.162 mol) and TBSCl (15.3 g, 0.102 mol) in sequence and then stirred at room temperature for 2 h (TLC indicated that the reaction was complete). The reaction was quenched with H_2O (5 mL), concentrated, treated with H_2O (150 mL), and extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with brine (100 mL), dried over anhydrous Na_2SO_4, and concentrated. The given residue was purified through silica gel chromatography (EtOAc/PE = 1:3) to afford intermediate **9** as yellow thick oil. To a solution of compound **9** in anhydrous CH_2Cl_2 (200 mL) was added TFA (12 mL) slowly. The color of the reaction mixture turned to red, then the reaction was stirred at room temperature until disappearance of compound **9**, as indicated by TLC. It was quenched with MeOH dropwise, until the color turned to orange, followed by the addition of aqueous ammonia until the mixture became nearly colorless. The resulting mixture was concentrated and then purified through silica gel chromatography (EtOAc/PE = 1:2) to afford intermediate **10** (11.8 g, 81% over three steps).

*5.1.5. Synthesis of Prodrug **1t**.* Compounds **10** (5.0 g, 13.9 mmol) and **7** (10.0 g, 20.8 mmol) were mixed in a 250 mL round-bottom flask and dried under vacuum at 50 °C for 15 min. Then the mixture was dissolved in anhydrous THF (50 mL), cooled to 0 °C, and treated with *t*BuMgCl (1 M in THF, 27.8 mL, 27.8 mmol). After addition it was stirred at room temperature for 5 h until TLC indicated that the reaction was complete. The reaction mixture was concentrated and then purified through silica gel chromatography ($MeOH/CH_2Cl_2$ = 1:40) to afford intermediate **11** as light-yellow thick oil. A solution of compound **11** in anhydrous THF (150 mL) was treated with TBAF (1 M in THF, 20.8 mL, 20.8 mmol) and stirred at room temperature for 4 h, until TLC indicated that the reaction was complete. The reaction mixture was concentrated and then purified through silica gel chromatography ($MeOH/CH_2Cl_2$ = 1:30) to afford product **1t** (6.8 g, 91% over two steps) as colorless solid. Enantiopure compound **1t** was obtained as white solid through recrystallization in a mixture of EtOH and H_2O (1:2.5). Mp 107−108 °C; $[\alpha]_D^{20}$ = +14 (c = 0.2, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD): 7.82 (d, J = 6.4 Hz, 1H), 7.34 (d, J = 7.6 Hz, 1H), 7.23−7.13 (m, 3H), 6.22−6.18 (m, 1H), 5.10 (d, J = 7.6 Hz, 2H), 4.96 (heptet, J = 6.4 Hz, 1H), 4.35 (quint, J = 4.0 Hz, 1H), 4.21−4.1 (m, 2H), 4.03−4.00 (m, 1H), 3.83−3.75 (m, 1H), 2.35 (s, 3H), 2.29−2.23 (m, 1H), 2.15−2.08 (m, 1H), 1.31 (dd, J = 7.2, 0.8 Hz, 3H), 1.20 (dd, J = 6.4, 1.6 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 173.3 (J_{C-P} = 4.8 Hz), 157.9 (J_{C-F} = 26.2 Hz), 149.2, 140.8 (J_{C-F} = 232.3 Hz), 136.6, 134.1 (J_{C-P} = 7.3 Hz), 129.9, 128.45, 128.38, 125.6, 124.3 (J_{C-F} = 34.2 Hz), 85.4, 85.2 (J_{C-P} = 7.8 Hz), 70.5, 68.7, 66.6 (J_{C-P} = 5.1 Hz), 65.8 (J_{C-P} = 5.4 Hz), 50.1 (J_{C-P} = 1.2 Hz), 39.4, 20.54, 20.48, 19.1 (J_{C-P} = 6.8 Hz), 17.5; IR (KBr) 3414, 2983, 1701, 1465, 1267, 1202, 1107, 1000, 894, 746; HRMS (m/z) $[M + Na]^+$ calcd for $C_{23}H_{31}FN_3O_9PNa$ 566.1674, found 566.1672.

5.2. Animals. Female KM mice (20 ± 2 g) and male Sprague−Dawley rats (220 ± 20 g) were purchased from Hunan SLAC laboratory animal Co. LTD (Hunan, China). The animals were maintained in a specific pathogen free (SPF) environment with a 12 h light/dark cycle at 20−26 °C with a relative humidity of 40−70% and received sterilized food and water freely available. All animals were treated according to the procedures outlined in the Guide for the Care and Use of Laboratory Animals (P. R. China), and experimental procedures were approved by the Animal Ethics Committee of Zhengzhou University.

5.3. Stability Assay in Plasma. *5.3.1. Stability of Prodrugs **1a**−**r**.* Stability of prodrugs **1a**−**r** in rat plasma was carried out by adding 4 μL of a 2.5 mM solution to 96 μL of rat plasma, which was incubated at 37 °C. At the desired times (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h), the reaction was stopped by the addition of 400 μL MeOH and centrifuged at 14000 rpm for 10 min at room temperature. Then 10 μL supernatant was analyzed by HPLC on a Thermo Fisher ACCELA HPLC system equipped with a Phenomenex Luna C8 column (150 mm × 4.6 mm, 5 μm) and UV detection at 267 nm. The mobile phase consisted of MeOH:H_2O = 68:32 (v/v). The flow rate was set at 0.80 mL/min, and the column temperature was maintained at 30 °C.

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J. Med. Chem. XXXX, XXX, XXX−XXX

5.3.2. Stability of Prodrugs 1s–t. A stock solution of prodrug **1t** (or **1s**) in MeOH was prepared at a concentration of 4 mg/mL, and a 25 μg/mL working solution was obtained by further dilution of the stock solutions with H_2O. 96 μL of rat (or dog, or human) plasma was used for the test. The reaction was started by adding 4 μL of a 25 μg/mL solution to give a final concentration of 1 μg/mL and incubated at 37 °C. At the desired times (0, 5, 10, 15, 20, and 25 min for rat plasma and 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h for dog and human plasma), the reaction was stopped by the addition of 400 μL ice-cold MeOH containing internal standard and centrifuged at 14000 rpm for 10 min at room temperature. Then 400 μL supernatant was evaporated to dryness under a stream of N_2. The residue was reconstituted with 100 μL H_2O, briefly vortexed, and again centrifuged. The supernatant was analyzed by HPLC-MS/MS: The HPLC was performed on a Thermo Fisher ACCELA LC system equipped with a Phenomenex PFP (2) column (100 mm × 2.0 mm, 3 μm). The mobile phase consisted of linear gradients of A (0.5 mM NH_4OAc solution) and B (MeOH): 0–1 min, 95% A (v/v); 1–3 min, 95–2% A; 3–5 min, 2% A; 5–5.1 min, 2–95% A; 5.1–9 min, 95% A. The flow rate was set at 0.18 mL/min, and the column temperature was maintained at 30 °C. Detection was achieved with a Thermo Fisher TSQ QUANTUM ULTRA triple-quadrupole mass spectrometer with an ESI interface in the negative-ion mode. The parameter settings were as follows: spray voltage, −3500 V; capillary temperature, 350 °C; sheath gas (N_2), 30 arbitrary units; auxiliary gas (N_2), 8 arbitrary units; vaporizer temperature, room temperature. Detection and quantification of **1t** (or **1s**) and metabolite **A** were performed as described in Section 5.8. The concentrations of compounds were determined for each time point, and the percentage remaining was calculated on the basis of the initial amount measured at 0 h. The half-life of each compound was calculated by using DAS 3.0 program (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

5.4. Activation in Rat Liver S9. After i.g. administration of phenobarbital and β-naphthoflavone for 3 days, two Sprague–Dawley rats were sacrificed by carbon dioxide inhalation, and the liver was harvested, rinsed with cold saline, and homogenized in 50 mM Tris-HCl, 150 mM KCl, pH 7.4. The homogenate was clarified by centrifugation and analyzed for protein content (BCA Protein Assay Kit). Reaction mixtures consisted of 100 mM KH_2PO_4, pH 7.4, 50 μM tested compounds and 5 mg/mL liver S9. After preincubation at 37 °C for 5 min, the reaction was initiated by adding 2 mM NADPH. The blank and negative control samples were incubated without prodrug or NADPH, respectively. At selected times (0, 10, 20, 40, and 60 min), aliquots were removed and extracted by addition of 5 volumes of MeOH containing internal standard. The extracts were clarified by centrifugation and dried under a stream of N_2. The residue was suspended in H_2O and analyzed for FdUMP (m/z 325 → 129) by HPLC-MS/MS.

5.5. Cell Viability Assays. SMMC7721 or a normal liver cell line (LO2) was cultured in RPMI-1640 medium (Gibco, Milano, Italy) supplemented with 100 units/mL penicillin G (Gibco), 150 μg/mL streptomycin (Invitrogen, Milano, Italy), and 10% fetal bovine serum (Invitrogen). Prodrug stock solutions (350 mM) were obtained by dissolving in DMSO. Individual wells of a 96-well culture plate were inoculated with 100 μL of complete medium containing 4×10^3 cells. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h prior to the experiments. After removal of the medium, 100 μL of fresh medium containing the test compound at different concentrations was added to each well and incubated at 37 °C for 72 h. The percentage of DMSO in the medium never exceeded 0.5%. Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test as previously described. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%, in comparison with cells treated with the maximum amount of DMSO (0.5%) and considered as 100% viable.

5.6. Assessment of Acute Toxicity in KM Mice. After overnight fasting, 10 KM mice were randomly divided into two groups and dosed orally by gavage with a solution of prodrug **1t** in 57% hydroxypropyl-β-cyclodextrin at a single dose of 2 or 3 g/kg, respectively. After administration, the mice were observed continuously for the first 4 h for any abnormal behavior and mortality changes, intermittently for the next 24 h, and occasionally thereafter for 15 days for weight, appetite, and behavior. All mice were sacrificed by carbon dioxide inhalation on day 15 after drug administration and were macroscopically examined for possible damage to the heart, liver, spleen, lung, and kidneys.

5.7. Assessment of Efficacy in KM Mice. The in vivo antitumor activity of prodrug **1t** was investigated using a syngeneic murine hepatocellular carcinoma cell line H22 in KM mice. According to the clinical oral dosage of fluorouracil (300 mg/d), the mouse equivalent dose of fluorouracil was 60 mg/kg based on body surface area (BSA),[29,30] and the equimolar oral dosage of **1t** was 250 mg/kg in mice. Through further dosage optimization, three oral doses of **1t** (60, 130, and 260 mg/kg) were employed. Similarly, the positive control group was administrated with 60 mg/kg of sorafenib.[25] Tumors were induced by a subcutaneous injection in the axillary region of 2×10^6 cells in 200 μL of 0.9% saline. Fifty KM mice were randomly divided into five groups, and starting on the second day, they were daily dosed by oral gavage with 200 μL/10g free vehicle (35% polyethylene glycol 400), prodrug **1t** (65, 130, and 260 mg/kg, dissolved in 35% polyethylene glycol 400), or sorafenib (60 mg/kg, suspended in 0.5% (w/v) CMC-Na) as the positive control. At the end of the study, the mice were sacrificed by carbon dioxide inhalation with tumor weights recorded.

5.8. Assessment of Tissue Distribution in Sprague–Dawley Rats. The assay was contracted and carried out by Shanghai ChemPartner (Shanghai) Co., Ltd. Dosing solutions of FDUR and prodrugs **1b** and **1t** were prepared in H_2O at a drug concentration of 4.72 mg/mL and in 20% hydroxypropyl-β-cyclodextrin at a drug concentration of 9.97 and 10.43 mg/mL (FDUR equivalents), respectively. Sprague–Dawley rats (n = 3 per time point) were treated with 23.62 mg/kg (0.096 mmol/kg) of FDUR, 49.87 mg/kg (0.096 mmol/kg) of **1b**, and 52.17 mg/kg (0.096 mmol/kg) of **1t**, respectively, by gavage. At specified times (0.25, 0.5, 1, 2, 4, 6, 12, and 24 h), blood samples were collected in EDTA-K_2 tubes via cardiac puncture under anesthesia with isoflurane. After centrifugation at 5000 rpm for 5 min at 4 °C, plasma was pipetted and stored at −80 °C. Rats were sacrificed by carbon dioxide inhalation, and the liver samples were freeze-clamped and homogenized with 3 volumes (v/w) of homogenizing solution (20 mM EDTA-K_2 in 70% MeOH). After centrifugation at 5000 rpm for 5 min at 4 °C, the supernatants were stored at −80 °C until HPLC-MS/MS analysis. 50 μL of plasma and 50 μL of liver homogenate were deproteinated, respectively, by addition of a solution of 0.1% formic acid in MeOH (200 μL) containing internal standard, vortexing, and centrifugation (14000 rpm, room temperature, 5 min). 200 μL supernatant was evaporated to dryness under a stream of N_2. The residue was reconstituted with 100 μL H_2O (containing 0.1% formic acid), briefly vortexed, and again centrifuged. The supernatant was analyzed by HPLC-MS/MS: The HPLC was performed on a Shimadzu LC system equipped with a Phenomenex PFP column (50 mm × 4.6 mm, 3 μm). The mobile phase consisted of linear gradients of solution A (0.1% formic acid in H_2O) and solution B (0.1% formic acid in MeOH): 0–0.4 min, 98% A (v/v); 0.4–1.6 min, 98–2% A; 1.6–2.4 min, 2% A; 2.4–2.41 min, 2–98% A; 2.41–3.5 min, 98% A. The flow rate was set at 0.8 mL/min, and the column temperature was maintained at 40 °C. Detection was achieved using an AB SCIEC API 4000 triple-quadrupole mass spectrometer with an ESI interface in the negative-ion mode. The parameter settings were as follows: spray voltage, −4500 V; temperature 500 °C; curtain gas, 20 psi; collisionally activated dissociation gas, 5 psi; gas 1, 50 psi; gas 2, 60 psi. Detection and quantification of the compounds were performed in the selected-reaction monitoring mode with the transitions of m/z 542 → 314 for **1t**, 129 → 42 for 5-FU, 500 → 245 for metabolite **A**, 245 → 129 for FDUR, 325 → 129 for FdUMP, and 145 → 42 for 5-chlorouracil (5-CU, internal standard), respectively.

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J. Med. Chem. XXXX, XXX, XXX–XXX

■ ASSOCIATED CONTENT

Supporting Information

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Characterization data and NMR spectra of prodrugs **1a**–**t** (PDF)

X-ray structure and data of prodrug **1t** (CIF)

Compound data (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HCC, hepatocellular carcinoma; FDUR, 5-fluoro-2′-deoxyuridine; FdUMP, 2′-deoxy-5′- fluorouridine-5′-monophosphate; HAI, hepatic arterial infusion; NMI, *N*-methylimidazole; DMTr, 4,4′-dimethoxytrityl; TBS, *t*-butyldimethylsilyl; TFA, trifluoroacetic acid; $t_{1/2}$, elimination half-life; IC_{50}, 50% inhibitory concentration; CC_{50}, 50% cytotoxic concentration; SD, standard deviation; 5-FU, 5-fluorouracil; C_{max}, maximum plasma/liver concentration; $AUC_{(inf)}$, area under the curve from time zero to infinity; ND, not detected; Hint1, histidine triad nucleotide-binding protein 1; i.g., intragastric gavage; NADPH, nicotinamide adenine dinucleotide phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SPF, specific pathogen free; BSA, body surface area; CMC-Na, sodium carboxyl methyl cellulose; 5-CU, 5-chlorouracil

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