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# Phosphoramidate ProTides of the Anticancer Agent FUDR Successfully Deliver the Preformed Bioactive Monophosphate in Cells and Confer Advantage over the Parent Nucleoside

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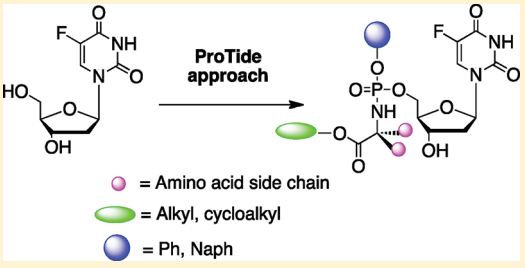
Supporting Information

**ABSTRACT:** The fluorinated pyrimidine family of nucleosides continues to represent major current chemotherapeutic agents for treating solid tumors. We herein report their phosphate prodrugs, ProTides, as promising new derivatives, which partially bypass the dependence of the current drugs on active transport and nucleoside kinase-mediated activation. They are also resistant to metabolic deactivation by phosphorolytic enzymes. We report 39 ProTides of the fluorinated pyrimidine FUDR with variation in the aryl, ester, and amino acid regions. Notably, only certain ProTide motifs are successful in delivering the nucleoside monophosphate into intact cells. We also find that the ProTides retain activity in mycoplasma infected cells, unlike FUDR. Data suggest these compounds to be worthy of further progression.




## INTRODUCTION

Chemotherapeutic agents, based largely on nucleoside analogues, continue to make a major contribution to the current chemotherapy of cancer. One of the first developed derivatives, which is still of major use, is the fluorinated pyrimidine 5-fluorouracil **1** (5-FU) (Figure 1). This drug was first introduced in 1957 by Heidelberger[1] and remains of major value in the treatment of ovary, breast, and gastrointestinal tumors in particular.[2] Besides the free base **1** the agent is also used as its 2′-deoxynucleoside FUDR (**2**) and prodrug capecitabine **3**. The 2′-deoxynucleoside **2** appears to be of particular value against liver metastases, as it is well metabolized in the liver.[3] Capecitabine has an improved ease of administration and may cause less systemic toxicity.[4]

By several metabolic routes each of these agents (**1**−**3**) leads to the generation of the corresponding nucleoside 5′-monophosphate **4** (FdUMP), which is considered to be the primary bioactive entity in this class. FdUMP acts as a potent suicide-type inhibitor of thymidylate synthase, a key enzyme in DNA synthesis, and this leads to a potent toxic event in the cell.[5]

Poor activity of this family of agents in vitro, which has sometimes been observed, and innate or acquired drug resistance in the clinic have been ascribed to several parameters, including reduced levels of the activating enzyme (i.e., thymidine kinase), required to phosphorylate **2** to **4**; overexpression of thymidylate synthase, the target of antitumor action of **4**; increased degradative cleavage of **2** to **1** by thymidine phosphorylase; and reduced transporter-mediated entry of **1** or **2** into cells.[6]

One approach to overcoming the imperative dependence of bioactive nucleoside analogues on kinase-mediated activation is to consider the preformed nucleotide as a clinical entity. However, in general this is not a useful solution because such polar nucleotides are poorly membrane soluble and subject to dephosphorylation.

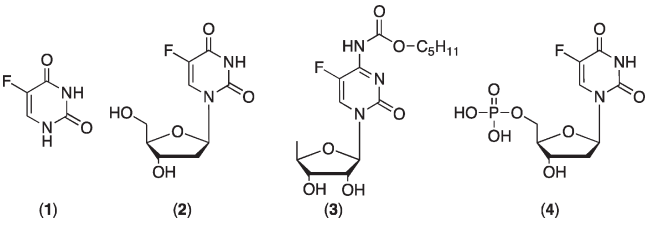



**Figure 1.** Some fluorinated pyrimidines.

A more successful approach is to mask the monophosphate creating a phosphate prodrug. Several methods exist to achieve this, and they have been reviewed.[7]

We have reported a phosphate prodrug ("ProTide") method, based on aryloxy phosphoramidates.[8] We initially applied this method to the anti-HIV agent d4T[9] and then to several other antivirals including abacavir[10] and more recently some anti hepatitis C virus agents.[11] We[12] and others[13] have also applied the method to the antiherpetic agent BVDU and revealed the interesting introduction of anticancer action of this antiviral compound upon ProTide formation. We have also reported the application of nucleoside ProTides to the antileukemic agent cladribine.[14]

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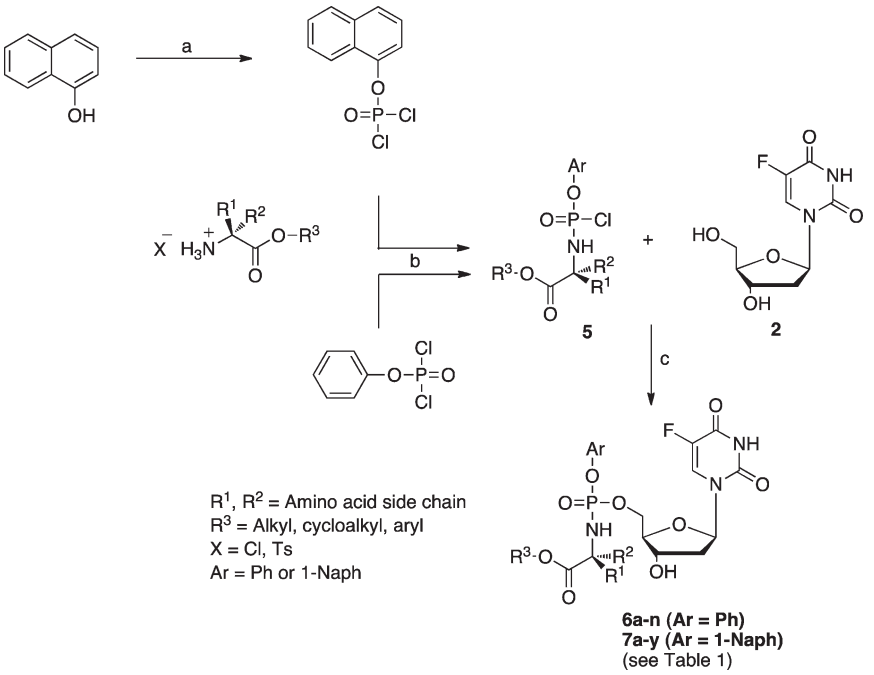
**Scheme 1. General Synthesis of FUDR ProTides**[a]




[a] Reagent and conditions: (a) $POCl_3$, $Et_3N$, anhydrous $Et_2O$, −78 °C for 1 h, then room temp for 1 h; (b) phenyl or 1-naphthyl phosphorodichloridate, $Et_3N$, anhydrous DCM, −78 °C, 1−3 h; (c) *tert*-BuMgCl (or) NMI, anhydrous THF, room temp, 16−18 h.

Thus, it was of interest to apply the ProTide method to the leading fluorinated pyrimidine family and **2** in particular. On the basis of prior art, we believed that ProTides of **2** could bypass their dependence on cell transporters and also upon thymidine kinase and that the ProTide should be resistant to the degradative pathway from **2** to **1**. We herein report the notable success of this enterprise.

## CHEMISTRY

The target ProTides of **2** were prepared using phosphorochloridate chemistry, as we have extensively reported.[15]

One component of the ProTides is an aryl unit, in this case either phenol or 1-naphthol. In the case of phenol, commercial phenyl phosphorodichloridate was used and its purity was checked by $^{31}P$ NMR prior to use. For the naphthyl analogue, 1-naphthol was allowed to react with phosphoryl chloride in dry diethyl ether in the presence of triethylamine at low temperature to give the required dichloridate (Scheme 1). The second component of the ProTide motif is an esterified amino acid. In some cases these are commercially available, but in most cases they are not and were prepared by esterification of the amino acids using standard methods.[16]

The key reagent to prepare ProTides is the arylaminoacyl phosphorochloridate **5**. These were prepared by allowing the aryl phosphorodichloridate to react with the amino acid ester in dichloromethane at low temperature (Scheme 1). The formation of the key phosphorochloridate was monitored and confirmed by $^{31}P$ NMR. In some cases the reagent was used crude, and in others it was subjected to rapid silica gel chromatography. Each of the compounds derived from a chiral amino acid was generated as a pair of diastereoisomers at the phosphate center, in roughly 1:1 ratio, as revealed by two closely spaced peaks by $^{31}P$ NMR.

Finally, each of the phosphorochloridates **5** were allowed to react with FUDR to generate the target ProTides **6a**−**n** (Ar = Ph) and **7a**−**y** (Ar = 1-Naph) in one step as presented in Scheme 1. Two sets of conditions were variously used for this coupling reaction: *N*-methylimidazole in THF or *tert*-butylmagnesium chloride in THF, both at room temperature for 16−18 h. In many cases byproducts with dual phosphorylation at the 3′- and 5′-hydroxyl groups were formed, and in some cases the 3′-mono phosphorylated species was also observed. This required extensive and repeated chromatographic purification of ProTides (**6**, **7**), leading to modest isolated yields. These were not optimized in the present report, since the primary goal was to establish biological activity at this stage.

As noted in Table 1, we varied the aryl unit from phenyl to 1-naphthyl, the amino acid from L-alanine to glycine, valine, leucine, isoleucine, phenylalanine, methionine, proline, and $\alpha,\alpha$-dimethylglycine and the ester rather extensively. In total 39 ProTides were prepared, purified, and fully characterized. In every case, multiple peaks in $^{31}P$ and $^{13}C$ NMR and HPLC confirmed the presence of phosphate diastereoisomers. These were not routinely separated in this study and were tested as mixtures of isomers, since chiral ProTide isomers frequently show rather similar biological profiles. In the great majority of cases, such ProTides progressed to the clinic as mixed diastereomers.[17]

## BIOLOGICAL ACTIVITY IN VITRO

The ProTides **6** and **7** described above were tested for their cytostatic activity against several established tumor cell lines, as presented in Table 1. Compounds **1** and **2** were included as positive controls. In particular, we first studied the compounds versus wild type L1210, CEM, and HeLa cells. In each case we also included a thymidine kinase deficient ($TK^-$) mutant of the parent cell line to probe the effect of TK deficiency on the

**Table 1. Cytostatic Activity of 5-FU, FUDR, and FUDR Prodrugs against Tumor Cell Lines**

| | | | | $IC_{50}$[a] ($\mu$M) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| compd | aryl | ester | AA | L1210/0 | L1210/TK$^-$ | Cem/0 | Cem/TK$^-$ | HeLa | HeLa/TK$^-$ |
| **1** | | | | 0.33 ± 0.17 | 0.32 ± 0.31 | 18 ± 5 | 12 ± 1 | 0.54 ± 0.12 | 0.23 ± 0.01 |
| **2** | | | | 0.0011 ± 0.0002 | 3.0 ± 0.1 | 0.022 ± 0.006 | 3.0 ± 0.4 | 0.050 ± 0.011 | 1.4 ± 0.4 |
| **6a** | Ph | Me | Ala | 0.022 ± 0.007 | 41 ± 3 | 0.70 ± 0.37 | 35 ± 12 | 0.28 ± 0.14 | 4.7 ± 0.4 |
| **6b** | Ph | Et | Ala | 0.13 ± 0.04 | 0.94 ± 0.18 | 0.92 ± 0.11 | 14 ± 0 | 0.48 ± 0.19 | 9.8 ± 1.4 |
| **6c** | Ph | *i*-Pr | Ala | 0.076 ± 0.022 | 1.1 ± 0.1 | 1.0 ± 0.1 | 30 ± 10 | 0.71 ± 0.15 | 25 ± 11 |
| **6d** | Ph | *c*-Hex | Ala | 0.039 ± 0.001 | 0.14 ± 0.02 | 0.17 ± 0.07 | 1.2 ± 0.01 | 0.18 ± 0.05 | 5.9 ± 0.4 |
| **6e** | Ph | Bn | Ala | 0.028 ± 0.007 | 13 ± 8 | 0.18 ± 0.03 | 22 ± 7 | 0.13 ± 0.01 | 19 ± 2 |
| **6f** | Ph | Et | Val | 0.16 ± 0.05 | 42 ± 2 | 1.0 ± 0.1 | >250 | 1.2 ± 0.3 | 27 ± 7 |
| **6g** | Ph | Bn | Leu | 0.044 ± 0.025 | 2.0 ± 0.3 | 0.24 ± 0.04 | 16 ± 1 | 0.067 ± 0.042 | 5.6 ± 0.3 |
| **6h** | Ph | Bn | Ile | 0.076 ± 0.022 | 1.1 ± 0.1 | 1.0 ± 0.1 | 30 ± 10 | 0.71 ± 0.15 | 25 ± 11 |
| **6i** | Ph | Bn | Phe | 0.036 ± 0.010 | 39 ± 4 | 0.25 ± 0.02 | 11 ± 3 | 0.014 ± 0.007 | 12 ± 2 |
| **6j** | Ph | Pnt | Met | 0.11 ± 0.06 | 2.2 ± 0.5 | 0.35 ± 0.13 | 13 ± 1 | 0.15 ± 0.00 | 7.1 ± 1.2 |
| **6k** | Ph | Bn | Met | 0.073 ± 0.035 | 4.1 ± 1.2 | 0.28 ± 0.03 | 25 ± 0 | 0.15 ± 0.02 | 11 ± 7 |
| **6l** | Ph | Bn | Pro | 0.35 ± 0.07 | 31 ± 5 | 0.98 ± 0.40 | 28 ± 8 | 1.1 ± 0.4 | 20 ± 11 |
| **6m** | Ph | Et | DMG | 0.039 ± 0.001 | 4.6 ± 0.0 | 0.65 ± 0.16 | 22 ± 1 | 0.59 ± 0.09 | 17 ± 2 |
| **6n** | Ph | Bn | DMG | 0.017 ± 0.003 | 0.18 ± 0.05 | 0.23 ± 0.04 | 4.8 ± 0.7 | 0.24 ± 0.07 | 3.7 ± 0.1 |
| **7a** | Nap | Et | Ala | 0.031 ± 0.005 | 0.36 ± 0.01 | 0.25 ± 0.04 | 1.6 ± 0.2 | 0.22 ± 0.04 | 2.8 ± 0.0 |
| **7b** | Nap | Pr | Ala | 0.021 ± 0.012 | 0.16 ± 0.07 | 0.14 ± 0.01 | 1.1 ± 0.2 | 0.11 ± 0.03 | 2.5 ± 0.1 |
| **7c** | Nap | butyl | Ala | 0.022 ± 0.004 | 0.11 ± 0.06 | 0.064 ± 0.007 | 0.84 ± 0.60 | 0.12 ± 0.02 | 2.7 ± 1.5 |
| **7d** | Nap | Pnt | Ala | 0.0028 ± 0.0010 | 0.13 ± 0.13 | 0.015 ± 0.006 | 0.28 ± 0.04 | 0.029 ± 0.023 | 0.44 ± 0.35 |
| **7e** | Nap | Hex | Ala | 0.0072 ± 0.0000 | 0.076 ± 0.015 | 0.080 ± 0.020 | 0.65 ± 0.34 | 0.039 ± 0.018 | 1.8 ± 0.1 |
| **7f** | Nap | *c*-Bu | Ala | 0.014 ± 0.003 | 0.088 ± 0.038 | 0.073 ± 0.018 | 1.5 ± 0.3 | 0.069 ± 0.003 | 1.5 ± 0.6 |
| **7g** | Nap | *c*-Pnt | Ala | 0.031 ± 0.010 | 0.13 ± 0.02 | 0.035 ± 0.025 | 0.92 ± 0.007 | 0.071 ± 0.036 | 2.2 ± 1.3 |
| **7h** | Nap | *c*-Hex | Ala | 0.043 ± 0.023 | 0.15 ± 0.00 | 0.057 ± 0.055 | 1.0 ± 0.1 | 0.090 ± 0.014 | ND |
| **7i** | Nap | $CH_2$-*t*-Bu | Ala | 0.27 ± 0.11 | 1.2 ± 0.7 | 0.49 ± 0.05 | 6.7 ± 1.0 | 0.70 ± 0.11 | 32 ± 26 |
| **7j** | Nap | $CH_2CH_2$-*t*-Bu | Ala | 0.016 ± 0.006 | 0.062 ± 0.009 | 0.053 ± 0.021 | 0.19 ± 0.04 | 0.078 ± 0.018 | 1.3 ± 0.9 |
| **7k** | Nap | $CH_2$-*c*-Pr | Ala | 0.017 ± 0.007 | 0.12 ± 0.06 | 0.059 ± 0.017 | 1.1 ± 0.2 | 0.068 ± 0.001 | 1.4 ± 0.4 |
| **7l** | Nap | 2-Ind | Ala | 0.021 ± 0.002 | 40 ± 0 | 0.079 ± 0.018 | 1.0 ± 0.2 | 0.10 ± 0.06 | 7.1 ± 2.1 |
| **7m** | Nap | Bn | Ala | 0.011 ± 0.007 | 0.045 ± 0.027 | 0.068 ± 0.035 | 0.31 ± 0.06 | 0.065 ± 0.013 | 2.5 ± 1.3 |
| **7n** | Nap | THP | Ala | 0.038 ± 0.014 | 27 ± 6 | 0.11 ± 0.02 | 43 ± 12 | 0.13 ± 0.04 | 15 ± 7 |
| **7o** | Nap | *c*-Hex | Val | 1.1 ± 0.5 | 35 ± 8 | 0.80 ± 0.28 | 46 ± 14 | 0.67 ± 0.03 | 27 ± 6 |
| **7p** | Nap | Pnt | Leu | 0.017 ± 0.001 | 1.2 ± 0.4 | 0.071 ± 0.008 | 15 ± 4 | 0.039 ± 0.014 | 7.5 ± 0.4 |
| **7q** | Nap | Bn | Leu | 0.028 ± 0.004 | 1.5 ± 0.6 | 0.13 ± 0.00 | 30 ± 6 | 0.080 ± 0.022 | 9.4 ± 1.4 |
| **7r** | Nap | Pnt | Ile | 0.22 ± 0.12 | 12 ± 2 | 0.46 ± 0.11 | 17 ± 1 | 0.30 ± 0.02 | 11 ± 1 |
| **7s** | Nap | Pnt | Phe | 0.026 ± 0.001 | 2.9 ± 1.2 | 0.10 ± 0.00 | 8.3 ± 1.0 | 0.040 ± 0.000 | 6.6 ± 0.5 |
| **7t** | Nap | Bn | Phe | 0.012 ± 0.007 | 5.6 ± 1.3 | 0.10 ± 0.03 | 7.2 ± 0.1 | 0.16 ± 0.08 | 6.8 ± 1.5 |
| **7u** | Nap | Bn | Met | 0.072 ± 0.001 | 1.9 ± 0.2 | 0.19 ± 0.10 | 11 ± 1 | 0.087 ± 0.017 | 8.3 ± 0.0 |
| **7v** | Nap | Bn | Pro | 0.21 ± 0.08 | 25 ± 8 | 0.89 ± 0.35 | 32 ± 9 | 1.2 ± 0.0 | 26 ± 1 |
| **7w** | Nap | Et | DMG | 0.064 ± 0.008 | 0.82 ± 0.16 | 0.36 ± 0.05 | 6.9 ± 1.8 | 0.20 ± 0.12 | 3.2 ± 0.0 |
| **7x** | Nap | Pnt | DMG | 0.037 ± 0.010 | 0.30 ± 0.13 | 0.14 ± 0.00 | 5.4 ± 1.1 | 0.12 ± 0.03 | 2.3 ± 0.1 |
| **7y** | Nap | Bn | DMG | 0.011 ± 0.005 | 0.13 ± 0.04 | 0.16 ± 0.02 | 2.4 ± 0.8 | 0.078 ± 0.020 | 3.1 ± 0.6 |

[a] $IC_{50}$ or compound concentration required to inhibit tumour cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

cytostatic activity of **1** and **2** and the degree to which the ProTides could bypass this dependence. Previous examples of this type of study have revealed ProTides to be highly efficient at bypassing the dependence on nucleoside kinases.[8,9]

Thus, in 2 of the 3 cell lines studied (i.e., L1210 and HeLa) 5-FU showed activity at ~0.5 $\mu$M, being rather poorly active against CEM cells ($IC_{50}$ = 18 $\mu$M). Compound **1** largely retained activity in the TK$^-$ cells, indicating that it must be primarily activated to **4** by other metabolic routes such as phosphoribosylation. Activation of 5-FU by phosphoribosylation is catalyzed by the enzyme orotate phosphoribosyl transferase (OPRT) responsible for the direct conversion of the nucleobase to the nucleoside monophosphate.[18,19] On the other hand **2** was more active in the wild-type cell lines, being active at 1−50 nM and thus 10−800 times more potent than 5-FU. But **2** is highly dependent on TK activity, being 30- to 3000-fold less active in the TK-deficient tumor cells than in the parent cell lines. The L1210 cells were particularly striking in this regard. These data clearly show the presence of TK as a prerequisite for **2** to exert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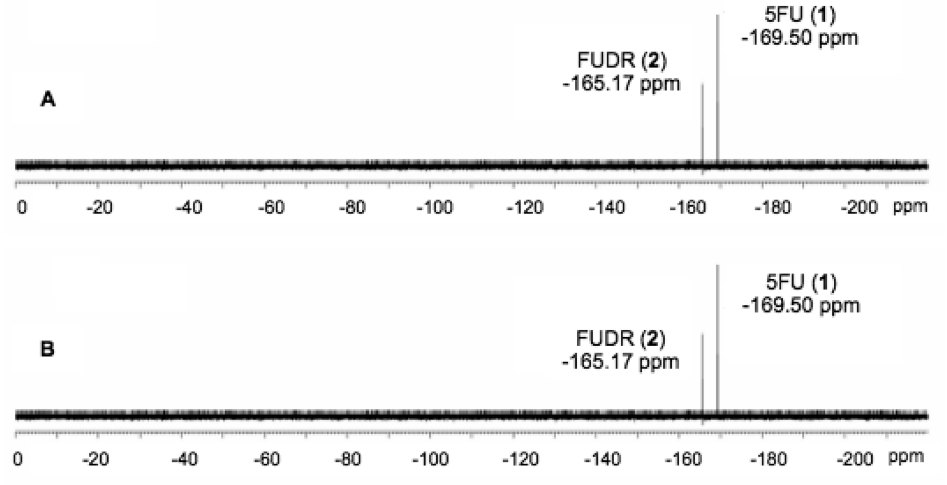



**Figure 2.** $^{19}$F NMR spectra: (A) FUDR and 5-FU in methanol-$d_4$, 25 °C; (B) FUDR submitted to the thymidine phosphorylase assay, 25 °C, 5 min.

cytostatic activity. The first ProTide prepared, the L-Ala-OMe phenyl derivative **6a**, is approximately 5- to 30-fold less active than the nucleoside **2** against the parent cell lines (Table 1). Moreover and in notable contrast to our prior ProTide work,[8−12] **6a** was very significantly reduced in cytostatic activity against the TK-deficient cells: 2000-fold difference in cytostatic activity against the L1210 wild-type and TK-deficient cells, for example. Although the cytostatic reduction was less in the HeLa cells, the agent still lost significant activity in the absence of thymidine kinase (∼15-fold). These data clearly show that **6a** requires TK to exert biological activity, most probably through efficient liberation of **2** as liberation of **1** should lead to TK-independence according to the data for **1** in Table 1. The notable success of the ProTide approach on other deoxypyrimidine nucleosides such as BVDU[12] makes this especially surprising. However, we have earlier observed the need to optimize the ProTide motif for each nucleoside we have studied,[10,11] and so we varied the ProTide motifs of **6a**. In the first instance we retained the phenyl unit and the L-alanine motif, as the latter in particular often appears to be beneficial.[8] Thus, the methyl ester in **6a** was lengthened to ethyl (**6b**), branched to isopropyl (**6c**), and cyclized to cyclohexyl (**6d**). We also prepared the benzyl analogue (**6e**), which has often been found by us to be a highly preferred ProTide motif.[8] In general each of these esters maintained similar potency in the parent cell lines compared to **6a**. Compound **6d** was also the compound that retained the highest potency in the TK-deficient cells, particularly in L1210/TK$^-$ where it was only 3-fold reduced and thus 21-fold more active than FUDR. Thus, among the family of phenyl FUDR ProTides, **6d** emerged as the most successful compound in our study to date. Interestingly, the "preferred" benzyl compound (**6e**) hardly retained activity in the TK$^-$ cells and thus demonstrated a very low degree of effectiveness as a phosphate prodrug. This highlights the need to optimize and tune the ProTide motif for every nucleoside, as already mentioned above.

We next studied amino acid variation. L-Val-OEt (**6f**), which showed a somewhat similar profile compared to the L-alanine analogues (**6b**), was in general less active as a cytostatic agent. Also, the L-Leu-OBn (**6g**) and L-Ile-OBn (**6h**) were similar to the L-Ala-OBn analogue (**6e**). Reasonably similar data were noted for L-phenylalanine (**6i**), L-methionine (**6j**, **6k**), and L-proline (**6l**) derivatives. In general, the compounds were active at submicromolar concentrations in TK-competent cells but significantly less active in the TK-deficient cells, as FUDR. All of these data demonstrated a poor degree of effectiveness as ProTides. Notably, our generally observed preference for L-alanine was not apparent in this series, and indeed little amino acid SAR could be discerned in contrast to our prior work.[20] Finally in this series we prepared analogues of the actual unnatural amino acid $\alpha$, $\alpha$-dimethylglycine as its −OEt (**6m**) and −OBn (**6n**) esters, but these derivatives showed no distinct advantage over earlier analogues.

We have recently reported that in some cases we can achieve a modest potency boost for some ProTides on replacing the phenyl unit by 1-naphthyl.[11,21] Thus, we prepared a series of 25 naphthyl ProTides **7a**−**y** with variation in the amino acid and ester moieties. In general, each of the naphthyl analogues was more potent than its phenyl equivalent across the range of cell lines. However, by comparison to the phenyl series **6a**−**n**, the naphthyl family tended to display a more significant retention of activity in the TK$^-$ panel of tumor cell lines. Compounds emerging as most potent in L1210/TK$^-$, for example, were the L-Ala-OHex (**7e**) and L-Ala-OBn (**7m**) and also an extended L-alanine ester (**7j**). By comparison and in marked contrast to the phenyl series here, the usual amino acid SARs emerged with L-alanine strongly preferred. Naphthyl ProTides of other amino acids were all significantly more dependent on TK for their cytostatic activity as demonstrated in L1210/TK$^-$ cells. Thus, a number of naphthyl L-Ala ProTides with a variety of esters emerged as reasonably effective in bypassing the TK dependence of FUDR. The L-Ala-OBn (**7m**) and L-Ala-OPnt (**7d**) derivatives were among the most potent, being active at an $IC_{50}$ of 11 and 2.8 nM in L1210, respectively. Thus, compounds **7m** and **7d** were only 10-fold and 2.5-fold less active than the parent FUDR but 30 times and 100 times more active than 5-FU, respectively. In L1210/TK$^-$, compound **7m** retained significant cytostatic potency, being only 5-fold reduced, versus FUDR which was 3000-fold diminished, whereas compound **7d** has shown a 40-fold reduction of cytostatic activity in L1210/TK$^-$. The data were less dramatic for CEM/0 versus CEM/TK$^-$ cells but conveyed the same message. These data are in marked contrast to our prior phenyl/naphthyl comparisons where the replacement only caused modest increases in potency.

Although the data on the cytostatic activity of several ProTides of FUDR against a variety of tumor cell lines look interesting, one should be aware that all data were obtained from in vitro testing

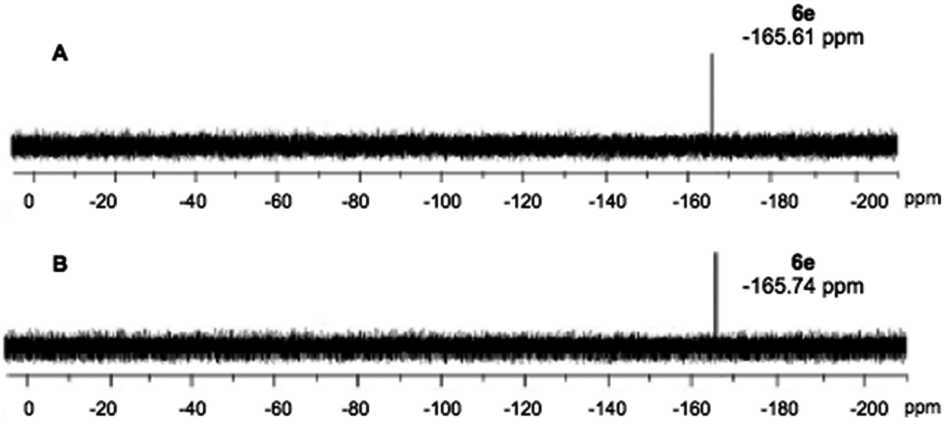



**Figure 3.** $^{19}$F NMR spectra of compound **6e** in phosphorylase assay: (A) **6e** in the absence of the enzyme (TP), 25 °C; (B) **6e** submitted to the action of thymidine phosphorylase (TP), spectra recorded after 72 h, 25 °C.

assays. Whereas the parent drug FUDR has a proven efficacy in in vivo animal models and in cancer patients, the in vivo efficacy of the FUDR ProTides need still to be established . In this respect, phosphoramidate prodrugs of acyclic nucleoside phosphonates have been shown to be quite effective in vivo. Therefore, such types of prodrug may well be efficacious in vivo when applied on FUDR as well. Experiments to demonstrate efficacy of the FUDR ProTides in a mouse model are under consideration.

As was previously noted, FUDR can be degraded to its nucleobase 5-fluorouracil in a phosphorolytic reaction catalyzed by thymidine phosphorylase (TP). This breakdown has been suggested to be one of the reasons for the limited therapeutic effectiveness of FUDR.[22] Therefore, in order to investigate the susceptibility of our FUDR ProTides to phosphorolysis, we have performed an enzymatic phosphorylase assay. This assay was carried out for comparison for both FUDR and one of our first synthesized model compound **6e**, using TP (purified from *Escherichia coli*) in the presence of potassium phosphate buffer (300 mM solution, pH 7.4). A potential phosphorolysis reaction and hence potential formation of 5-FU were monitored by in situ $^{19}$F NMR experiments. Thus, we first recorded the individual $^{19}$F NMR spectra of FUDR and 5-FU (spectra not shown) and the additional $^{19}$F NMR spectra of compounds **2** and **1** together (Figure 2A). The single peak at $\delta_F$ −165.17 is assigned to the nucleoside **2**, whereas the single peak at $\delta_F$ −169.50 ppm is assigned to the nucleobase **1**. The phosphorylase assay was then carried out by dissolving **2** in methanol-$d_4$ in the presence of potassium phosphate buffer and recording the blank $^{19}$F NMR spectrum prior to addition of the enzyme (spectra not shown). A single peak at $\delta_F$ −165.17 ppm was observed. After 5 min from the addition of thymidine phosphorylase (20.7 UNI), the $^{19}$F NMR spectrum (Figure 2B) revealed the appearance of an additional peak at $\delta_F$ −169.50 ppm due to the release of 5-FU from the FUDR. Two single peaks with the same chemical shifts as have been observed for the first experiment were found; therefore, these data might confirm that the signal at $\delta_F$ −169.50 ppm can be assigned to 5-FU which was formed upon phosphorolytic action of the enzyme in the assay.

The $^{19}$F NMR spectrum of compound **6e** under conditions of the phosphorolysis assay (Figure 3B) was recorded after 5 min, 14 h, and 72 h and did not show any evidence of phosphorolysis at these three time points. These experiments confirmed that, in contrast to nucleoside **2**, the ProTide **6e** is at best a very poor, if any, substrate for thymidine phosphorylase.

We have recently reported that mycoplasma infection of cells can significantly alter the metabolism of nucleoside analogues, partly through the expression of mycoplasma-derived enzymes such as TP.[23] FUDR is known to be subject to TP-mediated deactivation, and we thus expected that **2** would be less cytostatic in the presence of mycoplasmas. If ProTides were able to deliver bioactive **4** directly and were resistant to TP, then they may also be less subject to mycoplasma-induced catabolic degradation. We present the data from such a study in Table 2. Interestingly, whereas the cytostatic activity of FUDR was heavily compromised in mycoplasma infected cells (a drop of cytostatic activity by 378-fold was observed), the prodrugs generally kept a significant cytostatic activity under similar experimental conditions. In general, the 1-naphthyl prodrug derivatives (**7**) markedly kept their cytostatic potential in the mycoplasma-infected tumor cell cultures. They often lost only 2- to 3-fold antiproliferative activity (Table 2).

Thus, we herein demonstrate that the ProTides of FUDR, in contrast with the parent nucleoside, are resistant to the phosphorolytic activity of mycoplasma-encoded thymidine phosphorylase but also cellular phosphorylases. This property may give the ProTides of FUDR a therapeutic edge when exposed not only to mycoplasma-infected tumor tissue but also to any TP-expressing tumor in general. It is indeed well-known that tumors often show an increased TPase activity to allow a better angiogenesis in the tumor tissue. Such activity should result in an increased rate of hydrolysis (inactivation) of parent FUDR that is a known substrate for TPase[23] but should not affect the FUDR ProTides, shown to be resistant to this phosphorolytic cleavage.

The eventual cytostatic activity of FUDR also highly depends on its efficient transport into the tumor cells. Both FUDR and FdUMP show a 60- to 70-fold decreased cytostatic activity against CEM cells that lack the hENT1 transporter (designated Cem/hEnt-0) (Table 3). Importantly, the FUDR prodrugs proved to be less dependent on the presence of the hENT1 transporter, since they lost only 7- to 15-fold antiproliferative activity against the hENT1-deficient CEM cells. These observations are in agreement with an only 2- to 7-fold decreased cytostatic activity of the ProTides in the presence of transport inhibitors (i.e., dipyridamole and NBMPR), compared to a 20- to 60-fold loss of antiproliferative activity of FUDR and FdUMP under similar experimental conditions.

With the aim of investigating the chemical and enzymatic stability of FUDR ProTides to ester hydrolysis under biologically relevant conditions, we performed several stability studies at

**Table 2. Cytostatic Activity of FUDR and FUDR Prodrugs in Wild Type Murine Leukemia L1210 Cell Cultures (L1210/0) and L1210 Cell Cultures, Infected with *Mycoplasma hyorhinis* (L1210.Hyor)**

| | | | | $IC_{50}$[a] ($\mu$M) | | |
|---|---|---|---|---|---|---|
| compd | aryl | ester | AA | L1210/0 | L1210.Hyor | $IC_{50}$(L1210.Hyor)/$IC_{50}$(L1210/0) |
| **2** | | | | 0.0009 ± 0.0003 | 0.34 ± 0.13 | 378 |
| **6a** | Ph | Me | Ala | 0.040 ± 0.016 | 0.87 ± 0.28 | 22 |
| **6b** | Ph | Et | Ala | 0.11 ± 0.0021 | 0.54 ± 0.12 | 5 |
| **6c** | Ph | *i*-Pr | Ala | 0.050 ± 0.013 | 0.70 ± 0.10 | 14 |
| **6d** | Ph | *c*-Hex | Ala | 0.032 ± 0.0050 | 0.040 ± 0.016 | 1.25 |
| **6e** | Ph | Bn | Ala | 0.026 ± 0.008 | 0.15 ± 0.006 | 5.8 |
| **6f** | Ph | Et | Val | 0.20 ± 0.033 | 4.4 ± 1.1 | 22 |
| **6g** | Ph | Bn | Leu | 0.054 ± 0.0021 | 0.17 ± 0.047 | 3.2 |
| **6h** | Ph | Bn | Ile | 0.98 ± 0.39 | 2.2 ± 0.031 | 2.2 |
| **6i** | Ph | Bn | Phe | 0.016 ± 0.0014 | 0.56 ± 0.023 | 35 |
| **6j** | Ph | Pnt | Met | 0.13 ± 0.0078 | 0.41 ± 0.21 | 3.2 |
| **6k** | Ph | Bn | Met | 0.058 ± 0.035 | 0.76 ± 0.18 | 13 |
| **6l** | Ph | Bn | Pro | 0.35 ± 0.022 | 18 ± 0.71 | 51 |
| **6m** | Ph | Et | DMG | 0.030 ± 0.0005 | 0.26 ± 0.01 | 8.7 |
| **6n** | Ph | Bn | DMG | 0.029 ± 0.001 | 0.02 ± 0.002 | 0.69 |
| **7a** | Naph | Et | Ala | 0.028 ± 0.0021 | 0.095 ± 0.0028 | 3.4 |
| **7b** | Naph | Pr | Ala | 0.030 ± 0.00035 | 0.036 ± 0.0064 | 1.2 |
| **7c** | Naph | butyl | Ala | 0.0095 ± 0.0021 | 0.021 ± 0.0071 | 2.2 |
| **7d** | Naph | Pnt | Ala | 0.0021 ± 0.00007 | 0.006 ± 0.0014 | 2.9 |
| **7e** | Naph | Hex | Ala | 0.0032 ± 0.00035 | 0.0022 ± 0.00028 | 0.69 |
| **7f** | Naph | *c*-Bu | Ala | 0.011 ± 0.0014 | 0.024 ± 0.00014 | 2.2 |
| **7g** | Naph | *c*-Pnt | Ala | 0.016 ± 0.0007 | 0.024 ± 0.005 | 1.5 |
| **7h** | Naph | *c*-Hex | Ala | 0.036 ± 0.017 | 0.049 ± 0.004 | 1.4 |
| **7i** | Naph | $CH_2$-*t*-Bu | Ala | 0.093 ± 0.033 | 0.18 ± 0.069 | 1.9 |
| **7j** | Naph | $CH_2CH_2$-*t*-Bu | Ala | 0.012 ± 0.0018 | 0.032 ± 0.0088 | 2.7 |
| **7k** | Naph | $CH_2$-*c*-Pr | Ala | 0.014 ± 0.0042 | 0.031 ± 0.0064 | 2.2 |
| **7l** | Naph | 2-Ind | Ala | 0.039 ± 0.019 | 0.042 ± 0.040 | 1.08 |
| **7m** | Naph | Bn | Ala | 0.011 ± 0.009 | 0.025 ± 0.01 | 2.27 |
| **7n** | Naph | THP | Ala | 0.041 ± 0.0028 | 0.48 ± 0.11 | 11.7 |
| **7o** | Naph | *c*-Hex | Val | 1.2 ± 0.17 | 1.29 ± 0.29 | 1.08 |
| **7p** | Naph | Pnt | Leu | 0.031 ± 0.0020 | 0.035 ± 0.010 | 1.13 |
| **7q** | Naph | Bn | Leu | 0.029 ± 0.0021 | 0.048 ± 0.020 | 1.7 |
| **7r** | Naph | Pnt | Ile | 0.42 ± 0.021 | 0.70 ± 0.074 | 1.67 |
| **7s** | Naph | Pnt | Phe | 0.030 ± 0.0039 | 0.14 ± 0.007 | 4.67 |
| **7t** | Naph | Bn | Phe | 0.021 ± 0.0061 | 0.23 ± 0.078 | 11 |
| **7u** | Naph | Bn | Met | 0.054 ± 0.013 | 0.20 ± 0.098 | 3.7 |
| **7v** | Naph | Bn | Pro | 0.26 ± 0.055 | 0.65 ± 0.070 | 2.5 |
| **7w** | Naph | Et | DMG | 0.056 ± 0.04 | 0.17 ± 0.03 | 3 |
| **7x** | Naph | Pnt | DMG | 0.045 ± 0.0021 | 0.019 ± 0.0028 | 0.42 |
| **7y** | Naph | Bn | DMG | 0.019 ± 0.004 | 0.045 ± 0.004 | 2.4 |

[a] $IC_{50}$ or compound concentration required to inhibit tumor cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

different pH values, in the presence of human serum and carboxypeptidase Y.

A chemical hydrolysis of L-Ala-OBn phenyl ProTide **6e** was evaluated under experimental conditions at pH 1 and pH 8 and monitored by $^{31}P$ NMR. During the assay (14 h) under acidic conditions (pH 1) only two peaks representing two diastereoisomers of **6e** were recorded (Figure 4). Lack of formation of new signals in the $^{31}P$ NMR spectrum indicates that the tested compound **6e** is highly stable in acidic medium. The same result was observed when the ProTide **6e** was subjected to the assay under mild basic conditions (pH 8; data not shown).

In order to explore whether FUDR ProTides can be activated via our putative mechanism,[24,25] we carried out an enzymatic study using a carboxypeptidase Y assay following the protocol already described.[26] As depicted in Figure 5, the mechanism of activation of phosphoramidates begins with the hydrolysis of the carboxylic ester moiety (a) hypothesized to be mediated by a carboxyesterase-type enzyme to give the intermediate **8**. In the

**Table 3. Cytostatic Activity of FUDR and Several FUDR Prodrugs in CEM Cell Cultures Containing (Cem/hEnt-1) or Lacking (Cem/hEnt-0) the hEnt1 Transporter**

| | | | | $IC_{50}$ [a] ($\mu$M) | | | |
|---|---|---|---|---|---|---|---|
| compd | aryl | ester | AA | Cem/hEnt-1 | Cem/hEnt-0 | Cem/hEnt-1 + dipyridamole | Cem/hEnt-1 + NBMPR |
| 5-FdUMP | | | | 0.05 ± 0.02 | 3.6 ± 0.69 | 1.74 | 1.06 |
| **2** | | | | 0.04 ± 0.02 | 2.5 ± 0.65 | 1.36 | 0.80 |
| **6e** | Ph | Bn | Ala | 0.13 ± 0.05 | 1.4 ± 0.65 | 0.66 | 0.72 |
| **6m** | Ph | Et | DMG | 0.37 ± 0.14 | 5.8 ± 0.50 | 2.35 | 2.56 |
| **6n** | Ph | Bn | DMG | 0.17 ± 0.06 | 1.2 ± 0.11 | 0.26 | 0.61 |
| **7m** | Naph | Bn | Ala | 0.05 ± 0.02 | 0.6 ± 0.11 | 0.13 | 0.26 |
| **7w** | Naph | Et | DMG | 0.21 ± 0.07 | 1.4 ± 0.20 | 0.52 | 0.62 |
| **7y** | Naph | Bn | DMG | 0.05 ± 0.03 | 0.4 ± 0.13 | 0.16 | 0.28 |

[a] $IC_{50}$ or compound concentration required to inhibit tumor cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

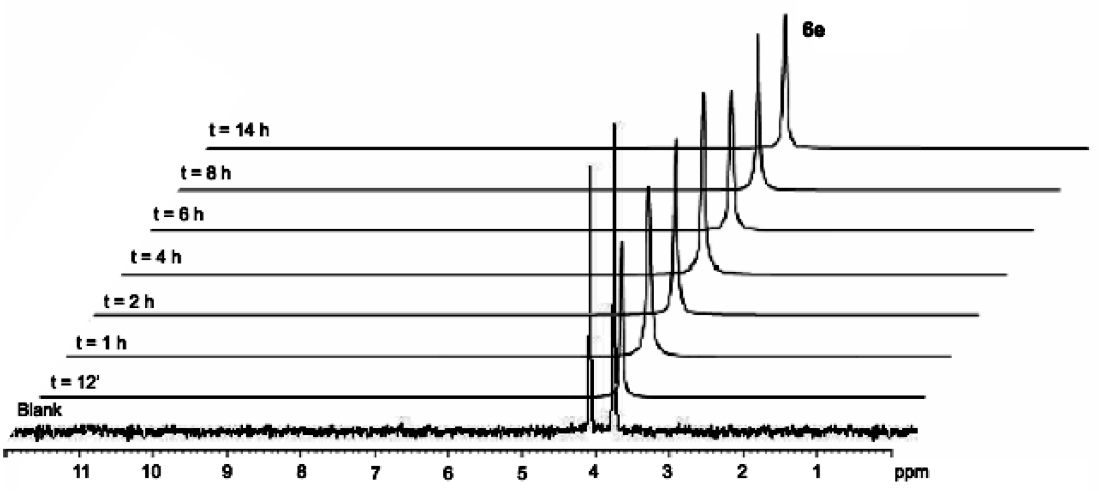



**Figure 4.** Convoluted and deconvoluted $^{31}P$ NMR specta of phosphoramidate **6e** (buffer, pH 1).

second step (b) a spontaneous cyclization occurs through an internal nucleophilic attack of the carboxylate residue on the phosphorus center following a displacement of the aryl moiety to yield **9**. The third step (c) is the opening of the unstable cyclic mixed anhydride mediated by water with the release of the intermediate **10**, which upon the cleavage of the P−N bond (d) mediated by a hypothesized phosphoramidase-type enzyme gives the corresponding monophosphate **4**. Among our large family of FUDR ProTides, the enzymatic assay was applied to one of our lead compounds **7d**. Thus, the L-Ala-OPnt naphthyl derivative **7d**, carboxypeptidase Y, and Trizma buffer (pH 7.6) were dissolved in acetone-$d_6$ and $^{31}P$ NMR (202 MHz) spectra were recorded at regular intervals (every 7 min) over 14 h (Figure 6). According to the results, the parent ProTide **7d** (represented as two signals at $\delta_P$ 4.03 and 4.31 ppm) was rapidly hydrolyzed to the first metabolite **8** lacking the ester moiety shown in the $^{31}P$ NMR spectrum at $\delta_P$ 4.99 and 5.13 ppm. Noteworthy, both diastereoisomers of **7d** were processed at roughly similar rate. A further processing of **8** led to the formation of metabolite **10** shown as a single peak at $\delta_P$ 6.82 ppm. During the enzymatic process, compound **7d** was fully converted to the metabolite **10** within approximately 45 min with an estimated half-life of less than 5 min. In fact, this assay showed that the rate of the initial activation step might be considered in general as one of requirements for good biological activity of phosphoramidates. In order to support the proposed putative mechanism and the results from the enzymatic assay, the intermediate **10** was prepared via a synthetic route (Scheme 2). Therefore, chemical hydrolysis of compound **7m** in the presence of triethylamine and water was performed. Product **10′** obtained as a diammonium salt was then added to the final assay sample **7m** (containing only the enzymatic metabolite **10** in Trizma). Its $^{31}P$ NMR spectrum has exclusively shown one peak at $\delta_P$ 6.85 ppm, strongly supporting this part of the metabolic pathway and activation of the ProTides.

The stability of the prodrug **7a** in the presence of human serum was investigated using in situ $^{31}P$ NMR. The aim of this experiment was to identify the formation of any metabolites of **7a** (Figure 5), which would appear as new peaks in the $^{31}P$ NMR spectrum. Thus, after the first (control) $^{31}P$ NMR data of **7a** in DMSO and $D_2O$ were recorded, the NMR sample was treated with human serum (0.3 mL) and immediately subjected to further $^{31}P$ NMR experiments at 37 °C. The $^{31}P$ NMR data were recorded every 15 min over 14 h and are reported in Figure 7. In order to improve a visualization of results, all the spectra were further processed using the Lorentz−Gauss deconvolution method. The spectra displayed a single peak inherent to the human serum at ~$\delta_P$ 2.00 ppm and two

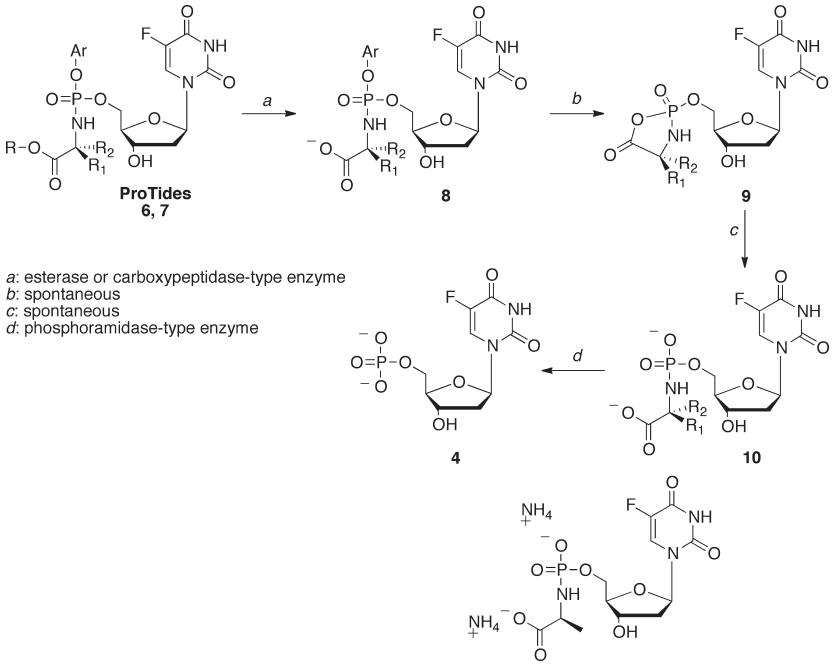



**Figure 5.** Proposed activation pathway of FUDR ProTides.

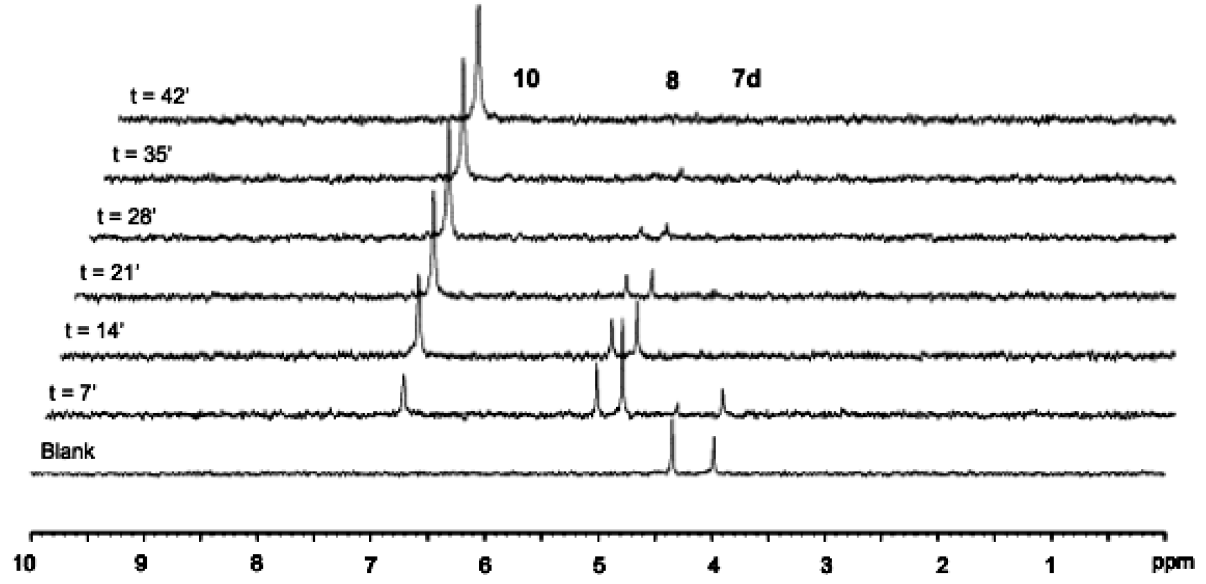
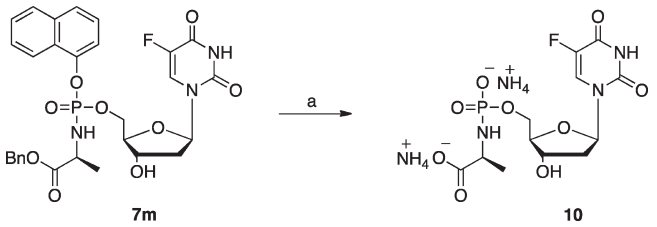



**Figure 6.** Carboxypeptidase Y assay applied on ProTide **7d** and monitored by $^{31}$P NMR, 25 °C.

**Scheme 2. Synthesis of Intermediate 10**[a]




[a] Reagents and conditions: (a) $Et_3N/H_2O$ (1:1), 35 °C, 16 h.

peaks corresponding to **7a** at ~$\delta_P$ 4.59 and 4.84 ppm. After about 6 h and 45 min the compound was hydrolyzed partly to the intermediate **8** shown as a single peak at $\delta_P$ 5.79 ppm. After 11 h and 30 min, the formation of the second metabolite **10** shown as a single peak at $\delta_P$ 7.09 ppm was observed. After 13 h and 30 min the reaction mixture contained 96% of the parent compound **7a** together with the proposed metabolites **8** (3%) and **10** (1%).

## CONCLUSIONS

We herein report the successful application of ProTide technology to the anticancer agent FUDR. Several ProTides emerged that

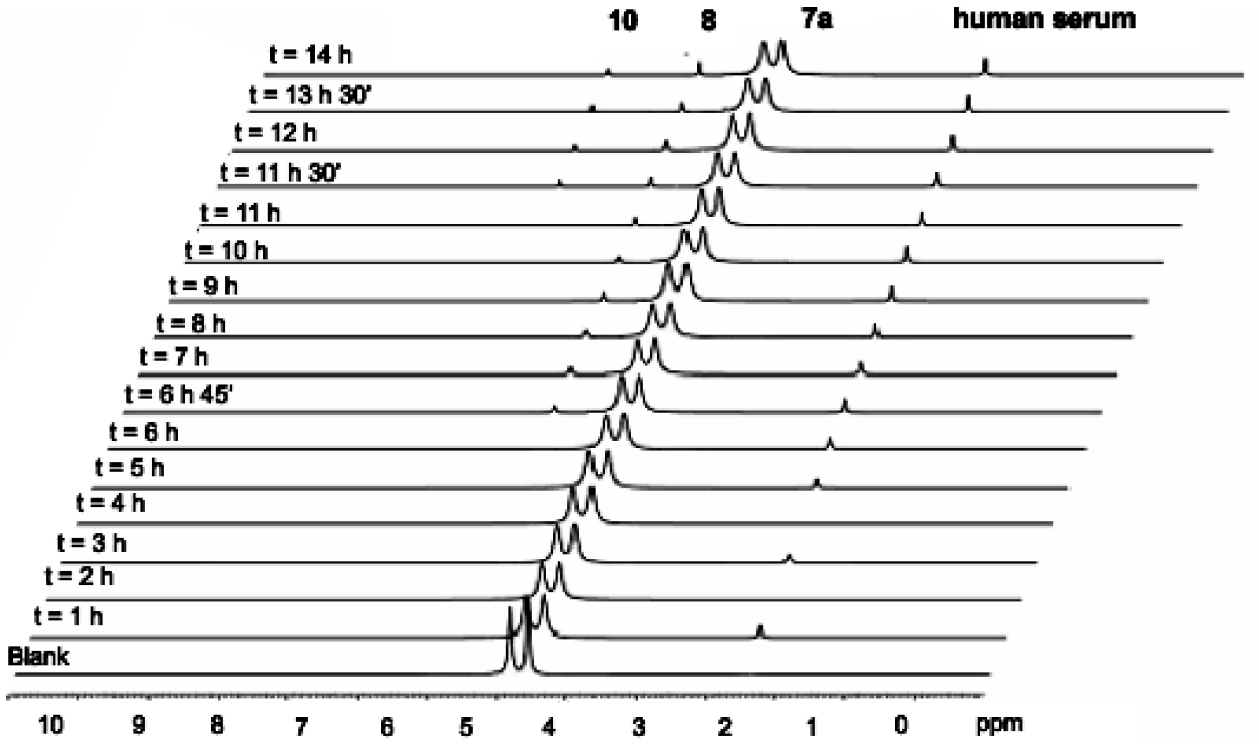



**Figure 7.** Human serum assay applied on ProTide **7a** and monitored by $^{31}$P NMR, 37 °C.

retain the high potency of FUDR in vitro and in addition partially bypass the high dependence of the parent nucleoside on kinase-mediated activation and on cell transporter-mediated uptake.

The compounds are also resistant to thymidine phosphorylase and do not show significant loss of activity as displayed by FUDR upon mycoplasma infection of the tumor cell cultures. The ProTides are stable in acid and at neutral pH and in plasma but are activated by intracellular carboxypeptidase. The ability of the ProTides to overcome several of the sources of resistance of FUDR in the clinic suggests that these agents should be further progressed to (pre)clinical trials.

## EXPERIMENTAL SECTION

**Cell Cultures and Cytostatic Assays.** Murine leukemia L1210/0, human T-lymphocyte CEM/0, and human cervix carcinoma HeLa/0 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Thymidine kinase deficient CEM/TK$^-$ cells were a kind gift from Prof. S. Eriksson (currently at Uppsala University, Uppsala, Sweden) and Prof. A. Karlsson (Karolinska Institute, Stockholm, Sweden), and CEM/hENT-0 samples were obtained from Prof. Cass (Cross Cancer Institute, Edmonton, Alberta, Canada). Thymidine kinase deficient L1210/TK$^-$ and HeLa/TK$^-$ cells were derived from L1210/0 and HeLa/0 cells, respectively, after selection for resistance against 5-bromo-2′-dUrd. The HeLa/TK$^-$ cells were kindly provided by Prof. Y.-C. Cheng, Yale University, New Haven, CT. Infection of the cell lines with *Mycoplasma hyorhinis* (ATCC) resulted in chronically infected cell lines further referred to as L1210.Hyor. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), 10 mM Hepes, and 1 mM sodium pyruvate (Invitrogen). Cells were grown at 37 °C in a humidified incubator with a gas phase of 5% $CO_2$.

Monolayer cells (HeLa/0 and HeLa/TK$^-$) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark) at 10 000 cells/well. After 24 h, an equal volume of fresh medium containing the test compounds was added. On day 5, cells were trypsinized and counted in a Coulter counter (Analis, Suarlée, Belgium). Suspension cells (L1210/0, L1210/TK$^-$, L1210.Hyor, CEM/0, CEM/TK$^-$, CEM/hEnt-1, CEM/hEnt-0) were seeded in 96-well microtiter plates (Nunc) at 60 000 cells/well in the presence of a given amount of the test compounds. The cells were allowed to proliferate for 48 h (L1210) or 72 h (CEM) and were then counted in a Coulter counter. The 50% inhibitory concentration ($IC_{50}$) was defined as the compound concentration required to reduce cell proliferation by 50%. In the nucleoside transporter inhibition experiments, dipyridamole (10 $\mu$M) and NBMPR (10 $\mu$M) were added to the CEM/hEnt-1 cells in the presence of different concentrations of the test compounds. The cytostatic activity of the compounds was determined after 3 days, as outlined above.

**Phosphorylase Assay Using Thymidine Phosphorylase Purified from *Escherichia coli*.** The experiment was carried out by dissolving FUDR ProTide **6e** (6.0 mg) in methanol-$d_4$ (0.05 mL), followed by addition of 300 mM potassium phosphate buffer (pH 7.4, 0.45 mL). The resulting cloudy solution was submitted to the $^{19}$F NMR experiment at 25 °C, and the data were recorded as a control. Then to that sample was added thymidine phosphorylase (17 $\mu$L). The resulting sample was submitted for $^{19}$F NMR experiment. Additional $^{19}$F NMR experiments for the same sample was repeated after 72 h.

**$^{31}$P NMR Stability Experiments in Acidic and Basic pH.** *Buffer pH 1.* The stability assay toward hydrolysis by aqueous buffer at pH 1 was conducted using in situ $^{31}$P NMR (202 MHz). The experiment was carried out by dissolving FUDR ProTide **6e** (2.6 mg) in methanol-$d_4$ (0.10 mL) and then adding buffer, pH 1 (prepared from equal parts of 0.2 M HCl and 0.2 M KCl). Next, the sample was subjected to $^{31}$P NMR experiments at 37 °C and the spectra were recorded every 12 min over 14 h.

*Buffer pH 8.* The stability assay toward hydrolysis by aqueous buffer at pH 8 was conducted using in situ $^{31}$P NMR (202 MHz). The experiment was carried out by dissolving FUDR ProTide **6e** (4.5 mg) in methanol-$d_4$ (0.10 mL) and then adding buffer, pH 8 (prepared from solution of 0.1 M $Na_2HPO_4$ and adjusted to the appropriate pH using 0.1 M HCl).

Next, the sample was subjected to $^{31}$P NMR experiments at 37 °C and the spectra were recorded every 12 min over 14 h.

**Carboxypeptidase Y (EC 3.4.16.1) Assay.** The experiment was carried out by dissolving FUDR ProTide **7d** (3.0 mg) in acetone-$d_6$ (0.15 mL) and by adding 0.30 mL of Trizma buffer (pH 7.6). After the $^{31}$P NMR data were recorded at 25 °C as a control, a previously defrosted carboxypeptidase Y (0.1 mg dissolved in 0.15 mL of Trizma) was added to the sample. Next, the sample was submitted to $^{31}$P NMR experiments (at 25 °C) and the spectra were recorded every 7 min over 14 h. $^{31}$P NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

**Stability Assay in Human Serum.** The experiment was carried out by dissolving FUDR ProTide **7a** (5.0 mg) in DMSO (0.050 mL) and $D_2O$ (0.15 mL). After the $^{31}$P NMR data were recorded at 37 °C as a control, a previously defrosted human serum (0.30 mL) was added to the sample. Next, the sample was submitted to $^{31}$P NMR experiments at 37 °C and the spectra were recorded every 15 min over 14 h. $^{31}$P NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

**Chemistry. General.** Anhydrous solvents were obtained from Aldrich and used without further purification. Amino acid esters were purchased from Carbosynth. Carboxypeptidase Y, human serum, and buffers were from Sigma-Aldrich. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with $^{31}$P NMR spectra. Column chromatography was performed on silica gel (35−70 $\mu$M). Proton ($^1$H), carbon ($^{13}$C), phosphorus ($^{31}$P), and fluorine ($^{19}$F) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak, and all $^{13}$C NMR and $^{31}$P NMR were proton-decoupled. The purity of final compounds was verified to be >95% by HPLC analysis using Varian Polaris C18-A (10 $\mu$M) as an analytic column with a gradient elution of $H_2O$/MeOH from 100/0 to 0/100 in 45 min (method 1) and with a gradient elution of $H_2O$/$CH_3CN$ from 100/0 to 0/100 in 35 min (method 2). The HPLC analysis was conducted by Varian Prostar (LC Workstation-Varian prostar 335 LC detector). Low and high resolution mass spectra were performed as a service by Birmingham University, Birmingham, U.K., using electrospray mass spectrometry (ESMS). CHN microanalysis was performed as a service by MEDAC Ltd., Surrey, U.K.

**General Method for the Preparation of Phosphorochloridates (5).** Anhydrous triethylamine (2.0 mol equiv) was added dropwise at −78 °C to a stirred solution of the appropriate aryl dichlorophosphate (1.0 mol equiv) and an appropriate amino acid ester (1.0 mol equiv) in anhydrous DCM under argon atmosphere. Following the addition, the reaction mixture was allowed to slowly warm to room temperature and was stirred for 1−2 h. The formation of desired compound was monitored by $^{31}$P NMR. After the reaction was completed, the solvent was evaporated under reduced pressure and the resulting residue was redissolved in anhydrous $Et_2O$ and filtered. The filtrate was reduced to dryness to give a crude product as an oil, which was in some cases used without further purification in the next step. Most of aryl phosphorochloridates, in particular those obtained from the amino acid tosylate salt, were purified by flash column chromatography using EtOAc/hexane (7:3) as an eluent.

**1-Naphthyl (Benzyl-L-alaninyl)phosphorochloridate (5m).** Yellowish oil; yield, 47% (1.82 g). $R_f$ = 0.90 (hexane−EtOAc, 7:3). $^{31}$P NMR (202 MHz, $CDCl_3$, mixture of diastereoisomers): $\delta_P$ 7.92, 8.14 (int, 1.00:1.00). $^1$H NMR (500 MHz, $CDCl_3$, mixture of diastereoisomers with a ratio of 1:1): $\delta_H$ 8.12−7.97 (m, 1H, Ar*H*), 7.73−7.09 (m, 11H, Ar*H*), 5.09 (s, 2H, OC$H_2$Ph), 4.81−4.78 (m, 1H, N*H*), 4.23−4.20 (m, 1H, C*H*CH$_3$), 1.45−1.43 (m, 3H, CHC$H_3$).

**General Method for the Preparation of FUDR ProTides (6a−n and 7a−y).** To a solution of 5-fluoro-2′-deoxyuridine (0.25 g, 1.01 mmol) in dry THF (10 mL) at 0 °C under argon atmosphere was added dropwise NMI (0.40 mL, 5.07 mmol). The reaction mixture was allowed to stir for 30 min, and then a solution of appropriate phosphorochloridate (**5**) (3.04 mmol) dissolved in anhydrous THF (3 mL) was added dropwise. The reaction mixture was stirred at room temperature for 16−18 h and then evaporated in vacuo to give a residue that was redissolved in $CH_2Cl_2$ and washed twice with 0.5 M HCl (2 × 5 mL). The organic phase was purified by column chromatography on silica gel, eluting with $CH_2Cl_2$−MeOH as a gradient (0−5% MeOH) to afford the products as white solid.

**5-Fluoro-2′-deoxyuridine 5′-*O*-[1-Naphthyl(benzyl-L-alaninyl)] phosphate (7m).** **7m** was obtained from 5-fluoro-2′-deoxyuridine and **5m** as a white solid. Yield, 8% (47.0 mg). $R_f$ = 0.19 ($CH_2Cl_2$−MeOH, 95:5). (ES+) *m*/*z*, found: (M + Na$^+$) 636.1520. $C_{29}H_{29}N_3O_9FNaP$ required: (M$^+$), 613.15. Mixture of diastereoisomers (43%, 57%). $^{31}$P NMR (202 MHz, MeOD): $\delta_P$ 4.61, 4.25. $^{19}$F NMR (470 MHz, MeOD): $\delta_F$ −167.45, −167.25. $^1$H NMR (500 MHz, MeOD): $\delta_H$ 8.18−8.12 (m, 1H, Ar*H*), 7.90−7.86 (m, 1H, Ar*H*), 7.72−7.67 (m, 2H, Ar*H*, *H*-6), 7.55−7.47 (m, 3H, Ar*H*), 7.45−7.27 (m, 6H, Ar*H*), 6.16−6.06 (m, 1H, *H*-1′), 5.13, 5.08 (2 × AB system, 2H, *J* = 12.0 Hz, OC$H_2$Ph), 4.36−4.24 (m, 3H, 2 × *H*-5′, *H*-3′), 4.15−4.03 (m, 2H, C*H*CH$_3$, *H*-4′), 2.17−2.08 (m, 1H, *H*-2′), 1.79−1.67 (m, 1H, *H*-2′), 1.38−1.34 (m, 3H, CHC$H_3$). $^{13}$C NMR (125 MHz, MeOD): $\delta_C$ 174.9 (d, $^3J_{C-P}$ = 4.3 Hz, *C*═O, ester), 174.6 (d, $^3J_{C-P}$ = 5.0 Hz, *C*═O, ester), 159.3 (d, $^2J_{C-F}$ = 26.1 Hz, *C*═O, base), 150.5 (d, $^4J_{C-F}$ = 4.0 Hz, *C*═O, base), 147.9 (d, $^2J_{C-P}$ = 7.4 Hz, *C*-Ar, Naph), 147.8 (d, $^2J_{C-P}$ = 7.7 Hz, OC-Naph), 141.7, 141.6 (2d, $^1J_{C-F}$ = 234.0 Hz, *C*F-base), 137.2, 137.1, 136.2 (*C*-Ar), 129.7, 129.6, 129.5, 129.4, 129.0, 128.9, 128.1, 128.0 (*C*H-Ar), 127.9, 127.8 (*C*-Ar), 127.7, 127.6, 126.6, 126.5, 126.2 (*C*H-Ar), 125.6, 125.5 (2*d*, $^2J_{C-F}$ = 34.0 Hz, *C*H-base), 122.6 (*C*H-Ar), 116.5, 116.2 (2d, $^3J_{C-P}$ = 3.5 Hz, *C*H-Ar), 87.0, 86.9 (*C*-1′), 86.8, 86.7 (2d, $^3J_{C-P}$ = 8.1 Hz, *C*-4′), 72.1, 72.0 (*C*-3′), 68.1, 68.0 (C$H_2$Ph), 67.8, 67.6 (2d, $^2J_{C-P}$ = 5.2 Hz, *C*-5′), 51.9, 51.8 (CHCH$_3$), 40.9, 40.8 (*C*-2′), 20.5 (d, $^3J_{C-P}$ = 6.5 Hz, CHCH$_3$), 20.3 (d, $^3J_{C-P}$ = 7.6 Hz, CHCH$_3$). Reverse HPLC, eluting with $H_2O$/MeOH from 100/0 to 0/100 in 45 min, showed two peaks of the diastereoisomers with $t_R$ = 34.23 min and $t_R$ = 34.59 min (47%, 51%). Anal. Calcd for $C_{29}H_{29}FN_3O_9P$: C, 56.77; H, 4.76; N, 6.85. Found: C, 56.57; H, 5.06; N, 6.72. UV (0.05 M phosphate buffer, pH 7.4) $\lambda_{max}$ = 271 nm ($\varepsilon_{max}$ = 7050). log *P* measured: 1.74.

**5-Fluoro-2′deoxyuridine 5′-*O*-[(L-Alaninyl)]phosphate Ammonium Salt (10′).** 5-Fluoro-2′deoxyuridine 5′-*O*-[1-naphthyl(benzyl-L-alaninyl)]phosphate (**7m**) (0.08 g, 0.130 mmol) was dissolved in a solution of triethylamine (5 mL) and water (5 mL). The reaction mixture was stirred at 35 °C for 16 h, and then the solvents were removed under reduced pressure. The residue was treated with water and extracted with dichloromethane. The aqueous layer was concentrated and evaporated under reduced pressure. Then the resulting crude material was purified by column chromatography on silica, eluting with 2-propanol−$H_2O$−$NH_3$ (8:1:1) to afford the title compound **10′** as a white solid. Yield, 30% (15.0 mg). $R_f$ = 0.04 (2-propanol−$H_2O$−$NH_3$ (8:1:1)). $^{31}$P NMR (202 MHz, $D_2O$): $\delta_P$ 7.13. $^{19}$F NMR (470 MHz, $D_2O$): $\delta_F$ −168.00. $^1$H NMR (500 MHz, $D_2O$): $\delta_H$ 7.93 (d, 1H, $^3J_{H-F}$ = 6.1 Hz, *H*-6), 6.30−6.25 (m, 1H, *H*-1′), 4.49−4.44 (m, 1H, *H*-3′), 4.11−4.06 (m, 1H, *H*-4′), 3.94−3.83 (m, 2H, *H*-5′), 3.53 (q, 1H, *J* = 7.5 Hz, C*H*CH$_3$), 2.37−2.28 (m, 2H, *H*-2′), 1.25−1.19 (m, 3H, CHC$H_3$). $^{13}$C NMR (125 MHz, MeOD): $\delta_C$ 174.8 (d, $^3J_{C-P}$ = 4.6 Hz, *C*═O), 159.2 (d, $^2J_{C-F}$ = 26.2 Hz, *C*═O, base), 150.3 (d, $^4J_{C-F}$ = 4.0 Hz, *C*═O, base), 141.8 (d, $^1J_{C-F}$ = 233.8 Hz, *C*F-base), 125.6 (d, $^2J_{C-F}$ = 34.0 Hz, *C*H-base), 87.0 (*C*-1′), 86.7 (d, $^3J_{C-P}$ = 7.5 Hz, *C*-4′), 71.1 (*C*-3′), 67.2 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5′), 51.0 (CHCH$_3$), 40.2 (*C*-2′), 20.3 (d, $^3J_{C-P}$ = 7.2 Hz, CHCH$_3$). *m*/*z* (ES) 396.1 (M − 2 NH$_4^-$ + H]$^-$, 100%). Reverse-phase HPLC, eluting with $H_2O$/MeOH from 100/0 to 0/100 in 45 min, 1 mL/min, $\lambda$ = 275 nm, showed a peak of the diastereoisomer with $t_R$ = 3.65 min (95%).

## ■ ASSOCIATED CONTENT

**Supporting Information.** Preparative methods and spectroscopic and analytical data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS USED

TLC, thin layer chromatography; FUDR, 2′-deoxy-5-fluorouridine; TP, thymidine phosphorylase; $TK^-$, thymidine kinase deficient; OPRT, orotate phosphoribosyl transferase; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CEM, human T-lymphocyte; NBMPR, *S*-(4-nitrobenzyl)-6-thioinosine; ESMS, electrospray mass spectrometry; HPLC, high performance liquid chromatography; ClogP, calculated logarithm of the octanol/water partition

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