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Application of ProTide Technology to Gemcitabine: A successful approach to overcome the key cancer resistance mechanisms leads to a new agent (NUC-1031) in clinical development.

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## ABSTRACT

Gemcitabine is a nucleoside analogue commonly used in cancer therapy but with limited efficacy due to a high susceptibility to cancer cell resistance. The addition of a phosphoramidate motif to the gemcitabine can protect it against many of the key cancer resistance mechanisms. We have synthesized a series of gemcitabine phosphoramidate prodrugs and screened for cytostatic activity in a range of different tumor cell lines. Among the synthesized compounds, one in particular (NUC-1031, **6f**) was shown to be potent *in vitro*. Importantly, compared with gemcitabine, **6f** activation was significantly less dependent on deoxycytidine kinase and on nucleoside transporters, and it was resistant to cytidine deaminase-mediated degradation. Moreover, **6f** showed a significant reduction in tumor volumes *in vivo* in pancreatic cancer xenografts. The ProTide **6f** is now in clinical development with encouraging efficacy signals in a Phase I/II study, which strongly supports the ProTide approach to generate promising new anti-cancer agents.

## INTRODUCTION

Nucleoside analogues and nucleobases constitute a major class of chemotherapeutic agents and are widely used for the treatment of patients with cancer. This group of agents, known as anti-metabolites, includes a variety of pyrimidine and purine nucleoside derivatives with cytotoxic activity in both hematological and solid tumors. Gemcitabine<sup>1</sup> (2',2'- difluoro-2'-deoxycytidine) is a pyrimidine nucleoside analogue, shown to be active against several solid tumor types.<sup>2</sup> Following FDA approval in 1996, gemcitabine has become the standard of care for the treatment of pancreatic cancer.<sup>3</sup> More recently, the compound has also gained approval for treating non-small cell lung, ovarian, bladder, and breast cancer.<sup>4</sup>

At a molecular level, the mechanisms underpinning the anti-cancer effect of gemcitabine rely on the sequential conversion of the compound into the monophosphate (dFdCMP), diphosphate (dFdCDP) and then triphosphate (dFdCTP) forms. Gemcitabine triphosphate replaces deoxycytidine during DNA replication, which leads to cell arrest.<sup>5</sup> Failure to repair the DNA subsequently triggers apoptosis and blocks tumor growth. In addition, several self-potentiating mechanisms have been reported, such as inhibition of DNA synthesis by dFdCTP and of ribonucleotide reductase (RNR) by dFdCDP.<sup>6</sup> Notably all of these mechanisms require phosphorylation of gemcitabine at least to the monophosphate level.

Both innate and acquired resistance to nucleoside analogues (such as gemcitabine, 5-fluorouracil, cytarabine and fludarabine) is a common problem in the treatment of cancer and is regarded as a key driver of poor patient survival outcomes. Gemcitabine, like all nucleoside analogues, faces numerous inherent and acquired cancer resistance mechanisms that dramatically limit its effectiveness. These include: (i) poor conversion of the drug into the active dFdCDP and dFdCTP forms; (ii) rapid degradation into toxic byproducts; and (iii) limited uptake by cancer cells. These effects are due to: (i) down-regulation of the key initial phosphorylating enzyme deoxycytidine kinase (dCK)<sup>7</sup> required to convert gemcitabine into the monophosphate form, considered the key rate-limiting step in its activation process<sup>8</sup>; (ii) expression of the key

deactivating enzyme cytidine deaminase<sup>9</sup>; and (iii) deficiency of nucleoside transporter<sup>10</sup> proteins. In addition, increased expression and/or activity of cytidine deaminase (CDA) increases the degradation of gemcitabine into the toxic metabolite 2',2'-difluoro-2'-deoxyuridine (dFdU).<sup>9</sup> Because of these processes, single agent gemcitabine has limited activity in cancer treatment, with response rates of less than 10% in pancreatic cancer.<sup>11</sup>

Various strategies to improve the treatment efficiency of gemcitabine have been developed, including the chemical modifications and functionalization of its 4-(*N*) and 5'-sites (reviewed in Moysan *et al.*).<sup>12</sup> In 2009, Bender *et al.* reported an orally active prodrug of gemcitabine, LY2334737 which is significantly less prone to degradation by CDA due to a valproic acid linkage at the 4-(*N*)-position.<sup>13</sup> Based on *in vivo* data in the HCT-116 human colon xenograft, LY2334737 has been further developed and advanced into Phase I clinical studies, either alone or in combination therapy with other agents.<sup>14</sup> As an alternative approach, CP-4126 was developed<sup>15</sup> to enhance the cellular uptake of gemcitabine, by attaching a fatty acid chain to the ribose 5'-position, but this failed to show superiority over gemcitabine in clinical trials.<sup>16</sup> While these strategies may offer theoretical benefits, they only address one or two of the three known resistance mechanisms associated with gemcitabine. Notably, these approaches do not overcome the inability of the resistant cancer cell to activate gemcitabine through phosphorylation. This obligatory first-phosphorylation step is likely to limit the therapeutic effectiveness of these prodrugs, as is established for the parent nucleoside analogue. Therefore, a different approach, utilizing a pre-activated monophosphate form of these nucleoside analogues has emerged as a therapeutic approach.<sup>17</sup>

A major strategy currently applied in the modulation of several antiviral nucleoside analogues is the ProTide technology developed in 1995 by our laboratories.<sup>18</sup> This method is designed to overcome the key resistance mechanisms associated with nucleoside analogues in general. Bearing one phosphate group protected with biolabile groups (termed the phosphoramidate motif), the ProTides are pre-activated, thereby bypassing the initial rate-limiting step of kinase phosphorylation into the monophosphate form. Importantly, the cellular

uptake of these compounds is independent of nucleoside transporters and their resistance to deactivation by deaminases and other enzymes is superior to nucleoside analogues.<sup>19</sup> The addition of the phosphoramidate motif will also change the pharmacokinetic/dynamic properties, that may be favorable for therapy. Overcoming cancer resistance mechanisms should lead to significant clinical benefits.

In our laboratory we have extensively investigated the phosphoramidate ProTide approach and successfully applied this technology to many active antiviral agents such as d4T,<sup>20</sup> abacavir,<sup>21</sup> and various anti-hepatitis C virus agents.<sup>22</sup> Similarly, we generated ProTides based on several anti-cancer nucleosides such as cladribine,<sup>23</sup> clofarabine<sup>24</sup> and recently to 5-fluoro-2'-deoxyuridine (FUDR),<sup>25</sup> with very encouraging results. Here we report the application of the ProTide technology to the cornerstone of pancreatic cancer treatment, gemcitabine, and the screening process that has identified a novel lead candidate, now in clinical development.

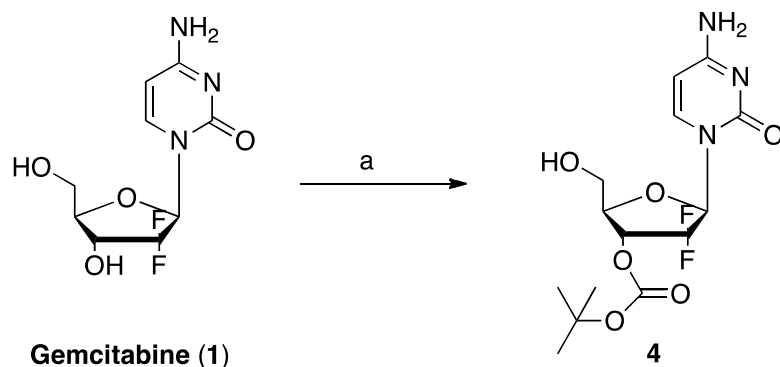
The potency of the compounds varies with the individual components (aryl, ester and amino acid) of the phosphoramidate moiety and therefore we examined a panel of gemcitabine ProTides. Thus, we herein report for the first time in the open literature the design, synthesis, anti-tumor evaluation and metabolic studies of a panel of L-alanine-based gemcitabine phosphoramidates. **NUC-1031 (6f)**, first patented by us in 2005 as CPF-31<sup>26</sup>) emerged as the most promising compound. The compound is currently making progress through the clinical development stage and has already shown very encouraging activity against a range of solid tumors and a favorable safety profile in a Phase I study.<sup>27</sup>

## CHEMISTRY

The synthesis of gemcitabine ProTides is largely based on phosphorochloridate chemistry and requires the preparation of two components: a regioselectively protected gemcitabine on the 3'-OH group and an aryl aminoacyl phosphorochloridate.

First, gemcitabine (**1**) was selectively protected on the 3'-OH group<sup>28</sup> in order to favor the

5'-regio-selectivity of the phosphorylation with the aryl aminoacyl phosphorochloridates (Scheme 1). Thus, gemcitabine was allowed to react with *tert*-butyl dicarbonate in 1,4-dioxane and water (4:1 v/v) at room temperature for 48h to give the corresponding intermediate (**4**) with a 75% yield after column chromatography.



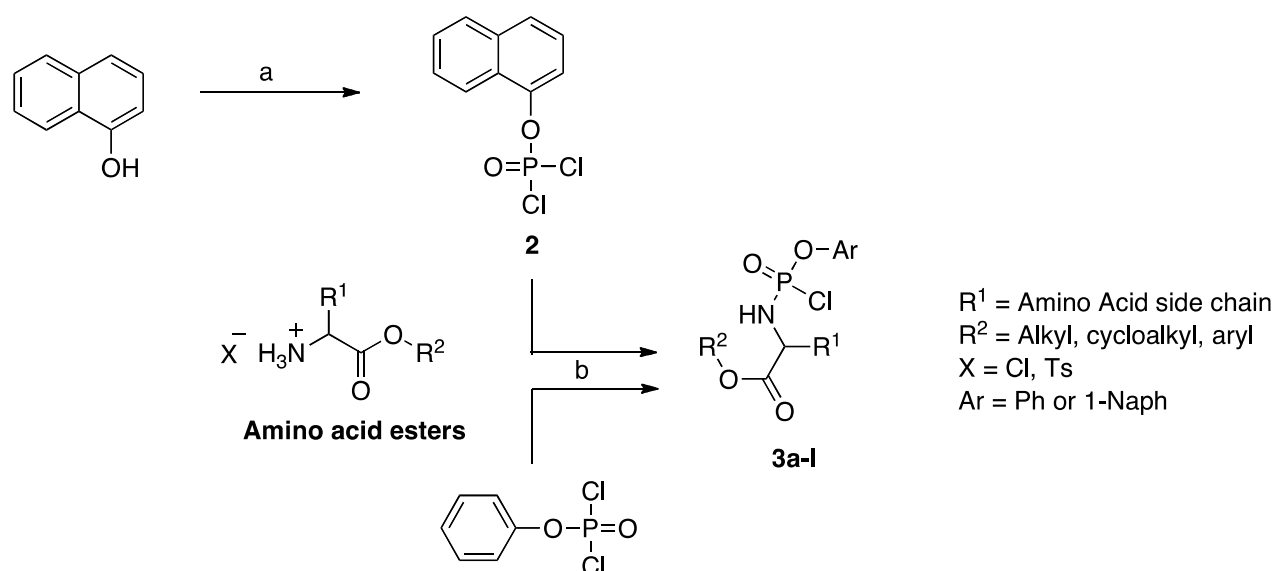
**Scheme 1.** Selective protection of gemcitabine (**1**)

Reagents and conditions: a) DBOC, Na<sub>2</sub>CO<sub>3</sub>, 1,4-dioxane/H<sub>2</sub>O (4:1 v/v), rt, 48h.

In parallel, the aryl aminoacyl phosphorochloridates (**3**), which are key reagents in the ProTide methodology, are prepared using two reagents: an esterified amino acid and an aryloxy phosphorodichloridate (Scheme 2).<sup>29</sup> The first reagent, an amino acid ester, if not commercially available, can be prepared by esterification of the appropriate amino acid via standard esterification methods.<sup>30</sup> The second reagent is an aryl (phenyl or 1-naphthyl) phosphorodichloridate; the phenyl phosphorodichloridate is commercially available, whereas the 1-naphthyl analogue (**2**) can be obtained by reacting 1-naphthol with phosphorus oxychloride and triethylamine following published procedures.<sup>31</sup>

The aryl aminoacyl phosphorochloridates (**3**) were formed by reaction of phenyl or 1-naphthyl phosphorodichloridates with appropriate amino acid esters in the presence of triethylamine, as outlined in Scheme 2. Chiral amino acid esters generated a pair of diastereoisomers at the phosphate center, in about 1:1 ratio, visible by two close peaks in the <sup>31</sup>P NMR spectra. In most cases, the aryl aminoacyl phosphorochloridate (**3**) syntheses were completed within two hours as monitored by <sup>31</sup>P NMR. Because of their limited stability, these products (**3**) were then used as crude materials, or after rapid silica gel chromatography, in the

ProTide syntheses.



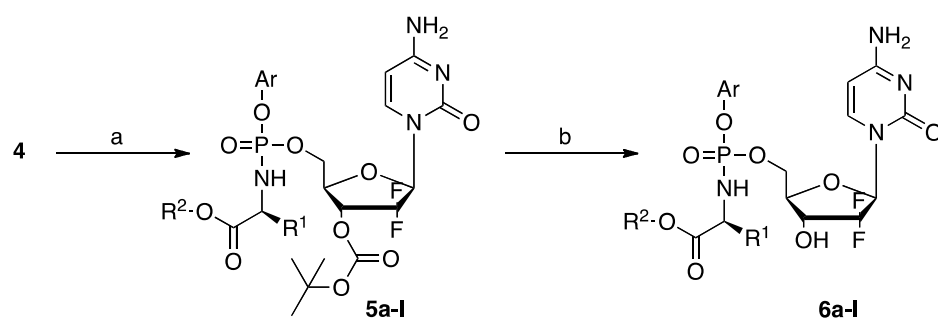
**Scheme 2.** Synthesis of aryl aminoacyl phosphorochloridates (**3a-l**)

Reagents and conditions: a) POCl<sub>3</sub>, Et<sub>3</sub>N, dry Et<sub>2</sub>O, -78 °C for 1h then rt for 1h; b) (**2**) or phenyl phosphorodichloridate, Et<sub>3</sub>N, dry DCM, -78 °C, 1-3h.

Next, each aminoacyl phosphorochloridates (**3**) were reacted with the 3'-protected gemcitabine (**4**) in the presence of *tert*-butylmagnesium chloride (1.0 M solution in THF) as shown in Scheme 3. Overnight reactions at ambient temperature generated crude materials that were purified by column chromatography to provide good yields of 3'-Boc-protected phosphoramidates **5a-l**. Finally, the end products **6a-l** were obtained through acidic deprotection, typically 2-4 hours reaction at 0 °C in TFA/DCM (1:1 v/v), followed by basic work-up and column purification on silica gel. As a result of the phosphate chirality, ProTides **6a-l** were isolated as diastereoisomeric mixtures, evidenced by <sup>31</sup>P NMR, HPLC (two peaks), <sup>1</sup>H NMR, and <sup>13</sup>C NMR (with splitting of many nucleoside signals). Extensive Structure/Activity Relationship (SAR) studies,<sup>32</sup> and prior work suggested that the L-alanine phosphoramidates were the most potent gemcitabine ProTides.<sup>26</sup> Thus, we further explored this ProTide family bearing the L-alanine residue by altering the aryl and ester moieties. This led us to prepare both series of phenyl and 1-naphthyl L-alanine gemcitabine ProTides with branched (isopropyl,



neopentyl), linear (pentyl, hexyl), cyclic (cyclopentyl, cyclohexyl) and benzyl ester motifs.



- 5.6a:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = iPro, Ar = Ph  
**5.6b:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = Pentyl, Ar = Ph  
**5.6c:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = Hexyl, Ar = Ph  
**5.6d:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = CH<sub>2</sub>tBu, Ar = Ph  
**5.6e:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = cHex, Ar = Ph  
**5.6f:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = Bn, Ar = Ph  
**5.6g:** R<sup>1</sup> = Dimethylglycine side chain, R<sup>2</sup> = Pentyl, Ar = 1-Naph  
**5.6h:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = Hexyl, Ar = 1-Naph  
**5.6i:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = cPentyl, Ar = 1-Naph  
**5.6j:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = cHexyl, Ar = 1-Naph  
**5.6k:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = Bn, Ar = 1-Naph  
**5.6l:** R<sup>1</sup> = L-Ala side chain, R<sup>2</sup> = Pentyl, Ar = 1-Naph

### Scheme 3. Preparation of gemcitabine ProTides (**5a-l**, **6a-l**) under coupling conditions

Reagents and conditions: a) (ArO)(R<sup>2</sup>OC(O)CR<sup>1</sup>NH)P(O)Cl (**3a-l**), *tert*-BuMgCl, THF, rt, 16h;

b) TFA/DCM (1:1 v/v), 0 °C, 2-4h.

### BIOLOGICAL ACTIVITY *IN VITRO*

Nucleoside analogues face numerous inherent and acquired cancer resistance mechanisms that can dramatically limit their effectiveness. ProTides are specifically designed to overcome these key cancer resistance pathways and thereby achieve a superior anti-neoplastic effect. Over the years, our laboratory has created a large library of over 3,500 ProTides and built considerable know-how as to structure/activity relationships through multiple rounds of pharmacomodulation and functional tests. By applying the ProTide chemistry to gemcitabine, more than 80 different compounds were generated with various natural amino acids (data not published). Early chemical and biological screens during the development of this ProTide family, led to the conclusion that L-alanine-based molecules best enhanced functional activity. This is in keeping with previous experience. Several L-alanine phosphoramidate compounds were synthesized, with numerous variations on the aryl and ester groups, and these were screened *in vitro* for

cytotoxic activity against a range of cancer cell lines. Of these, six compounds showed activity comparable to or better than gemcitabine and the results are summarised in Table 1. ProTides showed a broad spectrum of activity levels around the gemcitabine IC<sub>50</sub> reference value, which was used as a positive control. Notably, within the series of compounds tested, ProTides **6f**, **6i** and **6k** demonstrated better (2 to 4 fold increase) potency than gemcitabine in pancreatic (MiaPaCa-2, BxPC-3) cell lines. Because gemcitabine is the cornerstone of pancreatic cancer treatment, these data were particularly encouraging.

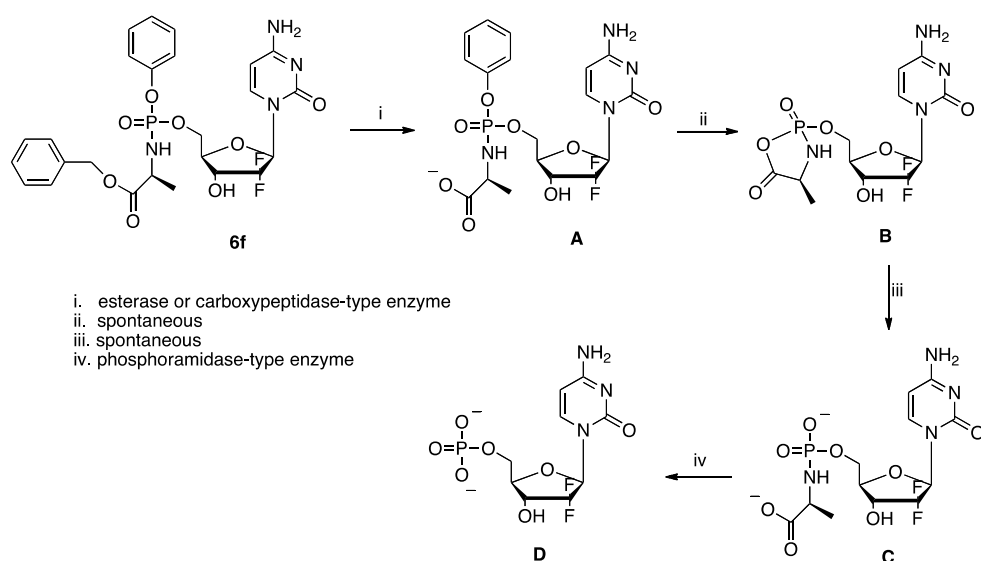
**Table 1.** IC<sub>50</sub> (μM) values of selected prodrug derivatives against four cancer cell lines

Entry	Amino acid	Ester	Aryl	IC <sub>50</sub> (μM) <sup>a</sup>			
				L1210	CEM	MP-2	BXPC-3
<b>6a</b>	L-Ala	iPrO	Ph	>5	>5	>5	>5
<b>6b</b>	L-Ala	Pentyl	Ph	0.024 ± 0.009	0.08 ± 0.011	N.D.	N.D.
<b>6c</b>	L-Ala	Hexyl	Ph	0.016 ± 0.005	0.081 ± 0.008	N.D.	N.D.
<b>6d</b>	L-Ala	CH <sub>2</sub> tBu	Ph	0.39 ± 0.04	0.44 ± 0.04	1.41 ± 0.57	0.36 ± 0.13
<b>6e</b>	L-Ala	cHexyl	Ph	0.6 ± 0.06	0.42 ± 0.17	N.D.	N.D.
<b>6f</b>	L-Ala	Bn	Ph	0.035 ± 0.02	0.1 ± 0.03	0.44 ± 0.06	0.15 ± 0.04
<b>6g</b>	DMG <sup>b</sup>	Pentyl	1-Naph	0.16 ± 0.03	0.13 ± 0.01	N.D.	N.D.
<b>6h</b>	L-Ala	Hexyl	1-Naph	0.022 ± 0.005	0.075 ± 0.005	N.D.	N.D.
<b>6i</b>	L-Ala	cPentyl	1-Naph	0.18 ± 0.03	0.11 ± 0.00	0.46 ± 0.24	0.15 ± 0.05
<b>6j</b>	L-Ala	cHexyl	1-Naph	0.32 ± 0.03	0.14 ± 0.02	N.D.	N.D.
<b>6k</b>	L-Ala	Bn	1-Naph	0.035 ± 0.03	0.10 ± 0.03	0.57 ± 0.22	0.24 ± 0.05
<b>6l</b>	L-Ala	Pentyl	1-Naph	0.03 ± 0.01	0.073 ± 0.004	N.D.	N.D.
<b>dFdC (1)</b>				0.013 ± 0.02	0.086 ± 0.003	1.04 ± 0.71	0.67 ± 0.34

<sup>a</sup> IC<sub>50</sub> (50% Inhibitory Concentration) is the compound concentration required to inhibit tumor cell proliferation by 50%. <sup>b</sup> DMG is dimethylglycine. Data shown are the mean of at least two independent experiments.

To exert their anti-cancer activity the ProTides should be metabolised to release the monophosphate form, which will then generate the active compounds, gemcitabine di- and tri-phosphate. The proposed intracellular activation route of the ProTides has been described<sup>33</sup> for other ProTide families, such as the FUDR ProTides, and is depicted in Scheme 4. The hypothesised metabolic route would begin with the hydrolysis of the ProTide ester (i), mediated

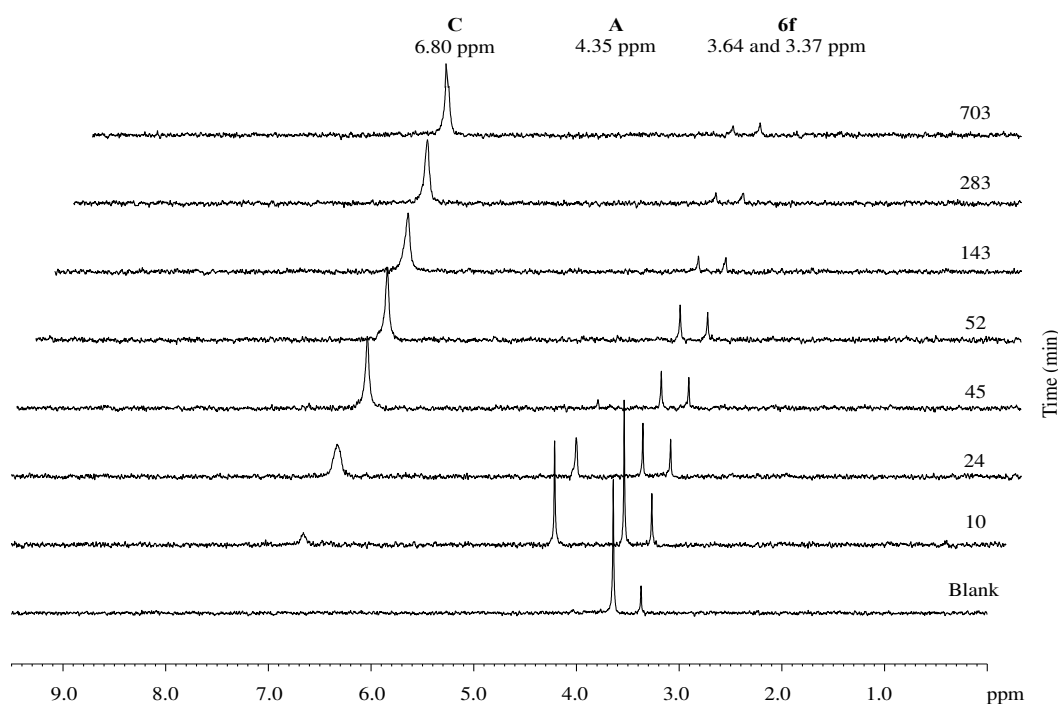
by a carboxyesterase-type enzyme to form the intermediate (**A**). This step is followed by a spontaneous cyclisation (ii) displacing the aryl moiety via an internal nucleophilic attack of the carboxylate residue on the phosphorus centre to yield (**B**). In a third step (iii) the unstable cyclic mixed anhydride would be hydrolysed to release the intermediate (**C**). The final step (iv) would then involve a phosphoramidase-type enzyme, which would cleave off the amino acid to generate the corresponding monophosphate (**D**).



**Scheme 4.** Proposed activation pathway of gemcitabine ProTide **6f**.

To support the proposed activation pathway ProTide **6f** was incubated with carboxypeptidase Y. This assay<sup>34</sup> was designed to verify whether an enzymatic cleavage of the ester motif would be sufficient to trigger the first steps of the activation route and generate the intermediate (**C**). Compound **6f** was dissolved in acetone-*d*<sub>6</sub> in the presence of Trizma buffer (pH 7.6) and treated with carboxypeptidase Y to monitor the metabolic conversion of the ProTide by <sup>31</sup>P NMR spectroscopy over time (Figure 1). Two peaks at  $\delta$  3.64; 3.37 ppm in the blank spectrum recorded at 25 °C correspond to the diastereoisomers of the parent compound **6f**. Within 10 minutes of incubation with carboxypeptidase Y, a single peak at  $\delta$  4.35 ppm started appearing, consistent with the chemical shift of the metabolite (**A**) lacking the ester moiety. A

further processing of the intermediate (**A**) led to the formation of a third peak  $\delta$  6.80 ppm after 10 minutes, which is consistent with the single chemical shift expected for the achiral intermediate (**C**). The peak increased over time and persisted, suggesting the accumulation of a stable product. Therefore, the spectra recorded during the enzymatic reaction suggest that within 12 hours of assay **6f** was almost fully converted into the metabolite (**C**) via the intermediate (**A**) and strongly support the proposed pathway as the actual ProTide activation route.



**Figure 1.**  $^{31}\text{P}$  NMR spectra of ProTide **6f** over time after carboxypeptidase Y digestion showing the signals of metabolites.

The optimal metabolism of these ProTides depends on their chemical stability. Upon administration, the circulating ProTides are exposed to challenging conditions, such as catalytic enzymes in the liver and should resist long enough to enter tumor cells before being degraded. Conversely, ProTides unaffected by enzymatic digestion might not be efficiently converted into active compounds inside the cancer cells. To further select the lead candidates with suitable

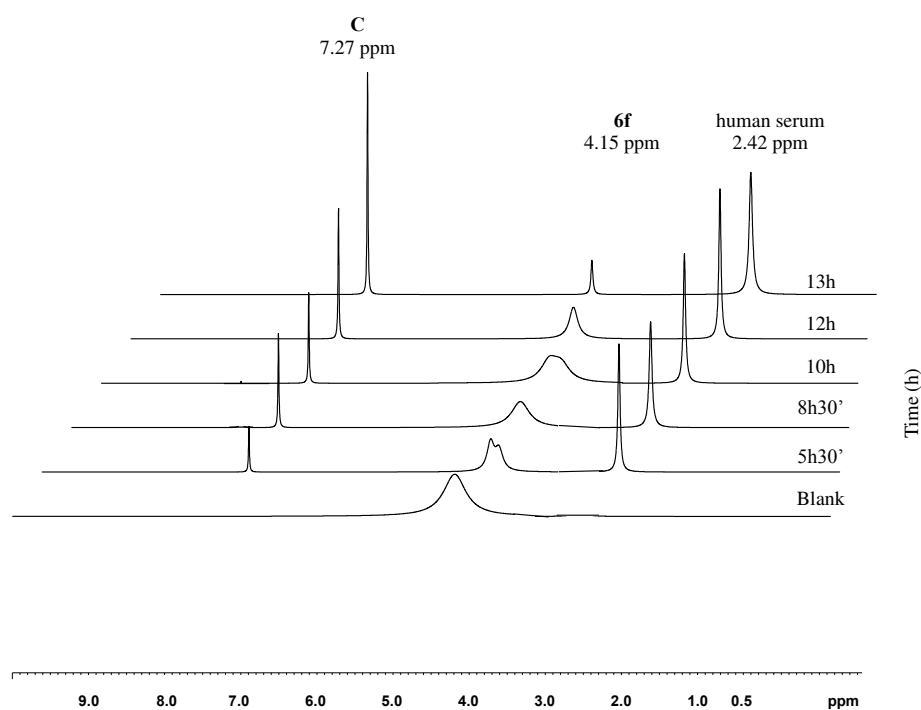
chemical stability, six ProTides were incubated with human hepatocytes and liver microsomes. The half-lives and the percentage of ProTide remaining after one hour were measured and are summarised in Table 2. In the human hepatocyte extracts the half-life of the six compounds varied from 79 minutes for **6k**, up to >1000 minutes for **6d**. The selection criteria included identifying a compound with a mid range half-life in human hepatocytes. Importantly, compounds **6f** and **6j** met this criterion. Next, the stability of ProTides was further investigated using liver microsomes. After one hour incubation 18% of **6f** remained detectable, while **6j** had entirely been processed. These results, together with the cytotoxic data shown earlier, pointed to ProTide **6f** (L-Ala-OBn phenyl) as the lead candidate for further analyses.

**Table 2.** Metabolic stability of ProTides incubated with human hepatocytes and liver microsomes.

Entry	Human hepatocytes half-life (min.)	Liver microsomes % remain after 1h
<b>6a</b>	219	64
<b>6d</b>	1065	27
<b>6e</b>	N.D	2
<b>6f</b>	139	18
<b>6j</b>	93	0
<b>6k</b>	79	1

Next, the stability of the lead compound, ProTide **6f** in the presence of human serum was investigated using *in situ*  $^{31}\text{P}$  NMR (Figure 2). The ProTide was dissolved in deuterated solvent and scanned at 37 °C at regular intervals over 13 hours to monitor the formation of metabolites. The resolution of the recordings was improved using the Lorentz-Gauss deconvolution method. The spectra initially displayed a single peak due to human serum at  $\delta$  2.42 ppm and a broad peak at  $\delta$  4.15 ppm, which can be assigned to the ProTide **6f**. After 5 h 30 minutes incubation, a third signal appeared at  $\delta$  7.27 ppm consistent with the chemical shift expected for metabolite **C**

(Scheme 4). The reaction mixture contained 59% of the original compound **6f** after 13 hours together with 41% of the putative intermediate **C**. This suggests that in the presence of human serum the ProTide **6f** was converted to the metabolite **C** with an estimated half-life of over 14 hours. Also, these results indicate that the stability of the compound should be suitable for clinical studies.



**Figure 2.** Deconvoluted <sup>31</sup>P NMR spectra of ProTide **6f** over time after incubation with human serum showing the signals of metabolites.

The ProTides are specifically designed to overcome the key drug resistance mechanisms associated with nucleoside analogue treatments against cancer. At the cellular level, gemcitabine resistance in particular is dependent on three parameters: (i) the down-regulation of deoxycytidine kinase, necessary for the activation into the phosphorylated moiety; (ii) the reduced expression of nucleoside transporters in particular hENT1 required for uptake by cancer cells and (iii) the up-regulation of catalytic enzymes especially cytidine deaminase that degrades gemcitabine. Thus, the lead ProTide **6f** was evaluated for its ability to bypass these three key

resistance mechanisms. Because the ProTide is synthesised as a masked monophosphate it is expected to bypass the dCK-dependent phosphorylation event for its activation, which is the rate-limiting step to generate the di- and tri-phosphate active moieties. To test this hypothesis, the cytotoxicity of **6f** and gemcitabine on RT112 cancer cells was measured in the presence of a substrate for the dCK enzyme, 2'-deoxycytidine (Figure 3A). This compound was shown to compete with gemcitabine for dCK activity<sup>7b,9b</sup>, which mimics a down-regulation of the enzyme. In the presence of deoxycytidine the gemcitabine concentration required to block cell proliferation, EC<sub>50</sub> increased 75-fold from 1.4nM to 104.9nM, as determined in the colorimetric MTT assay measuring the number of viable cells in culture. By contrast, **6f** was more cytotoxic than gemcitabine (i.e. **6f**: EC<sub>50</sub> lower than gemcitabine EC<sub>50</sub>) and its activity was not significantly affected by deoxycytidine (**6f**: EC<sub>50</sub> = 0.7nM in the presence of deoxycytidine as opposed to 0.2nM without deoxycytidine). This result indicates that **6f** can bypass dCK-mediated monophosphorylation step to block tumor cell growth.

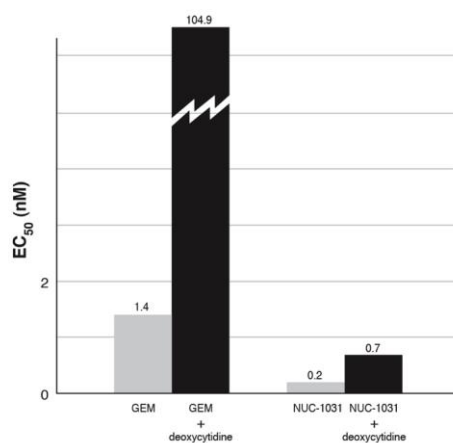
Next, we explored whether the ProTide could overcome the requirement for nucleoside analogue transporters to enter the cancer cells. In a similar assay, the growth inhibition effect of gemcitabine and **6f** was studied on PANC1 pancreatic cancer cells in the presence of an inhibitor of nucleoside transport, dipyridamole (Figure 3B). This inhibitor blocks hENT1 transporter activity to resemble cancers where the transporters responsible for nucleoside uptake are down-regulated. When gemcitabine was incubated with dipyridamole, its cytotoxic activity was markedly reduced, with the EC<sub>50</sub> significantly increasing from 611μM to >2000μM. In contrast, the ProTide, which had a baseline activity ~3 fold superior to gemcitabine (190μM compared to 611μM for gemcitabine), maintained a strong cytotoxic level in the presence of the inhibitor (EC<sub>50</sub> = 162μM). These data suggest that **6f** is not reliant on the nucleoside transporter hENT1 to enter the cells and exert its anti-cancer effect.

Cytidine deaminase found in the serum and inside the cells can break down gemcitabine and thereby limit its activity. Expression of this enzyme is thought to play a key role in the resistance to the drug<sup>35</sup>. Unprotected hydroxyl groups at positions 3' and 5' of the nucleoside

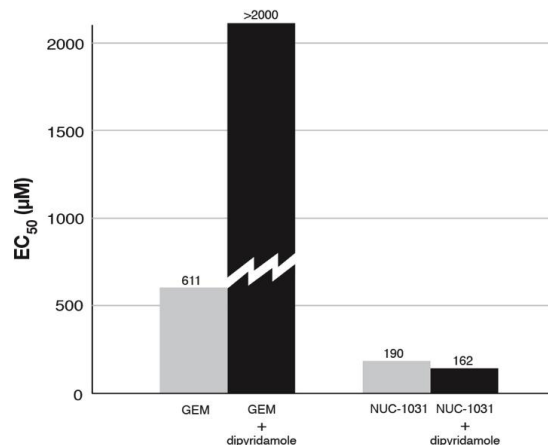
analogue were shown to be necessary for the cleavage to occur.<sup>36</sup> In addition, the amino acid ester prodrugs of gemcitabine have been reported previously to be resistant to deamination by cytidine deaminase *in vitro*.<sup>37</sup>

Because **6f** bears a phosphoramidate moiety on the 5'-carbon of the ribose it was predicted to resist deamination. To assess their resistance to enzymatic breakdown, the ProTide and gemcitabine were incubated with cytidine deaminase and monitored by UV absorption spectroscopy at wavelengths between 220 to 350 nm (Figure 3C). The absorbance of gemcitabine shifted from 267 nm to 257 nm within the first 2 minutes of the UV assay, suggesting breakdown of gemcitabine and conversion to the corresponding dFdU toxic metabolite. The spectrum of **6f** remained unchanged (Abs 267 nm) at 30 minutes and demonstrates that the ProTide is resistant to deaminase breakdown. Because **6f** is less susceptible to degradation, it is anticipated that it should release less harmful deaminated catabolite, dFdU, and have a more favorable safety profile in patients. Together, the cytotoxicity and absorbance data support a model where the ProTide **6f** is able to overcome the three key resistance mechanisms associated gemcitabine.

A



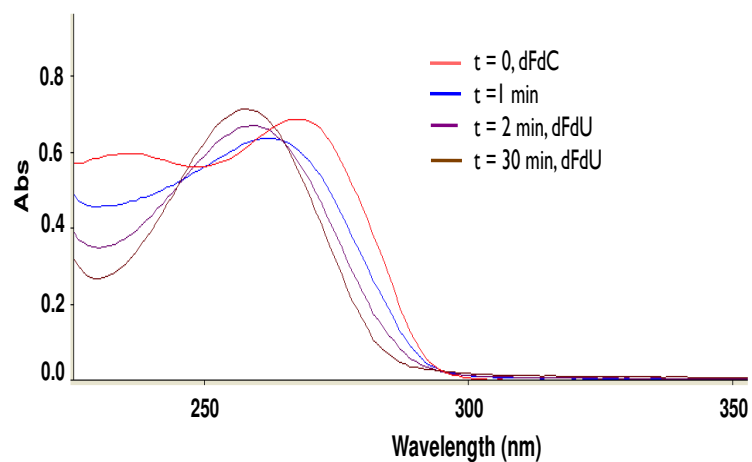
B



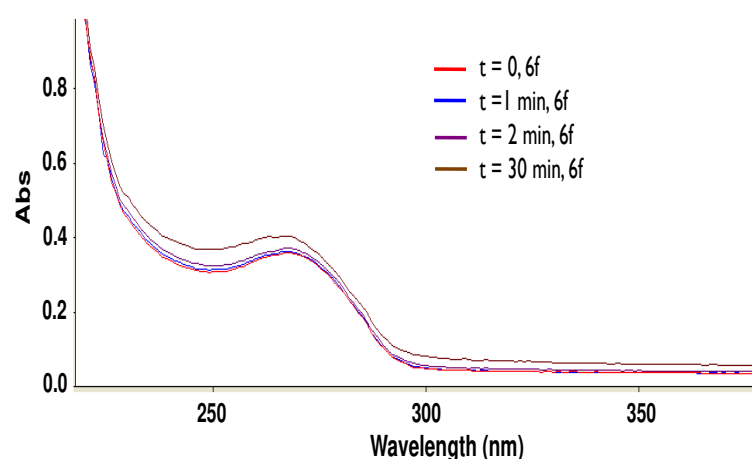
Bladder cancer cell line (RT112); 100µM deoxycytidine. Pancreatic cancer cell line (PANC1), 100µM dipyridamole



C



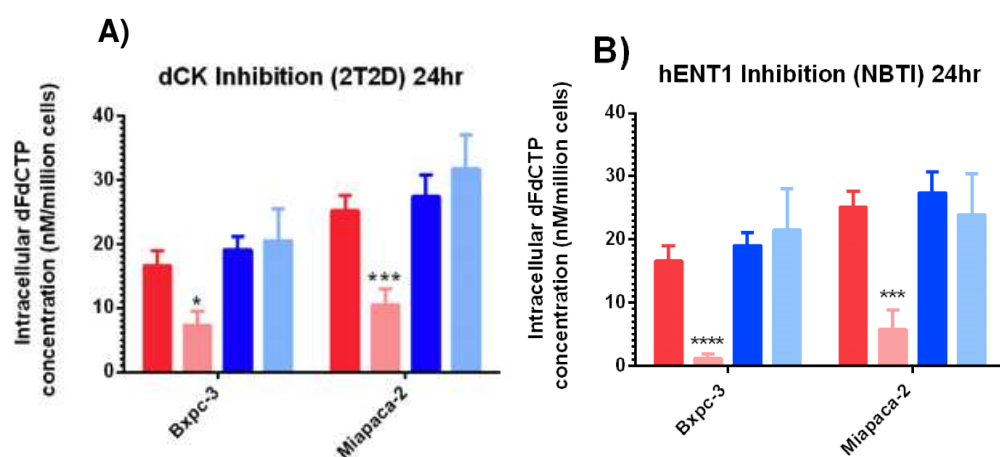
D



**Figure 3.** The ProTide **6f** overcomes the key cancer resistance mechanisms associated with gemcitabine *in vitro*. (A) Effect of deoxycytidine-mediated dCK inhibition on gemcitabine (GEM) and **6f** cytotoxicity in RT112 cells. (B) Effect of dipyridamole-mediated nucleoside transport inhibition on gemcitabine (GEM) and **6f** cytotoxicity in PANC1 cells. (C) and (D) Absorbance spectra of gemcitabine and ProTide **6f** exposed to cytidine deaminase over 30 minutes.

Gemcitabine is a prodrug, which requires further processing into the di- and tri-phosphate active moieties (dFdCDP and dFdCTP, respectively) to block cancer cell growth. The triphosphate form is considered the main driver of the anti-cancer effect, directly interfering with DNA replication, while the diphosphate derivative is thought to further potentiate the activity.<sup>5</sup>

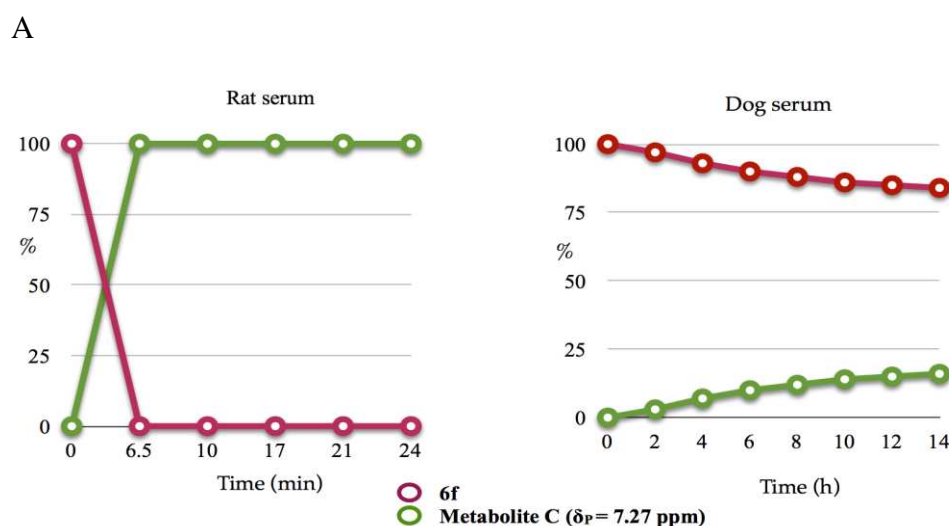
To test whether **6f** cytotoxic activity correlates with high levels of intracellular dFdCTP, the ProTide was tested in a cell growth assay for 24 hours before measuring the intracellular dFdCTP levels (Figure 4). The cells were again exposed to different inhibitors to recreate the drug resistance conditions associated with nucleoside analogue treatment (*i.e.* dCK kinase and hENT1 transporter down-regulation). As expected, when the pancreatic cancer cells (BxPC-3 and MiaPaCa-2) were exposed to gemcitabine, the dFdCTP levels were significantly decreased upon 2T2D and NBTI treatments (Figure 4A and 4B, compare RED and PINK for both cell lines). In contrast, the ProTide (in dark and light blue) maintained steady levels of the triphosphate derivative even in the presence of the inhibitors. These results demonstrate the capacity of **6f** to bypass the requirement for dCK-mediated activation and hENT1-dependent uptake to generate high concentrations of intracellular dFdCTP. Together, these analyses along with the cytotoxicity results strongly suggest that the ProTide **6f** is efficiently metabolised into the active anti-cancer moiety, overcoming the key cancer resistance mechanisms associated with gemcitabine and blocking tumor cell growth.



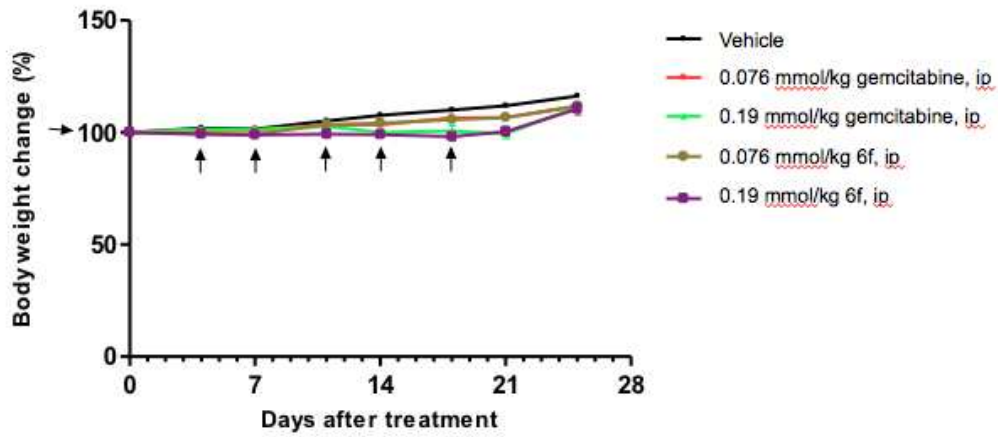
**Figure 4.** The ProTide **6f** generates high intracellular levels of the active moiety bypassing key drug resistance mechanisms *in vitro*. (A) Intracellular levels of dFdCTP (gemcitabine triphosphate) in pancreatic cell lines treated with gemcitabine (red and pink) or **6f** (dark and light blue) in the presence (pink and light blue) or absence (red and dark blue) of the kinase dCK inhibitor, 2T2D. (B) Intracellular levels of dFdCTP in pancreatic cell lines treated with gemcitabine (red and pink) or **6f** (dark and light blue) in the presence (pink and light blue) or

absence (red and dark blue) of the hENT1 (nucleoside transporter) inhibitor, NBTI.

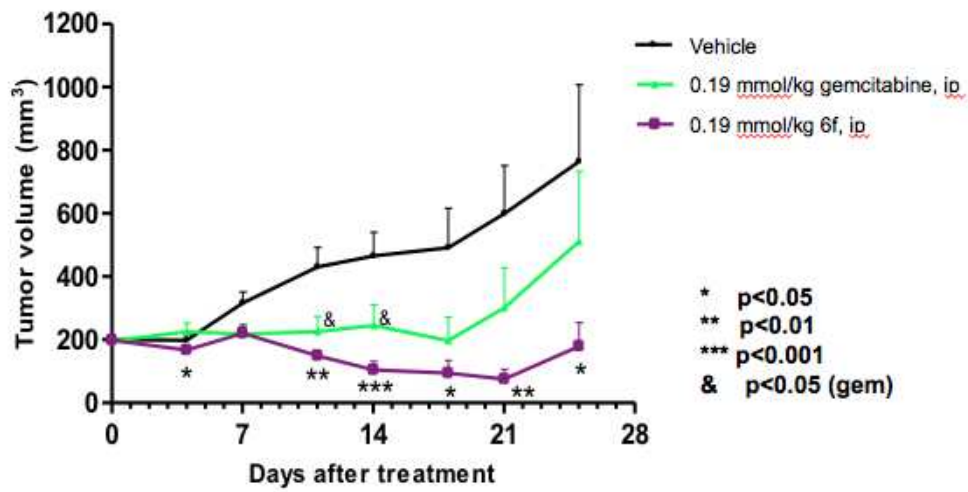
The data gathered *in vitro* prompted us to examine whether the ProTide **6f** would also have a strong anti-cancer activity *in vivo* against well-established mouse models with human tumor xenografts. First, because the ProTide had previously been shown to undergo rapid degradation in rodents due to blood circulating esterases, we examined **6f** stability in rat serum and compared it with dogs as a non-rodent specie reference (Figure 5A). As expected, when the ProTide was incubated with rat serum and monitored by  $^{31}\text{P}$  NMR over time, the spectra revealed the complete conversion of **6f** to the achiral metabolite **C** after 6.5 minutes whereas the compound was still stable after 14 hours in dog serum (>80% **6f**; 20% metabolite **C**). The estimated half-life of ProTide **6f** in rat serum was <5 minutes but exceeded 100 hours in dog serum. These analyses of ProTide **6f** and metabolite **C** confirmed the prior findings that rodent models are likely to reduce the advantages seen from the ProTides over the parent nucleoside analogue.



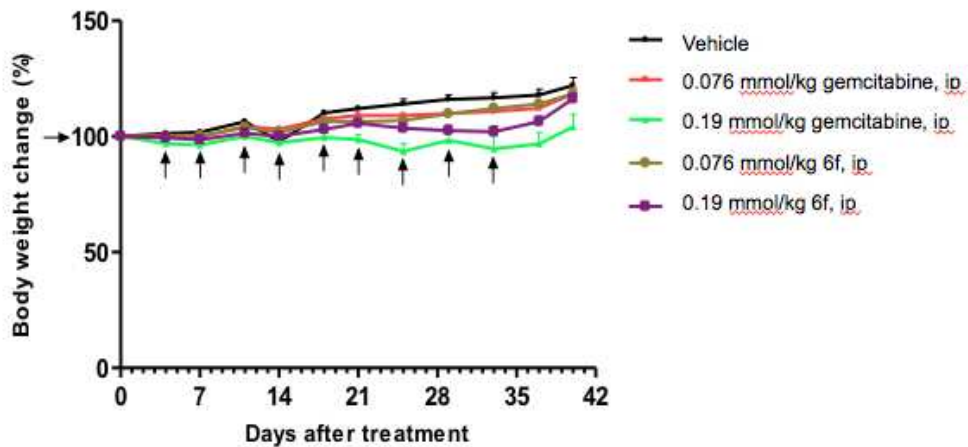
B



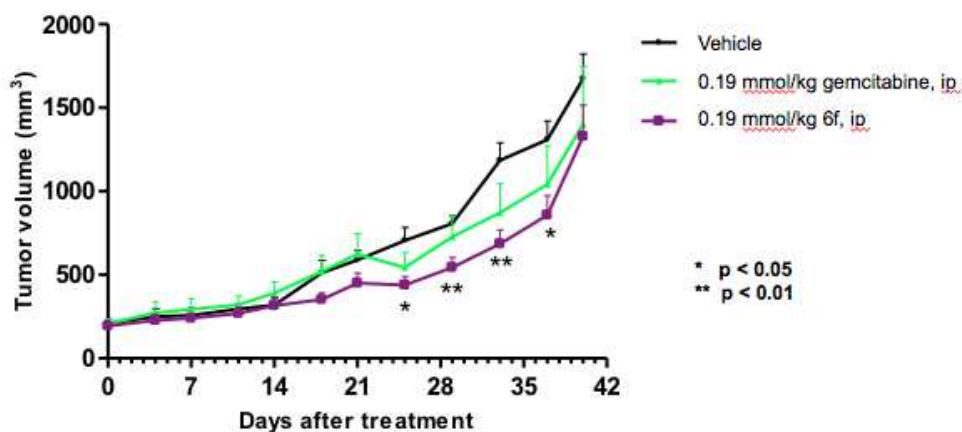
C



D



E



**Figure 5** (A) Metabolic stability of **6f** incubated with rat and dog serum. (B) Mean body weight change (%) of MiaPaCa-2 bearing nude mice following administration of vehicle (n=8), gemcitabine (n=8) and **6f** (0.19 mmol/kg, 2x/week; n=7). (C) Anti-tumor effect of gemcitabine and **6f** against MiaPaCa-2 pancreatic xenograft tumors in nude mice. (D) Mean body weight change (%) of BxPC-3 bearing nude mice following administration of vehicle (n=8), gemcitabine (n=8) and **6f** (0.19 mmol/kg, 2x/week; n=7). (E) Anti-tumor effect of gemcitabine and **6f** against BxPC-3 pancreatic xenograft tumors in nude mice.

To determine the effect of **6f** on tumor growth, nude mouse xenograft models of MiaPaCa-2 human pancreatic cancers were administered with equimolar doses of gemcitabine, **6f** and vehicle. The body weight was also recorded to assess the animal's tolerability of the compound. Over the course of the study, the observed change in body weight for the mice treated with **6f** was less than 4%, and similar to the gemcitabine treated mice (Figure 5B). After the treatment stopped, the mean body weight of both groups increased, indicating that the weight loss may have been due to the compound's toxicity. When examining **6f** and gemcitabine anti-cancer activities, both compounds proved effective, with **6f** significantly reducing tumor volume compared to control (vehicle) (Figure 5C). Importantly, **6f** (0.076 mmol/kg) achieved significantly greater reduction in tumor volume than gemcitabine on Day 7 after the first administration of the compounds. Moreover, tumor growth was inhibited following the first administration of the ProTide and continued to decrease until the dosing was stopped. These results were further confirmed with a second human pancreatic cancer cell line known to be resistant to gemcitabine, BxPC-3 where **6f** achieved a significant reduction in tumor growth compared to control unlike gemcitabine (Figure 5E). Interestingly, over the treatment period the mean body weight change was less in the mice treated with the ProTide than with gemcitabine, suggesting that **6f** was better tolerated than the nucleoside analogue (Figure 5D). Overall, even in rodents, where the ProTide is very unstable, the reported *in vivo* tolerability and efficacy of the lead compound **6f** were at least equivalent to or better than gemcitabine. These results are particularly encouraging because the ProTides are more stable in other species (*i.e.* no circulating rodent esterases), including humans and this is expected to translate into superior clinical efficacy.

## CONCLUSIONS

Tumor cells have numerous inherent and acquired resistance mechanisms that can render conventional chemotherapy with nucleoside analogues less effective. Here we describe the

successful application of the ProTide technology to generate new anti-cancer agents to overcome the key cancer resistance mechanisms associated with nucleoside analogues, and in particular, gemcitabine. The compounds synthesised through phosphoramidate chemistry demonstrated superior *in vitro* and *in vivo* efficacy compared to gemcitabine, even in experimental conditions designed to limit gemcitabine activity. By adding a specific phosphoramidate moiety onto the gemcitabine backbone, a series of novel and potent antimetabolite agents were created with superior efficacy and an improved safety profile, which were able to bypass the key cancer resistance mechanisms that limit gemcitabine activity. One ProTide in particular, **6f**, showed exceptional activity and was selected for clinical studies in patients with advanced cancers.

Gemcitabine is a prodrug with limited clinical utility due to its: reliance on deoxycytidine kinase expression for activation;<sup>7</sup> requirement on nucleoside transporters for cellular entry;<sup>10</sup> and susceptibility to degradation by deactivating enzymes such as cytidine deaminase.<sup>9</sup> *In vitro* the ProTides showed better cytotoxic activity than gemcitabine against a range of tumor cell lines. Importantly, the selected lead compound, **6f** generated much higher intracellular levels of the active moiety, gemcitabine triphosphate, in experimental conditions known to be associated with gemcitabine resistance. *In vivo*, the ProTide demonstrated a significant reduction in tumor size against pancreatic xenograft models compared with the gemcitabine treated group, and less adverse effects on body weight, indicating a better safety profile. Together these data strongly suggest that the ProTides are not reliant on kinases or nucleoside transporters to exert their activity inside tumor cells and remain stable in the presence of deaminases.

The ProTide **NUC-1031 (6f)** is currently advancing through Phase I/II clinical studies and has already generated strong pharmacokinetic data that confirm significantly higher intracellular levels of gemcitabine triphosphate, together with promising early efficacy signals and a favorable safety profile.<sup>27</sup> The phosphoramidate chemistry is potentially a great source of new and very effective anti-cancer agents, bringing a considerable array of advanced treatments specifically designed to overcome cancer resistance mechanisms that will benefit a greater proportion of patients.

## EXPERIMENTAL SECTION

**Cytostatic Activity Assays.** Murine leukemia L1210 and its dCK-deficient congener L1210/dCK<sup>-</sup>, and human lymphocyte CEM and its dCK-deficient congener CEM/dCK<sup>-</sup> were seeded at  $50$  to  $75 \times 10^3$  cells/200  $\mu$ l in the wells of a 96-well microtiter plate that contained a variety of drug concentrations. After 48 hours (L1210) or 72 hours (CEM) the cell number was determined by counting with a Coulter Counter. The IC<sub>50</sub> (50% inhibitory concentration) was determined as the drug concentration required to inhibit tumor cell proliferation by 50%.

**Metabolic Stability (Cryopreserved hepatocytes, human) Assay.** The assay was contracted and performed by Cerep (Seattle, WA, USA Laboratories 15318 N.E 95<sup>th</sup> Street Redmond, WA, 98052, USA) according to the published procedure (Cerep Ref. 1432).<sup>38</sup> Pooled cryopreserved hepatocytes were thawed, washed and re-suspended in Krebs-Heinslet buffer (pH 7.3). The reaction was initiated by adding the test compound (1  $\mu$ M final concentration) into cell suspension and incubated in a final volume of 100  $\mu$ L on a flat-bottom 96-well plate for 0 minute and 60 minutes, respectively, at 37 °C/ 5%CO<sub>2</sub>. The reaction was stopped by adding 100  $\mu$ L of acetonitrile into the incubation mixture. Samples were then mixed gently and briefly on a plate shaker, transferred completely to a 0.8 mL V-bottom 96-well plate, and centrifuged at 2550  $g$  for 15 minutes at room temperature. Each supernatant (150  $\mu$ L) was transferred to a clean cluster tube, followed by HPLC-MS/MS analysis on a Thermo Electron triplequadrupole system.

**Metabolic Stability (liver microsomes, human) Assay.** The stability assay was contracted and carried out by Cerep (Seattle, WA, USA Laboratories 15318 N.E 95<sup>th</sup> Street Redmond, WA, 98052, USA) according to the published procedure (Cerep Ref. 0607).<sup>39</sup> Pooled liver microsomes are pre-incubated with NADPH-regenerating system (1 mM NADP, 5 mM G6P, and 1U/mL G6PDHase) in phosphate buffer (pH 7.4) containing 3 mM MgCl<sub>2</sub> and 1 mM EDTA in a 2 mL-block 96-well plate for 10 minutes in a 37 °C shaking water-bath. The reaction was initiated by adding the test compound (final concentration 0.5  $\mu$ M) and incubated in a final volume of 700  $\mu$ L in the 37°C shaking water-bath. At 0, 15, 30, 45 and 60 minutes after reaction



initiation, 100  $\mu$ L of the incubation mixture is transferred to 100  $\mu$ L of acetonitrile/methanol (50/50, v/v) in a 0.8 mL V-bottom 96-well plate. Samples are then mixed on a plate shaker for 5 minutes and centrifuged at 2550  $xg$  for 15 minutes at room temperature. Each supernatant is transferred to a clean cluster tube, followed by HPLC-MS/MS analysis on a Thermo Electron triplequadrupole system.

**MTS Cell Viability Assay.** The assay was contracted and carried out by WuXi AppTec (Shanghai) Co., Ltd. The corresponding cell lines PC-3, T24/83, MiaPaCa-2, A549, H460, Bx-PC3, A2780, and HT29 were seeded at range of density 0.5 to  $100 \times 10^3$  cells/well in the wells of 96-well plate the day before incubation, then incubated for 72 hours with the different concentrations of compound tested. Then 50  $\mu$ L of MTS was added and cells were incubated for 4 h at 37  $^{\circ}$ C. The data were read and collected by Spectra Max 340 Absorbance Microplate Reader.

**Maximum Tolerated Dose (MTD) Assay** was contracted and performed by WuXi AppTec (Shanghai) Co., Ltd. Balb/c nude mouse (Slac Laboratory Animal Co., Shanghai, China): female, six to eight week old were intraperitoneally given compounds **6f** (i.p 0.228 mmol/kg, 132.3 mg/kg, 2x/WK) and **6j** (i.p 0.228 mmol/kg, 141.9 mg/kg, 2x/WK) or vehicle for 2 weeks. Compounds were dissolved in 40% Captisol<sup>®</sup> solution. (40% Captisol<sup>®</sup> was prepared by dissolving 20 mg of Captisol<sup>®</sup> with pure water, and made the final volume 50 mL. The solvent was filtered with 0.22  $\mu$ m filter). Mice were monitored daily for body weight change and clinical symptoms for 2 weeks. The results were expressed as means  $\pm$  SEM.

***In vivo* xenograft models of MiaPaCa-2 and BxPC-3 Bearing Mice Assay** was contracted and performed by WuXi AppTec (Shanghai) Co., Ltd. BxPC-3 cell line, MiaPaCa-2 cell line (ATCC, USA); RPMI 1640 medium (Invitrogen, USA); FBS (Invitrogen, Australia); Balb/c nude mouse (Slac Laboratory Animal Co., Shanghai, China): female, six to eight week old,  $20 \pm 2$  g; Captisol<sup>®</sup> (CyDex Pharmaceuticas Inc., USA). BxPC-3 and MiaPaCa-2 cell line cultures were performed according to the cell culture protocols of WuXi AppTec Shanghai) Co., Ltd. BxPC-3 and MiaPaCa-2 bearing mice were treated with test compounds or control for 3

weeks. Tumor volumes ( $\text{mm}^3$ ) and body weights were measured two times per week during the treatment. Behavioral and physical abnormal changes were observed and recorded. Mice continued to grow for one week without above treatment. Tumor volumes ( $\text{mm}^3$ ) and body weights were measured two times per week. Behavioral and physical abnormal changes were observed and recorded. The results were expressed as means  $\pm$  SEM. Data was compared using student's t-test. Significant differences were considered to exist for those probabilities below 5% ( $p < 0.05$ ).

**Carboxypeptidase Y (EC 3.4.16.1) Assay.** The experiment was carried out by dissolving ProTide **6f** (3.0 mg) in acetone- $d_6$  (0.15 mL) followed by addition of 0.30 mL of Trizma buffer (pH 7.6). After recording the control  $^{31}\text{P}$  NMR at 25 °C, a previously defrosted carboxypeptidase Y (0.1 mg dissolved in 0.15 mL of Trizma) was added to the sample, which was then immediately submitted to the  $^{31}\text{P}$  NMR experiments (at 25 °C). The spectra were recorded every 7 minutes over 14 hours.  $^{31}\text{P}$  NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

**Stability Assay in Serum.** The experiment was carried out by dissolving ProTide **6f** (5.0 mg) in DMSO (0.050 mL) and  $\text{D}_2\text{O}$  (0.15 mL). After recording the control  $^{31}\text{P}$  NMR at 37 °C, a previously defrosted human, rat or dog serum (0.30 mL) was added to the sample, which was next submitted to the  $^{31}\text{P}$  NMR experiments at 37 °C. The spectra were recorded every 30 minutes over 13 hours.  $^{31}\text{P}$  NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program. The Lorentz – Gauss deconvolution method was further used to improve a visualization of results.

**Cytidine deaminase (CDA) Assay.** CDA assays were performed using modified procedure.<sup>40</sup> Stock solution of gemcitabine and ProTide **6f** were dissolved in the assay buffer (50mM Tris-HCl buffer pH 7.5) to a final concentration of 100 $\mu\text{M}$ . Cytidine deaminase (100 $\mu\text{g}$  in 200 $\mu\text{L}$  20mM Tris-HCl buffer pH 8.0, containing 1mM DTT, 2mM EDTA, 100mM NaCl, 40% glycerol) was aliquoted for 4 portions and stored at -20 °C prior usage. 50 $\mu\text{L}$  aliquot of

cytidine deaminase was added to 150 $\mu$ L of assay buffer. For each assay, 1mL of 100 $\mu$ M of gemcitabine and **6f** was placed in a 1cm UV cuvet at 25 °C, and UV spectrum was recorded over the range 220 to 350nm. A portion of enzyme solution (200 $\mu$ L) was added, and spectra were recorded in 1 minute intervals for 30 minutes.

**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 from Sigma-Aldrich. Cytidine deaminase (recombinant human cytidine deaminase, His-tagged expressed in *E.coli*, in 20mM Tris-HCl buffer pH 8.0, containing 1mM DTT, 2mM EDTA, 100mM NaCl, 40% glycerol, concentration 0.5 mg/ml as determined by Bradford assay) was purchased from Creative BioMart. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on Silica Gel 60-F254 precoated aluminium plates and visualized under UV (254 nm) and/or with <sup>31</sup>P NMR spectra. Column chromatography was performed on silica gel (35–70  $\mu$ M). Proton (<sup>1</sup>H), carbon (<sup>13</sup>C), phosphorus (<sup>31</sup>P) and fluorine (<sup>19</sup>F) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were auto-calibrated to the deuterated solvent peak and all <sup>13</sup>C NMR and <sup>31</sup>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<sub>2</sub>O/MeOH from 100/0 to 0/100 in 40 min. (method 1), and with a gradient elution of H<sub>2</sub>O/CH<sub>3</sub>CN 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 Cardiff University, using electrospray spectrometry (ESMS). CDA assays were performed with Varian Carry 50 Bio UV/Vis spectrophotometer. UPLC-MS/MS Analysis: dFdCTP was resolved using an ultra-performance liquid chromatography system (Accela UPLC, Thermo Scientific, UK) equipped with Ascentis Express 2.7  $\mu$ m C18 4.6  $\times$  100 mm HPLC column (Sigma Aldrich, UK). Mobile phase gradient was employed, comprising: buffer A (10mM Ammonium Acetate) = 98% and buffer B (acetonitrile) = 2% at 0 min. Buffer A from 98 to

30% over 6 minutes and from 30 – 98% over 2 minutes, all at a flow rate of 700  $\mu$ l/min. Eluting compounds of interest were detected using a triple stage quadrupole Vantage mass spectrometry system (Thermo Scientific, UK) equipped with heated electrospray (HESI) ion source. The ion source conditions for mixed ion mode (positive and negative) HESI analysis were as follows: ion spray voltage 3500V, vaporizer temperature 350°C, collision gas (argon) 1.5 mTorr, sheath and auxiliary gases (nitrogen) were 60 and 20 arbitrary units, respectively. The optimum transitional daughter ions mass and collision energy of dFdCTP was  $m/z$   $-p$  502.0  $\rightarrow$  159.0 (collision energy 37 V).

**General Method for the Preparation of phosphorochloridates (3a-l).** Anhydrous triethylamine (2.0 mol eq.) was added dropwise at -78 °C to a stirred solution of the appropriate aryl dichlorophosphate (1.0 mol eq.) and an appropriate amino acid ester (1.0 mol eq.) in anhydrous DCM under argon atmosphere. Following the addition, the reaction mixture was allowed to slowly warm to room temperature and stirred for 1-2 hours. A formation of a desired compound was monitored by  $^{31}\text{P}$  NMR. After the reaction was completed, the solvent was evaporated under reduced pressure and the resulting residue was re-dissolved in anhydrous  $\text{Et}_2\text{O}$  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.

**Phenyl(isopropoxy-L-alaninyl) phosphorochloridate (3a).** Yellowish oil; yield, 80% (2.94 g); mixture of diastereoisomers;  $^{31}\text{P}$  NMR (202 MHz,  $\text{CDCl}_3$ ):  $\delta_{\text{P}}$  8.08, 7.71 (int. 1.00:1.00).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta_{\text{H}}$  7.41 – 7.36 (m, 2H, ArH), 7.31 – 7.27 (m, 3H, ArH), 5.21 – 5.01 (m, 1H,  $\text{OCH}(\text{CH}_3)_2$ ), 4.21 – 4.06 (m, 1H,  $\text{CHCH}_3$ ), 1.51 (d,  $J = 6.9$  Hz, 3H,  $\text{CHCH}_3$ ), 1.26 – 1.19 (m, 6H,  $\text{OCH}(\text{CH}_3)_2$ ).

**3'-O-(tert-Butoxycarbonyl)-gemcitabine (4)** was prepared according to the published

procedure<sup>28</sup> and obtained as a white solid. Yield, 75% (1.03 g). <sup>19</sup>F NMR (470 MHz, MeOD):  $\delta_F$  – 115.74, – 115.75 (d,  $J = 243$  Hz, F), – 119.21 (broad signal, F). <sup>1</sup>H NMR (500 MHz, MeOD):  $\delta_H$  7.68 (d, 1H,  $J = 7.5$ ,  $H-6$ ), 6.18 (t, 1H,  $J = 8.5$ ,  $H-1'$ ), 5.84 (d, 1H,  $J = 7.5$ ,  $H-5$ ), 5.13 (q, 1H,  $J = 6.0$ ,  $H-3'$ ), 4.18 – 4.15 (m, 1H,  $H-4'$ ), 3.82 (dd, 1H,  $J = 13.0$ ,  $J = 3.0$ ,  $H-5'_a$ ), 3.68 (dd, 1H,  $J = 13.0$ ,  $J = 3.0$ ,  $H-5'_b$ ), 1.39 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>).

**General Method for the Preparation of Gemcitabine ProTides (5a-I).** To a solution of 3'-*O*-(*tert*-butoxycarbonyl)-gemcitabine **4** (1.0 eq) in dry THF (10 mL), *tert*-BuMgCl (1.0 M solution in THF, 1.2 eq.) was added in one portion followed by addition of appropriate phosphorochloridate (2.0 eq) dissolved in anhydrous THF (3 mL). The reaction mixture was stirred for 16 to 18 hours and then evaporated *in vacuo* to give a crude residue that was purified by column chromatography on silica gel, eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH as a gradient (0% to 5% MeOH) to afford the products **5a-I** as white solid.

**General Method for the Preparation of Gemcitabine ProTides (6a-I).** The 3'-*O*-(*tert*-butoxycarbonyloxy)-gemcitabine phosphates (**5a-I**) were treated with a mixture of TFA/DCM (1:1) (6mL) at 0 °C. The resulting reaction mixture was stirred at 0 °C for 2-4 hours. After the reaction was completed the solvents were evaporated and the residue was treated with saturated NaHCO<sub>3</sub>, and extracted with EtOAc. The organic layers were combined, dried (MgSO<sub>4</sub>), filtered, reduced to dryness and purified on silica gel with gradient of eluent MeOH/DCM (2% to 8% MeOH).

**2'-Deoxy-2',2'-difluoro-3'-*O*-(*tert*-butoxycarbonyloxy)-D-cytidine-5'-*O*-[phenyl (isopropoxy-L-alanyl)]phosphate (5a)** was obtained from 3'-*O*-(*tert*-butoxycarbonyl)-gemcitabine **4** (0.25 g, 0.69 mmol), the phosphorochloridate **3a** (0.42 g, 1.37 mmol) and *tert*-BuMgCl (0.83 mL, 0.83 mmol) as a white solid. Yield, 83% (0.36 g); mixture of diastereoisomers (45%, 55%). <sup>31</sup>P NMR (202 MHz, MeOD):  $\delta_P$  3.86, 3.79. <sup>1</sup>H NMR (500 MHz, MeOD):  $\delta_H$  7.56, 7.48 (2 x d,  $J = 7.50$  Hz, 1H,  $H-6$ ), 7.41 – 7.39 (m, 2H, ArH), 7.35 – 7.19 (m, 3H, ArH), 6.30 – 6.28 (m, 1H,  $H-1'$ ), 5.89, 5.85 (2 x d,  $J = 7.50$  Hz, 1H,  $H-5$ ), 5.28 – 5.22 (m, 1H,  $H-3'$ ), 5.04 – 4.98 (m, 1H,

OCH(CH<sub>3</sub>)<sub>2</sub>), 4.54 – 4.50 (m, 1H, *H*-5'<sub>a</sub>), 4.46 – 4.39 (m, 2H, *H*-5'<sub>b</sub>, *H*-4'), 4.01 – 3.98 (m, 1H, CHCH<sub>3</sub>), 1.51, 1.50 (2 x s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 1.38 (d, *J* = 6.30 Hz, 3H, CHCH<sub>3</sub>).

**2'-Deoxy-2',2'-difluoro-D-cytidine-5'-O-[phenyl(isopropoxy-L-alaninyl)] phosphate (6a)** was obtained from 2'-deoxy-2',2'-difluoro-3'-O-(*tert*-butoxycarbonyloxy)-D-cytidine-5'-O-[phenyl(isopropoxy-L-alaninyl)]phosphate (**5a**) (0.36 g, 0.57 mmol) as a white solid. Yield, 50% (0.15 g). (ES+) *m/z*: found: (M + Na<sup>+</sup>) 555.15. C<sub>21</sub>H<sub>27</sub>F<sub>2</sub>N<sub>4</sub>O<sub>8</sub>NaP required: (M<sup>+</sup>) 532.43. Mixture of diastereoisomers (45%, 55%). <sup>31</sup>P NMR (202 MHz, MeOD): δ<sub>P</sub> 3.80, 3.73. <sup>19</sup>F NMR (470 MHz, MeOD): δ<sub>F</sub> – 118.0 (d, *J* = 240 Hz), – 118.2 (d, *J* = 240 Hz, *F*), – 123.43 (– 123.74) (broad signal, *F*). <sup>1</sup>H NMR (500 MHz, MeOD): δ<sub>H</sub> 7.60, 7.55 (2 x d, *J* = 7.72 Hz, 1H, *H*-6), 7.41 – 7.36 (m, 2H, Ar*H*), 7.29 – 7.22 (m, 3H, Ar*H*), 6.30 – 6.25 (m, 1H, *H*-1'), 5.94, 5.89 (2 x d, *J* = 7.72 Hz, 1H, *H*-5), 5.03 – 4.97 (m, 1H, OCH(CH<sub>3</sub>)<sub>2</sub>), 4.56 – 4.35 (m, 2H, *H*-5'), 4.28 – 4.21 (m, 1H, *H*-3'), 4.14 – 4.09 (m, 1H, *H*-4'), 3.97 – 3.91 (m, 1H, CHCH<sub>3</sub>), 1.37 – 1.32 (m, 3H, CHCH<sub>3</sub>), 1.25 – 1.22 (m, 6H, OCH(CH<sub>3</sub>)<sub>2</sub>). <sup>13</sup>C NMR (125 MHz, MeOD): δ<sub>C</sub> 174.4 (C=O, ester), 167.62 (C-NH<sub>2</sub>), 157.80 (C=O base), 152.18, 152.12 (C-Ar), 142.5, 142.41 (CH-base), 130.88 (d, <sup>3</sup>*J*<sub>C-P</sub> = 3.75 Hz, CH-Ar), 126.33 (CH-Ar), 122.46 (d, <sup>1</sup>*J*<sub>C-F</sub> = 256 Hz, CF<sub>2</sub>), 121.42, 121.40 (2 x d, <sup>3</sup>*J*<sub>C-P</sub> = 2.5 Hz, CH-Ar), 96.8 (CH-base), 85.6 (broad signal, C-1'), 80.40 (C-4'), 71.62, 71.20 (2 x d, <sup>2</sup>*J*<sub>C-F</sub> = 37.5 Hz, C-3'), 70.24 (OCH(CH<sub>3</sub>)<sub>2</sub>), 65.71, 65.66 (2 x d, <sup>2</sup>*J*<sub>C-P</sub> = 5.0 Hz, C-5'), 51.89, 51.75 (CHCH<sub>3</sub>), 21.99, 21.91, 21.82 (OCH(CH<sub>3</sub>)<sub>2</sub>), 16.47 (CHCH<sub>3</sub>). Reverse HPLC, eluting with H<sub>2</sub>O/MeOH from 100/0 to 0/100 in 40 min., showed two peaks of the diastereoisomers with *t*<sub>R</sub> 22.73 min and *t*<sub>R</sub> 23.53 min (44%, 54%).

## ASSOCIATED CONTENT

**Supporting Information.** Preparative methods, spectroscopic and analytical data for target compounds, and xenograft data at low dose. This material is available free of charge via Internet at <http://pubs.org>.

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## ABBREVIATIONS

dFdC, 2',2'- difluoro-2'-deoxycytidine; dCyd, deoxycytidine; d4T, stavudine; dCK, deoxycytidine kinase; L1210, wild-type murine leukemia; CEM, human T-lymphocyte CEM; FUDR, 2'-deoxy-5-fluorouridine; NBTI, S-(4-nitrobenzyl)-6-thioinosine; hENT1, human equilibrative nucleoside transporter-1; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

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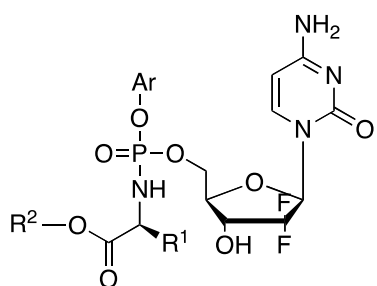
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## Table of Contents Graphic



### Gemcitabine ProTides

R<sup>1</sup> = Amino acid side chain

R<sup>2</sup> = Alkyl, cycloalkyl, aryl

Ar = Ph, 1-Naph

