Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

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

Magdalena Slusarczyk,[†] Monica Huerta Lopez,[†] Jan Balzarini,[‡] Malcolm Mason,[§] Wen G. Jiang,[∥] Sarah Blagden,[⊥] Emely Thompson,[#] Essam Ghazaly,[#] and Christopher McGuigan*[,†]

[†]Cardiff School of Pharmacy & Pharmaceutical Sciences, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, United Kingdom

[‡]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, Leuven B-3000, Belgium

[§]Section of Oncology & Palliative Medicine, Cardiff University School of Medicine, Velindre Hospital, Cardiff CF14 2TL, United Kingdom

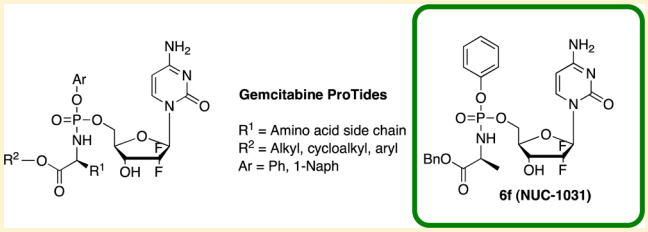
[∥]Metastasis Research Group, University Department of Surgery, Cardiff University, School of Medicine, Heath Park, Cardiff CF14 4XN, United Kingdom

[⊥]Department of Oncology, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0HS, United Kingdom

[#]Centre for Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, Charterhouse Square, London, EC1M 6BQ, United Kingdom

Supporting Information

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 anticancer 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 antimetabolites, includes a variety of pyrimidine and purine nucleoside derivatives with cytotoxic activity in both hematological and solid tumors. Gemcitabine[1] (2′,2′- difluoro-2′-deoxycytidine) is a pyrimidine nucleoside analogue, shown to be active against several solid tumor types.[2] Following FDA approval in 1996, gemcitabine has become the standard of care for the treatment of pancreatic cancer.[3] More recently, the compound has also gained approval for treating non-small cell lung, ovarian, bladder, and breast cancer.[4]

At a molecular level, the mechanisms underpinning the anticancer 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.[5] 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.[6] 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

Received: December 2, 2013
Published: January 28, 2014

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

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 the following: (i) down-regulation of the key initial phosphorylating enzyme deoxycytidine kinase (dCK)[7] required to convert gemcitabine into the monophosphate form, considered the key rate-limiting step in its activation process;[8] (ii) expression of the key deactivating enzyme cytidine deaminase;[9] and (iii) deficiency of nucleoside transporter[10] 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).[9] Because of these processes, single agent gemcitabine has limited activity in cancer treatment, with response rates of less than 10% in pancreatic cancer.[11]

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.).[12] 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.[13] 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.[14] As an alternative approach, CP-4126 was developed[15] 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.[16] 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 preactivated monophosphate form of these nucleoside analogues, has emerged as a therapeutic approach.[17]

A major strategy currently applied in the modulation of several antiviral nucleoside analogues is the ProTide technology developed in 1995 by our laboratories.[18] 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 preactivated, 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.[19] The addition of the phosphoramidate motif will also change the pharmacokinetic/dynamic properties, which 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,[20] abacavir,[21] and various anti-hepatitis C virus agents.[22] Similarly, we generated ProTides based on several anticancer nucleosides such as cladribine,[23] clofarabine,[24] and recently 5-fluoro-2′-deoxyuridine (FUDR),[25] 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.

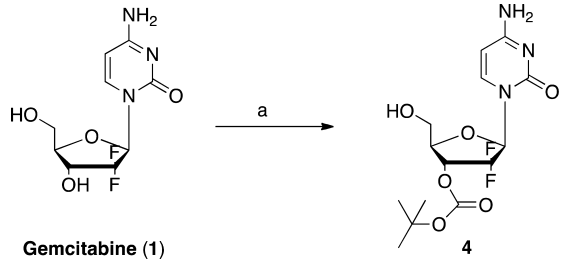
The potency of the compounds varies with the individual components (aryl, ester, and amino acid) of the phosphoramidate moiety, and therefore we examined a panel of gemcitabine ProTides. Thus, herein we report for the first time in the open literature the design, synthesis, antitumor evaluation, and metabolic studies of a panel of L-alanine-based gemcitabine phosphoramidates. **NUC-1031** (**6f**, first patented by us in 2005 as CPF-31[26]) 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.[27]

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[28] in order to favor the 5′-regioselectivity of the phosphorylation with the aryl aminoacyl phosphorochloridates (Scheme 1). Thus, gemcitabine was allowed to react with *tert*-

Scheme 1. Selective Protection of Gemcitabine (1)[a]



[a]Reagents and conditions: (a) DBOC, Na_2CO_3, 1,4-dioxane/H_2O (4:1 v/v), rt, 48 h.

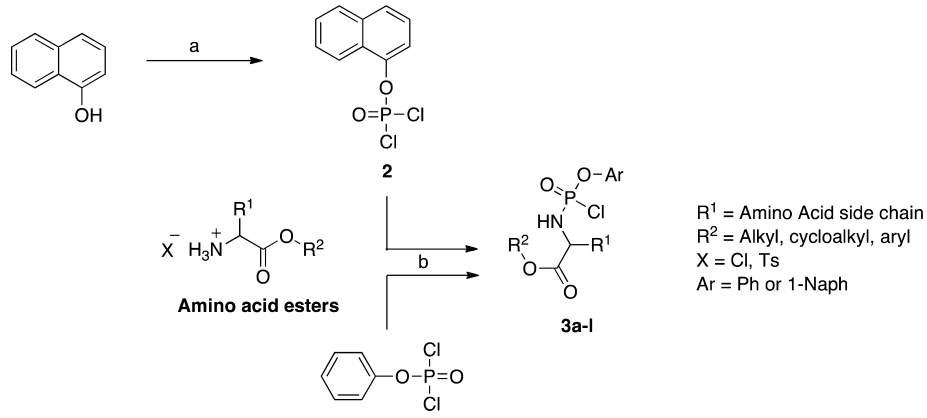
butyl dicarbonate in 1,4-dioxane and water (4:1 v/v) at room temperature for 48 h to give the corresponding intermediate (**4**) with a 75% yield after column chromatography.

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).[29] 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.[30] 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.[31]

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 ^{31}P NMR spectra. In most cases, the aryl aminoacyl phosphorochloridate (**3**) syntheses were completed within two hours as

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

Scheme 2. Synthesis of Aryl Aminoacyl Phosphorochloridates (3a–l)[a]

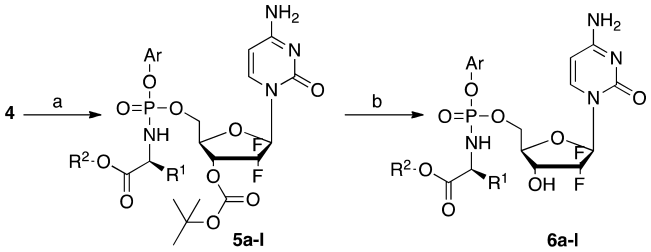


[a]Reagents and conditions: (a) $POCl_3$, Et_3N, dry Et_2O, −78 °C for 1 h, then rt for 1 h; (b) (**2**) or phenyl phosphorodichloridate, Et_3N, dry DCM, −78 °C, 1–3 h.

monitored by ^{31}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.

Next, each aminoacyl phosphorochloridate (**3**) was 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

Scheme 3. Preparation of Gemcitabine ProTides (5a–l, 6a–l) under Coupling Conditions[a]



5,6a: R^1 = L-Ala side chain, R^2 = iPro, Ar = Ph
5,6b: R^1 = L-Ala side chain, R^2 = Pentyl, Ar = Ph
5,6c: R^1 = L-Ala side chain, R^2 = Hexyl, Ar = Ph
5,6d: R^1 = L-Ala side chain, R^2 = CH_2tBu, Ar = Ph
5,6e: R^1 = L-Ala side chain, R^2 = cHex, Ar = Ph
5,6f: R^1 = L-Ala side chain, R^2 = Bn, Ar = Ph
5,6g: R^1 = Dimethylglycine side chain, R^2 = Pentyl, Ar = 1-Naph
5,6h: R^1 = L-Ala side chain, R^2 = Hexyl, Ar = 1-Naph
5,6i: R^1 = L-Ala side chain, R^2 = cPentyl, Ar = 1-Naph
5,6j: R^1 = L-Ala side chain, R^2 = cHexyl, Ar = 1-Naph
5,6k: R^1 = L-Ala side chain, R^2 = Bn, Ar = 1-Naph
5,6l: R^1 = L-Ala side chain, R^2 = Pentyl, Ar = 1-Naph

[a]Reagents and conditions: (a) $(ArO)(R^2OC(O)CR^1NH)P(O)Cl$ (**3a–l**), *tert*-BuMgCl, THF, rt, 16 h; (b) TFA/DCM (1:1 v/v), 0 °C, 2–4 h.

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 h reaction at 0 °C in TFA/DCM (1:1 v/v), followed by basic workup and column purification on silica gel. As a result of the phosphate chirality, ProTides **6a–l** were isolated as diastereoisomeric mixtures, evidenced by ^{31}P NMR, HPLC (two peaks), ^1H NMR, and ^{13}C NMR (with splitting of many nucleoside signals). Extensive structure/activity relationship (SAR) studies[32] and prior work suggested that the L-alanine phosphoramidates were the most potent gemcitabine ProTides.[26] 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.

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 antineoplastic effect. Over the years, our laboratory has created a large library of over 3500 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 summarized in Table 1. ProTides showed a broad spectrum of activity levels around the gemcitabine IC_{50} 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.

To exert their anticancer activity, the ProTides should be metabolized to release the monophosphate form, which will then generate the active compounds, gemcitabine di- and triphosphate. The proposed intracellular activation route of the ProTides has been described[33] for other ProTide families, such as the FUDR ProTides, and is depicted in Scheme 4. The

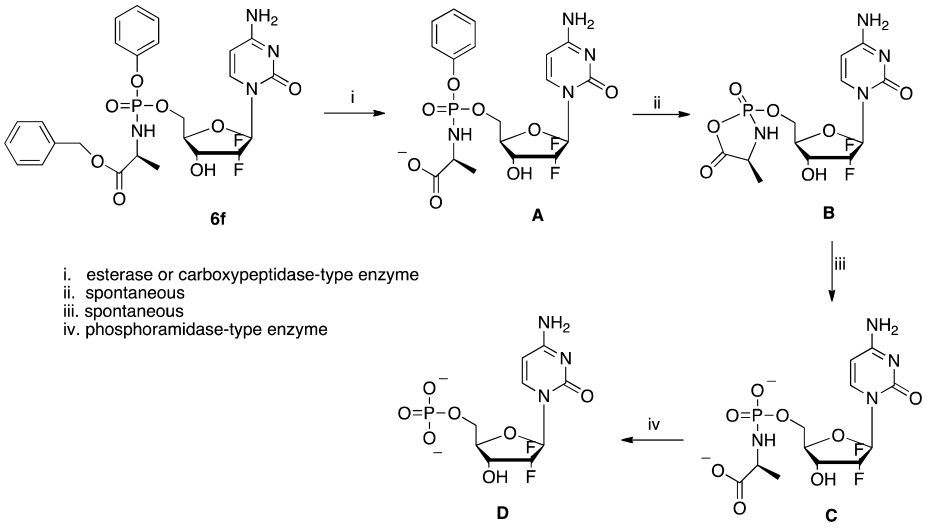
dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531–1542

Table 1. IC_{50} (μM) Values of Selected Prodrug Derivatives against Four Cancer Cell Lines

				IC_{50} (μM)[a]			
entry	amino acid	ester	aryl	L1210	CEM	MP-2	BxPC-3
6a	L-Ala	iPrO	Ph	>5	>5	>5	>5
6b	L-Ala	Pentyl	Ph	0.024 ± 0.009	0.08 ± 0.011	N.D.	N.D.
6c	L-Ala	Hexyl	Ph	0.016 ± 0.005	0.081 ± 0.008	N.D.	N.D.
6d	L-Ala	CH2tBu	Ph	0.39 ± 0.04	0.44 ± 0.04	1.41 ± 0.57	0.36 ± 0.13
6e	L-Ala	cHexyl	Ph	0.6 ± 0.06	0.42 ± 0.17	N.D.	N.D.
6f	L-Ala	Bn	Ph	0.035 ± 0.02	0.1 ± 0.03	0.44 ± 0.06	0.15 ± 0.04
6g	DMG[b]	Pentyl	1-Naph	0.16 ± 0.03	0.13 ± 0.01	N.D.	N.D.
6h	L-Ala	Hexyl	1-Naph	0.022 ± 0.005	0.075 ± 0.005	N.D.	N.D.
6i	L-Ala	cPentyl	1-Naph	0.18 ± 0.03	0.11 ± 0.00	0.46 ± 0.24	0.15 ± 0.05
6j	L-Ala	cHexyl	1-Naph	0.32 ± 0.03	0.14 ± 0.02	N.D.	N.D.
6k	L-Ala	Bn	1-Naph	0.035 ± 0.03	0.10 ± 0.03	0.57 ± 0.22	0.24 ± 0.05
61	L-Ala	Pentyl	1-Naph	0.03 ± 0.01	0.073 ± 0.004	N.D.	N.D.
dFdC (1)				0.013 ± 0.02	0.086 ± 0.003	1.04 ± 0.71	0.67 ± 0.34

[a]IC_{50} (50% Inhibitory Concentration) is the compound concentration required to inhibit tumor cell proliferation by 50%. [b]DMG is dimethylglycine. Data shown are the means of at least two independent experiments.

Scheme 4. Proposed Activation Pathway of Gemcitabine ProTide 6f



hypothesized 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 cyclization (ii) displacing the aryl moiety via an internal nucleophilic attack of the carboxylate residue on the phosphorus center to yield (**B**). In a third step (iii), the unstable cyclic mixed anhydride would be hydrolyzed 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**).

To support the proposed activation pathway ProTide **6f** was incubated with carboxypeptidase Y. This assay[34] 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_6 in the presence of Trizma buffer (pH 7.6) and treated with carboxypeptidase Y to monitor the metabolic conversion of the ProTide by ^{31}P NMR spectroscopy over time (Figure 1). Two peaks at δ 3.64 and 3.37 ppm in the blank spectrum recorded at 25 °C correspond to the diastereoisomers of the parent compound **6f**. Within 10 min of incubation with carboxypeptidase Y, a single peak at δ 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 δ 6.80 ppm after 10 min, 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 h 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.

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

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

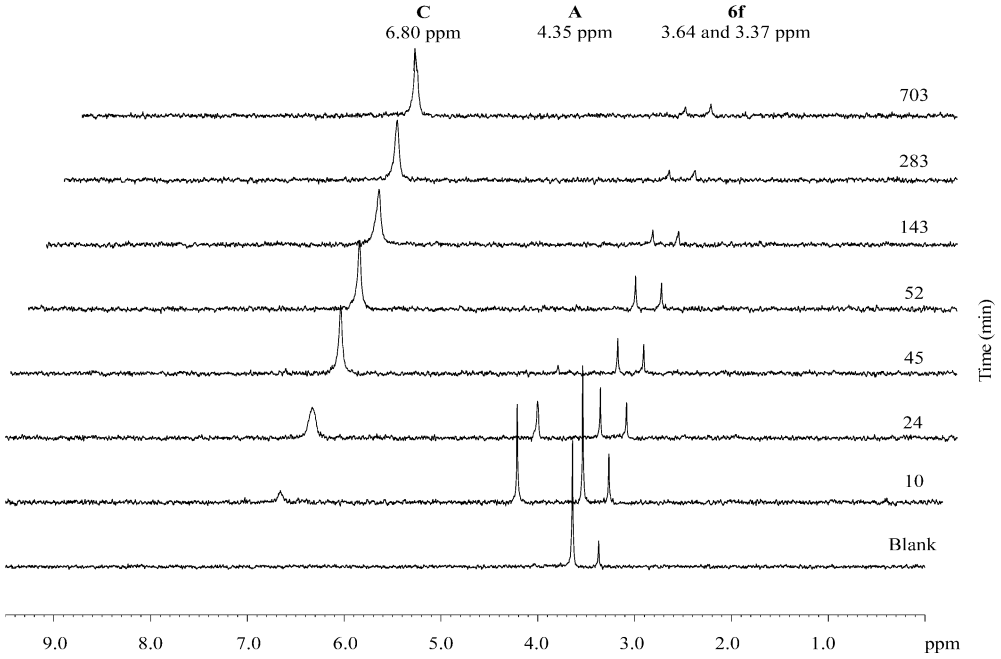


Figure 1. ^{31}P NMR spectra of ProTide **6f** over time after carboxypeptidase Y digestion showing the signals of metabolites.

and are summarized in Table 2. In the human hepatocyte extracts the half-lives of the six compounds varied from 79 min

Table 2. Metabolic Stability of ProTides Incubated with Human Hepatocytes and Liver Microsomes

entry	human hepatocytes half-life (min)	liver microsomes % remain after 1 h
6a	219	64
6d	1065	27
6e	N.D.	2
6f	139	18
6j	93	0
6k	79	1

for **6k** up to >1000 min 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.

Next, the stability of the lead compound, ProTide **6f** in the presence of human serum was investigated using *in situ* ^{31}P NMR (Figure 2). The ProTide was dissolved in deuterated solvent and scanned at 37 °C at regular intervals over 13 h 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 δ 2.42 ppm and a broad peak at δ 4.15 ppm, which can be assigned to the ProTide **6f**. After 5 h 30 min incubation, a third signal appeared at δ 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 h together with 41% of the putative intermediate **C**. This suggests that in the presence of human serum the ProTide

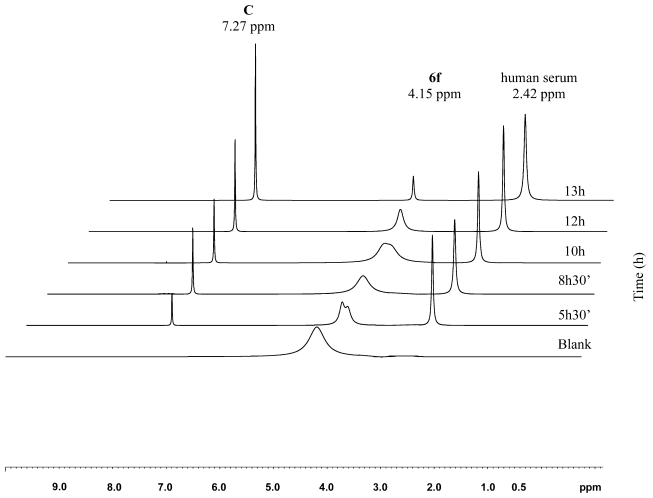


Figure 2. Deconvoluted ^{31}P NMR spectra of ProTide **6f** over time after incubation with human serum showing the signals of metabolites.

6f was converted to the metabolite **C** with an estimated half-life of over 14 h. Also, these results indicate that the stability of the compound should be suitable for clinical studies.

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 synthesized 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 triphosphate active

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

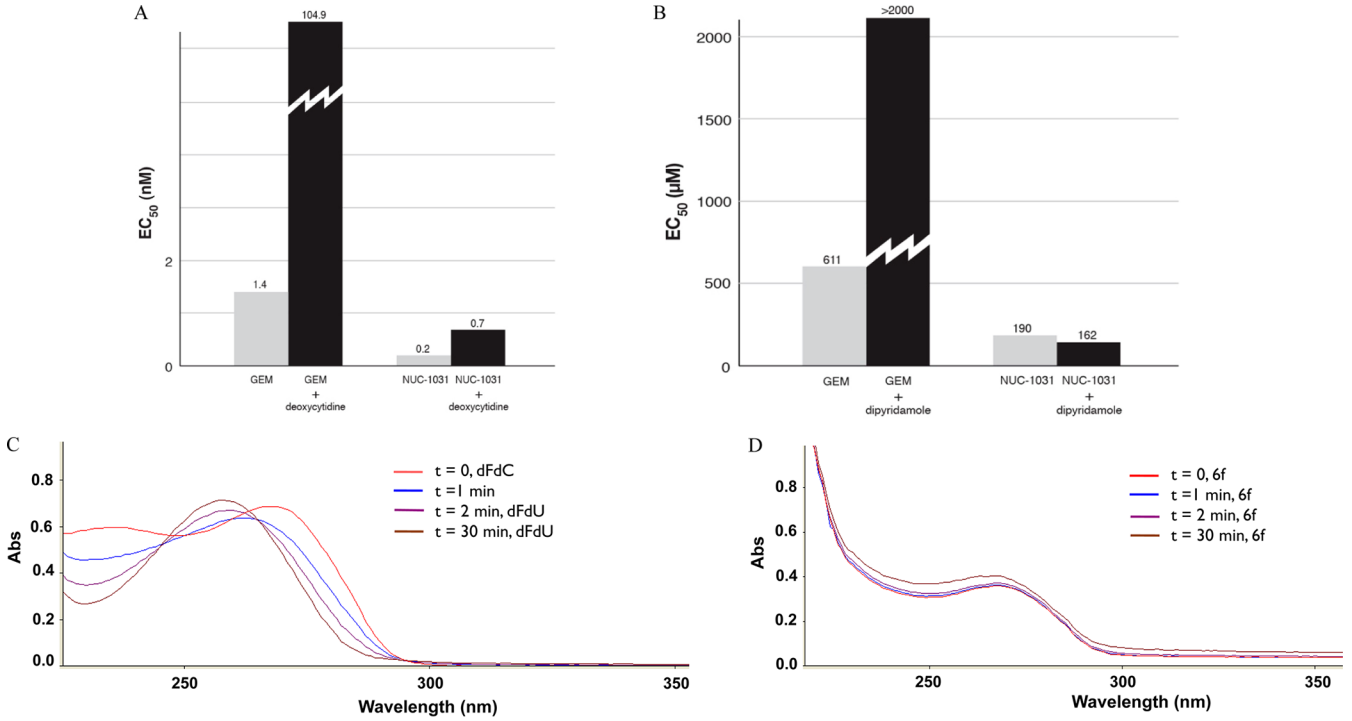


Figure 3. ProTide **6f** overcomes the key cancer resistance mechanisms associated with gemcitabine *in vitro*. (A) Effect of deoxycytidine-mediated dCK inhibition on gemcitabine (GEM) and **6f** cytotoxicity in RT112 cells. (B) Effect of dipyridamole-mediated nucleoside transport inhibition on gemcitabine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

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,[7b,9b] which mimics a down-regulation of the enzyme. In the presence of deoxycytidine, the gemcitabine concentration required to block cell proliferation, EC_{50}, increased 75-fold from 1.4 nM to 104.9 nM, 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_{50} lower than gemcitabine EC_{50}) and its activity was not significantly affected by deoxycytidine (**6f**: EC_{50} = 0.7nM in the presence of deoxycytidine as opposed to 0.2 nM without deoxycytidine). This result indicates that **6f** can bypass the 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_{50} significantly increasing from 611 μM to >2000 μM. In contrast, the ProTide, which had a baseline activity ∼3-fold superior to gemcitabine (190 μM compared to 611 μM for gemcitabine), maintained a strong cytotoxic level in the presence of the inhibitor (EC_{50} = 162 μM). These data suggest that **6f** is not reliant on the nucleoside transporter hENT1 to enter the cells and exert its anticancer 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.[35] Unprotected hydroxyl groups at positions 3′ and 5′ of the nucleoside analogue were shown to be necessary for the cleavage to occur.[36] In addition, the amino acid ester prodrugs of gemcitabine have been reported previously to be resistant to deamination by cytidine deaminase *in vitro*.[37]

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 and 350 nm (Figure 3C). The absorbance of gemcitabine shifted from 267 to 257 nm within the first 2 min 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 min 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.

Gemcitabine is a prodrug which requires further processing into the di- and triphosphate active moieties (dFdCDP and dFdCTP, respectively) to block cancer cell growth. The triphosphate form is considered the main driver of the anticancer effect, directly interfering with DNA replication, while the diphosphate derivative is thought to further potentiate the activity.[5] To test whether **6f** cytotoxic activity

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

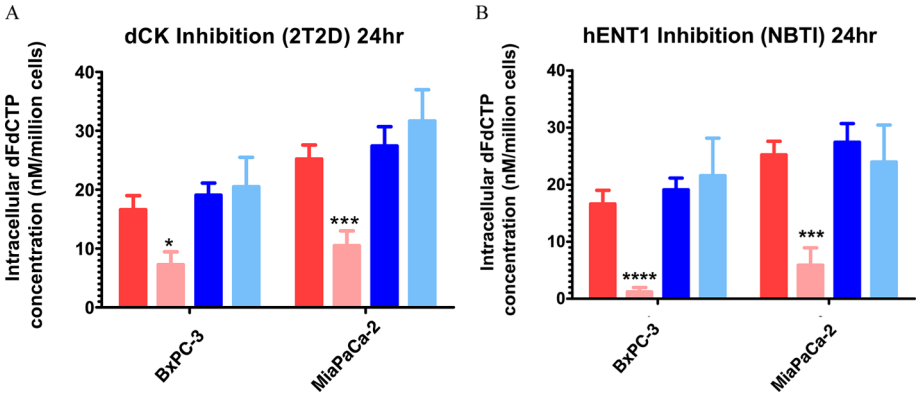


Figure 4. ProTide **6f** generates high intracellular levels of the active moiety bypassing key drug resistance mechanisms *in vitro*. (A) Intracellular levels of dFdCTP (gemcitabine triphosphate) in pancreatic cell lines treated with gemcitabine (red and pink) or **6f** (dark and light blue) in the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

correlates with high levels of intracellular dFdCTP, the ProTide was tested in a cell growth assay for 24 h 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 B; 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 metabolized into the active anticancer moiety, overcoming the key cancer resistance mechanisms associated with gemcitabine and blocking tumor cell growth.

The data gathered *in vitro* prompted us to examine whether the ProTide **6f** would also have a strong anticancer 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 nonrodent specie reference (Figure 5A). As expected, when the ProTide was incubated with rat serum and monitored by ^{31}P NMR over time, the spectra revealed the complete conversion of **6f** to the achiral metabolite **C** after 6.5 min, whereas the compound was still stable after 14 h in dog serum (>80% **6f**; 20% metabolite **C**). The estimated half-life of ProTide **6f** in rat serum was <5 min but exceeded 100 h 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.

To determine the effect of **6f** on tumor growth, nude mouse xenograft models of MiaPaCa-2 human pancreatic cancers were administrated with equimolar doses of gemcitabine, **6f**, and vehicle. The body weight was also recorded to assess the animal's tolerance 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 was 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 anticancer 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 anticancer agents to overcome the key cancer resistance mechanisms associated with nucleoside analogues, and in particular, gemcitabine. The compounds synthesized 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

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

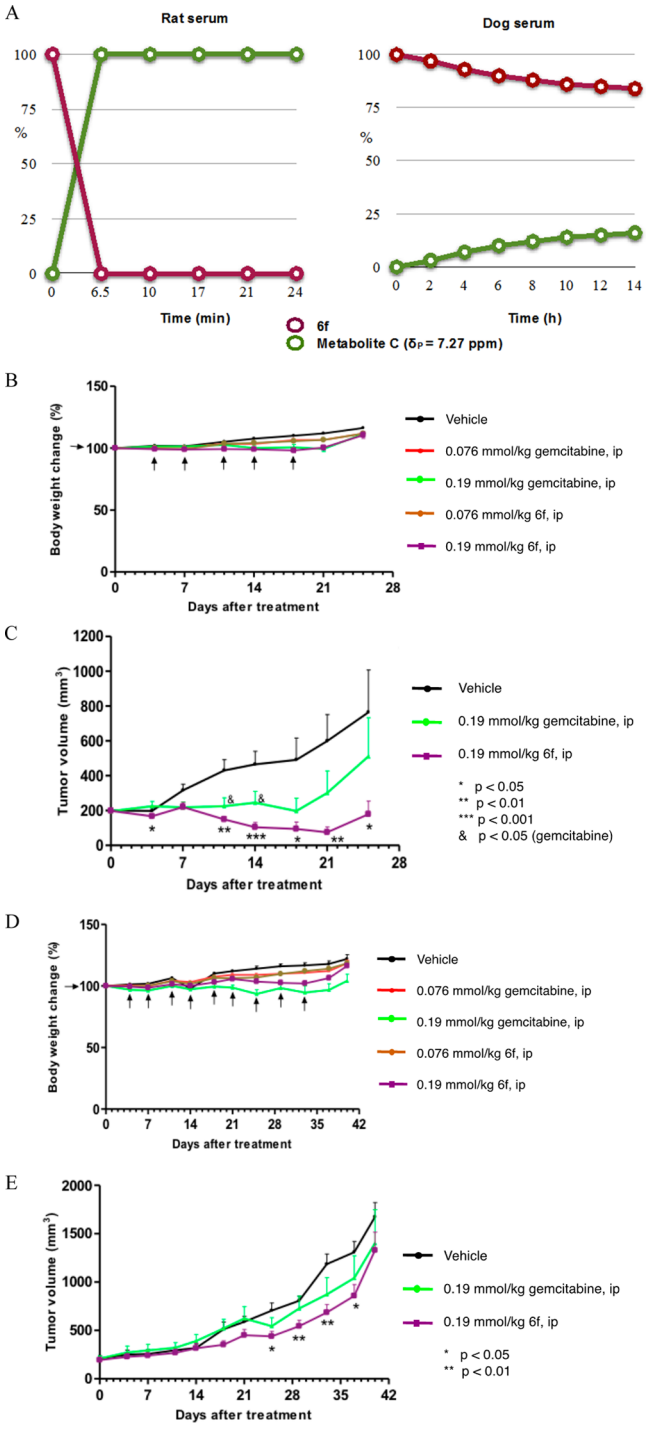


Figure 5. (A) Metabolic stability of **6f** incubated with rat and dog serum. (B) Mean body weight change (%) of MiaPaCa-2 bearing nude mice following administration of vehicle (n = 8), gemcitabine (n = 8), and **6f** (0.19 mmol/kg, 2×/week; n = 7). (C) Antitumor effect of gemcitabine and **6f** against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

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;[7] requirement on nucleoside transporters for cellular entry;[10] and susceptibility to degradation by deactivating enzymes such as cytidine deaminase.[9] *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.[27] The phosphoramidate chemistry is potentially a great source of new and very effective anticancer 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$^-$ and human lymphocyte CEM and its dCK-deficient congener CEM/dCK$^-$ were seeded at −50 to 75 × 10^3 cells/200 μL in the wells of a 96-well microtiter plate that contained a variety of drug concentrations. After 48 h (L1210) or 72 h (CEM) the cell number was determined by counting with a Coulter Counter. The IC_{50} (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 95th Street, Redmond, WA, 98052, USA) according to the published procedure (Cerep ref. 1432).[38] Pooled cryopreserved hepatocytes were thawed, washed, and resuspended in Krebs-Heinslet buffer (pH 7.3). The reaction was initiated by adding the test compound (1 μM final concentration) into cell suspension and incubated in a final volume of 100 μL on a flat-bottom 96-well plate for 0 and 60 min, respectively, at 37 °C/5% CO_2. The reaction was stopped by adding 100 μL 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 min at room temperature. Each supernatant (150 μL) 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 95th Street, Redmond, WA, 98052, USA) according to the published procedure (Cerep ref. 0607).[39] Pooled liver microsomes are preincubated with NADPH-regenerating system (1 mM NADP, 5 mM G6P, and 1 U/mL G6PDHase) in phosphate buffer (pH 7.4) containing 3 mM $MgCl_2$ and 1 mM EDTA in a 2 mL-block 96-well plate for 10 min in a 37 °C shaking water bath. The reaction was initiated by adding the test compound (final concentration 0.5 μM) and incubated in a final volume of 700 μL in the 37 °C shaking water-bath. At 0, 15, 30, 45, and 60 min after reaction initiation, 100 μL of the incubation mixture is transferred to 100 μL 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 min and

dx.doi.org/10.1021/jm401853a | J. Med. Chem. 2014, 57, 1531−1542

centrifuged at 2550 × *g* for 15 min 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 MiaPaCa-2 (MP2) and BxPC-3 were seeded at range of density 0.5 to 100 × 10^3 cells/well in the wells of 96-well plate the day before incubation, then incubated for 72 h with the different concentrations of compound tested. Then 50 μL of MTS was added and cells were incubated for 4 h at 37 °C. The data were read and collected by Spectra Max 340 Absorbance Microplate Reader.

Maximum Tolerated Dose (MTD) Assay. MTD was contracted and performed by WuXi AppTec (Shanghai) Co., Ltd. Balb/c nude mouse (Slac Laboraory 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, 2×/WK) and **6j** (i.p 0.228 mmol/kg, 141.9 mg/kg, 2×/WK) or vehicle for 2 weeks. Compounds were dissolved in 40% Captisol solution. (40% Captisol was prepared by dissolving 20 mg of Captisol with pure water, and made the final volume 50 mL. The solvent was filtered with 0.22 μm filter). Mice were monitored daily for body weight change and clinical symptoms for 2 weeks. The results were expressed as means ± SEM.

***In Vivo* Xenograft Models of MiaPaCa-2 and BxPC-3 Bearing Mice Assay.** 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 ± 2 g; Captisol (CyDex Pharmaceuticlas 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. Mice continued to grow for one week without the above treatment. The results were expressed as means ± SEM. Data was analyzed 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}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}P NMR experiments (at 25 °C). 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 Serum. The experiment was carried out by dissolving ProTide **6f** (5.0 mg) in DMSO (0.050 mL) and D_2O (0.15 mL). After recording the control ^{31}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}P NMR experiments at 37 °C. The spectra were recorded every 30 min over 13 h. ^{31}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.[40] Stock solution of gemcitabine and ProTide **6f** were dissolved in the assay buffer (50 mM Tris-HCl buffer pH 7.5) to a final concentration of 100 μM. Cytidine deaminase (100 μg in 200 μL 20 mM Tris-HCl buffer pH 8.0, containing 1 mM DTT, 2 mM EDTA, 100 mM NaCl, 40% glycerol) was aliquoted for 4 portions and stored at −20 °C prior to usage. 50 μL aliquot of cytidine deaminase was added to 150 μL of assay buffer. For each assay, 1 mL of 100 μM of gemcitabine and **6f** was placed in a 1 cm UV cuvette at 25 °C, and UV spectrum was recorded over the range 220 to 350 nm. A portion of enzyme solution (200 μL) was added, and spectra were recorded in 1 min intervals for 30 min.

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 20 mM Tris-HCl buffer pH 8.0, containing 1 mM DTT, 2 mM EDTA, 100 mM NaCl, 40% glycerol, concentration 0.5 mg/mL as determinated 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 aluminum plates and visualized under UV (254 nm) and/or with ^{31}P NMR spectra. Column chromatography was performed on silica gel (35−70 μM). 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 μM) as an analytic column with a gradient elution of H_2O/MeOH from 100/0 to 0/100 in 40 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 Cardiff University, using electrospray spectrometry (ESMS). CDA assays were performed with Varian Cary 50 Bio UV/vis spectrophotometer. UPLC-MS/MS Analysis: dFdCTP was resolved using an ultraperformance liquid chromatography system (Accela UPLC, Thermo Scientific, UK) equipped with Ascentis Express 2.7 μm C18 4.6 × 100 mm HPLC column (Sigma Aldrich, UK). Mobile phase gradient was employed, comprising buffer A (10 mM ammonium acetate) = 98% and buffer B (acetonitrile) = 2% at 0 min. Buffer A from 98% to 30% over 6 min and from 30% to 98% over 2 min, all at a flow rate of 700 μL/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 3500 V, 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 → 159.0 (collision energy 37 V).

General Method for the Preparation of Phosphorochloridates (3a−l). 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 stirred for 1−2 h. Formation of a 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.

Phenyl(isopropoxy-L-alaninyl) Phosphorochloridate (3a). Yellowish oil; yield 80% (2.94 g); mixture of diastereoisomers; ^{31}P NMR (202 MHz, $CDCl_3$): δ_P 8.08, 7.71 (int. 1.00:1.00). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.41 − 7.36 (m, 2H, Ar*H*), 7.31 − 7.27 (m, 3H, Ar*H*), 5.21 − 5.01 (m, 1H, OC*H*$(CH_3)_2$), 4.21 − 4.06 (m, 1H, C*H*CH_3), 1.51 (d, *J* = 6.9 Hz, 3H, CHC*H*$_3$), 1.26 − 1.19 (m, 6H, OCH$(CH_3)_2$).

3′-*O*-(*tert*-Butoxycarbonyl)-gemcitabine (4). Prepared according to the published procedure[28] and obtained as a white solid. Yield, 75% (1.03 g). ^{19}F NMR (470 MHz, MeOD): δ_F − 115.74, − 115.75 (d, *J* = 243 Hz, F), − 119.21 (broad signal, *F*). ^{1}H NMR (500 MHz, MeOD): δ_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$(CH_3)_3$).

General Method for the Preparation of Gemcitabine ProTides (5a−l). To a solution of 3′-*O*-(*tert*-butoxycarbonyl)-

dx.doi.org/10.1021/jm401853a | *J. Med. Chem.* 2014, 57, 1531−1542

gemcitabine **4** (1.0 equiv) in dry THF (10 mL), *tert*-BuMgCl (1.0 M solution in THF, 1.2 equiv) was added in one portion followed by addition of appropriate phosphorochloridate (2.0 equiv) dissolved in anhydrous THF (3 mL). The reaction mixture was stirred for 16 to 18 h and then evaporated *in vacuo* to give a crude residue that was purified by column chromatography on silica gel, eluting with CH_2Cl_2-MeOH as a gradient (0% to 5% MeOH) to afford the products **5a–l** as white solid.

General Method for the Preparation of Gemcitabine ProTides (6a–l). The 3′-*O*-(*tert*-butoxycarbonyloxy)-gemcitabine phosphates (**5a–l**) were treated with a mixture of TFA/DCM (1:1) (6 mL) at 0 °C. The resulting reaction mixture was stirred at 0 °C for 2–4 h. After the reaction was completed the solvents were evaporated and the residue was treated with saturated $NaHCO_3$, and extracted with EtOAc. The organic layers were combined, dried ($MgSO_4$), 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-alaninyl)]phosphate (5a). 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%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.86, 3.79. ^{1}H NMR (500 MHz, MeOD): δ_H 7.56, 7.48 (2*d*, *J* = 7.50 Hz, 1H, *H*-6), 7.41 − 7.39 (m, 2H, Ar*H*), 7.35 − 7.19 (m, 3H, Ar*H*), 6.30 − 6.28 (m, 1H, *H*-1′), 5.89, 5.85 (2*d*, *J* = 7.50 Hz, 1H, *H*-5), 5.28 − 5.22 (m, 1H, *H*-3′), 5.04 − 4.98 (m, 1H, OC*H*$(CH_3)_2$), 4.54 − 4.50 (m, 1H, *H*-5′$_a$), 4.46 − 4.39 (m, 2H, *H*-5′$_b$, *H*-4′), 4.01 − 3.98 (m, 1H, C*H*CH_3), 1.51, 1.50 (2*s*, 9H, C$(CH_3)_3$), 1.38 (d, *J* = 6.30 Hz, 3H, CHCH_3).

2′-Deoxy-2′,2′-difluoro-D-cytidine-5′-*O*-[phenyl(isopropoxy-L-alaninyl)] phosphate (6a). 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^+) 555.15. $C_{21}H_{27}F_2N_4O_8NaP$ required: (M^+) 532.43. Mixture of diastereoisomers (45%, 55%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.80, 3.73. ^{19}F NMR (470 MHz, MeOD): δ_F − 118.0 (d, *J* = 240 Hz), − 118.2 (d, *J* = 240 Hz, *F*), − 123.43 (− 123.74) (broad signal, *F*). ^{1}H NMR (500 MHz, MeOD): δ_H 7.60, 7.55 (2*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*d*, *J* = 7.72 Hz, 1H, *H*-5), 5.03 − 4.97 (m, 1H, OC*H*$(CH_3)_2$), 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, C*H*CH_3), 1.37 − 1.32 (m, 3H, CHCH_3), 1.25 − 1.22 (m, 6H, OCH$(CH_3)_2$). ^{13}C NMR (125 MHz, MeOD): δ_C 174.4 (C═O, ester), 167.62 (*C*-NH_2), 157.80 (C═O base), 152.18, 152.12 (*C*-Ar), 142.5, 142.41 (*C*H-base), 130.88 (d, $^3J_{C-P}$ = 3.75 Hz, CH−Ar), 126.33 (CH−Ar), 122.46 (d, $^1J_{C-F}$ = 256 Hz, CF_2), 121.42, 121.40 (2*d*, $^3J_{C-P}$ = 2.5 Hz, CH−Ar), 96.8 (CH-base), 85.6 (broad signal, *C*-1′), 80.40 (*C*-4′), 71.62, 71.20 (2*d*, $^2J_{C-F}$ = 37.5 Hz, *C*-3′), 70.24 (OCH$(CH_3)_2$), 65.71, 65.66 (2*d*, $^2J_{C-P}$ = 5.0 Hz, *C*-5′), 51.89, 51.75 (CHCH_3), 21.99, 21.91, 21.82 (OCH$(CH_3)_2$), 16.47 (CHCH_3). Reverse HPLC, eluting with H_2O/MeOH from 100/0 to 0/100 in 40 min, showed two peaks of the diastereoisomers with t_R 22.73 min and t_R 23.53 min (44%, 54%).

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +44 2920874537. E-mail: mcguigan@cardiff.ac.uk.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Helen Murphy and Lindsey Leven for secretarial assistance and NuCana BioMed Ltd and the KU Leuven (GOA 10/014) for financial support. We thank Sofiya Abramchuk and Lizette van Berckelaer for excellent laboratory assistance.

■ 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

■ REFERENCES

(1) Hertel, L. W.; Kroin, J. S.; Misner, J. W.; Tustin, J. M. Synthesis of 2-deoxy-2,2-difluoro-D-ribose and 2′-deoxy-2′,2′-difluoro-D-ribofuranosyl nucleosides. *J. Org. Chem.* **1988**, *53*, 2406–2409.

(2) (a) Hertel, L. W.; Boder, G. B.; Kroin, J. S.; Rinzel, S. M.; Poore, G. A.; Todd, G. C.; Grindey, G. B. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Res.* **1990**, *50*, 4417–4422. (b) Braakhuis, B. J.; Ruiz van Haperen, V. W.; Welters, M. J.; Peters, G. J. Schedule-dependent therapeutic efficacy of the combination of gemcitabine and cisplatin in head and neck cancer xenografts. *Eur. J. Cancer* **1995**, *31A*, 2335–2340. (c) Merriman, R. L.; Hertel, L. W.; Schultz, R. M.; Houghton, P. J.; Houghton, J. A.; Rutherford, P. G.; Tanzer, L. R.; Boder, G. B.; Grindey, G. B. Comparison of the antitumor activity of gemcitabine and ara-C in a panel of human breast, colon, lung and pancreatic xenograft models. *Invest. New Drugs* **1996**, *14*, 243–247. (d) Manegold, C. Gemcitabine (Gemzar) in non-small cell lung cancer. *Expert Rev. Anticancer Ther.* **2004**, *4*, 345–360. (e) Heinemann, V. Gemcitabine in metastatic breast cancer. *Expert Rev. Anticancer Ther.* **2005**, *5*, 429–443.

(3) Eckel, F.; Schneider, G.; Schmid, R. M. Pancreatic cancer: a review of recent advances. *Expert Opin. Invest. Drugs* **2006**, *15*, 1395–1410.

(4) Toschi, L.; Finocchiaro, G.; Bartolini, S.; Gioia, V.; Cappuzzo, F. Role of gemcitabine in cancer therapy. *Future Oncol.* **2005**, *1*, 7–17.

(5) (a) Huang, P.; Chubb, S.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res.* **1991**, *51*, 6110–6117. (b) Ruiz van Haperen, V. W.; Veerman, G.; Vermorken, J. B.; Peters, G. J. 2′,2′-Difluorodeoxycytidine (gemcitabine) incorporation into RNA and DNA of tumor cell lines. *Biochem. Pharmacol.* **1993**, *46*, 762–766.

(6) Heinemann, V.; Xu, Y. Z.; Chubb, S.; Sen, A.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. Cellular elimination of 2′,2′-difluorodeoxycytidine-5′-triphosphate: a mechanism of self-potentiation. *Cancer Res.* **1992**, *52*, 533–539.

(7) (a) Ruiz van Haperen, V. W.; Veerman, G.; Eriksson, S.; Boven, E.; Stegmann, A. P.; Hermsen, M.; Vermorken, J. B.; Pinedo, H. M.; Peters, G. J. Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. *Cancer Res.* **1994**, *54*, 4138–4143. (b) Kroep, J. R.; Loves, W. J.; van der Wilt, C. L.; Alvarez, E.; Talianidis, I.; Boven, E.; Braakhuis, B. J. M.; van Groeningen, C. J.; Pinedo, H. M.; Peters, G. J. Pretreatment deoxycytidine kinase levels predict *in vivo* gemcitabine sensitivity. *Mol. Cancer Ther.* **2002**, *1*, 371–376. (c) Galmarini, C. M.; Clarke, M. L.; Jordheim, L.; Santos, C. L.; Cros, E.; Mackey, J. R.; Dumontet, C. Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene. *BMC Pharmacol.* **2004**, *4*, 8.

(8) (a) Heinemann, V.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. *Cancer Res.* **1988**, *48*, 4024–4031. (b) Gandhi, V.; Plunkett, W. Modulatory activity of 2′,2′-difluorodeoxycytidine on the phosphorylation and

cytotoxicity of arabinosyl nucleosides. *Cancer Res.* **1990**, *50*, 3675−3680.
(9) (a) Beumer, J. H.; Eiseman, J. L.; Parise, R. A.; Joseph, E.; Covey, J. M.; Egorin, M. J. Modulation of gemcitabine (2′,2′-difluoro-2′-deoxycytidine) pharmacokinetics, metabolism, and bioavailability in mice by 3,4,5,6-tetrahydrouridine. *Clin. Cancer Res.* **2008**, *14*, 3529−3535. (b) Mini, E.; Nobili, S.; Caciagli, B.; Landini, I.; Mazzei, T. Cellular pharmacology of gemcitabine. *Ann Oncol.* **2006**, *17* (Suppl 5), v 7−12.
(10) (a) Mackey, J. R.; Mani, R. S.; Selner, M.; Mowles, D.; Young, J. D.; Belt, J. A.; Crawford, C. R.; Cass, C. E. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res.* **1998**, *58*, 4349−4357. (b) Gourdeau, H.; Clarke, M. L.; Ouellet, F.; Mowles, D.; Selner, M.; Richard, A.; Lee, N.; Mackey, J. R.; Young, J. D.; Jolivet, J.; Lafrenière, R. G.; Cass, C. E. Mechanisms of uptake and resistance to troxacitabine, a novel deoxycytidine nucleoside analogue, in human leukemic and solid tumor cell lines. *Cancer Res.* **2001**, *61*, 7217−7224.
(11) (a) www.GEMZAR.com. Locally advanced or metastatic pancreatic cancer. (b) Liau, S.-S.; Whang, E. HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma. *Clin. Cancer Res.* **2008**, *14*, 1470−1477.
(12) Moysan, E.; Bastiat, G.; Benoit, J. P. Gemcitabine versus modified gemcitabine: a review of several promising chemical modifications. *Mol. Pharmaceutics* **2013**, *10*, 430−444.
(13) Bender, D. M.; Bao, J.; Dantzig, A. H.; Diseroad, W. D.; Law, K. L.; Magnus, N. A.; Peterson, J. A.; Perkins, E. J.; Pu, Y. J.; Reutzel-Edens, S. M.; Remick, D. M.; Starling, J. J.; Stephenson, G. A.; Vaid, R. K.; Zhang, D.; McCarthy, J. R. Synthesis, crystallization, and biological evaluation of an orally active prodrug of gemcitabine. *J. Med. Chem.* **2009**, *52*, 6958−6961.
(14) Koolen, S. L. W.; Witteveen, P. O.; Jansen, R. S.; Langenberg, M. H. G.; Kronemeijer, R. H.; Nol, A.; Garcia-Ribas, I.; Callies, S.; Benhadji, K. A.; Slapak, C. A.; Beijnen, J. H.; Voest, E. E.; Schellens, J. H. M. Phase I study of oral gemcitabine prodrug (LY2334737) alone and in combination with erlotinib in patients with advanced solid tumors. *Clin. Cancer Res.* **2011**, *17*, 6071−6082.
(15) (a) Bergman, A. M.; Adema, A. D.; Balzarini, J.; Bruheim, S.; Fichtner, I.; Noordhuis, P.; Fodstad, O.; Myhren, F.; Sandvold, M. L.; Hendriks, H. R.; Peters, G. J. Antiproliferative activity, mechanism of action and oral antitumor activity of CP-4126, a fatty acid derivative of gemcitabine, in in vitro and in vivo tumor models. *Invest. New Drugs* **2011**, *29*, 456−466. (b) Adema, A. D.; Smid, K.; Losekoot, N.; Honeywell, R. J.; Verheul, H. M.; Myhren, F.; Sandvold, M. L.; Peters, G. J. Metabolism and accumulation of the lipophilic deoxynucleoside analogs elacytarabine and CP-4126. *Invest. New Drugs* **2012**, *30*, 1908−1916.
(16) Ahmed, T. Clavis and Clovis collaborate on anticancer drug CP-4126. *Pharma Deals Review* **2009**, *12*, 122. In 2012, Clavis Pharma and Clovis Oncology suspended further development of CP-4126 after demonstrating no difference in overall survival between CP-4126 and gemcitabine in hENT1-low patients.
(17) (a) Bazzanini, R.; Manfredini, S.; Durini, E.; Gröschel, B.; Cinatl, J.; Balzarini, J.; De Clercq, E.; Imbach, J. L.; Péirgaud, C.; Gosselin, G. Prodrugs of Ara-CMP and Ara-AMP with a S-acyl-2-thioethyl (SATE) biolabile phosphate protecting group: synthesis and biological evaluation. *Nucleoside Nucleotides* **1999**, *18*, 971−972. (b) Galmarini, C. M.; Warren, G.; Senanayake, M. T.; Vinogradov, S. V. Efficient overcoming of drug resistance to anticancer nucleoside analogs by nanodelivery of active phosphorylated drugs. *Int. J. Pharm.* **2010**, *395*, 281−289.
(18) Cahard, D.; McGuigan, C.; Balzarini, J. Aryloxy phosphoramidate triesters as protides. *Mini-Rev. Med. Chem.* **2004**, *4*, 371−381.
(19) McGuigan, C.; Shackleton, J. M.; Tollerfield, S. M.; Riley, P. A. Synthesis and evaluation of some novel phosphate and phosphinate derivatives of araA. Studies on the mechanism of action of phosphate triesters. *Nucleic Acids Res.* **1989**, *17*, 10171−10177.
(20) Balzarini, J.; Karlsson, A.; Aquaro, S.; Perno, C. F.; Cahard, D.; Naesens, L.; De Clercq, E.; McGuigan, C. Mechanism of anti-HIV action of masked alaninyl d4T-MP derivatives. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7295−7299.
(21) McGuigan, C.; Harris, S. A.; Daluge, S. M.; Gudmundsson, K. S.; McLean, E. W.; Burnette, T. C.; Marr, H.; Hazen, R.; Condreay, L. D.; Johnson, L.; De Clercq, E.; Balzarini, J. Application of phosphoramidate pronucleotide technology to abacavir leads to a significant enhancement of antiviral potency. *J. Med. Chem.* **2005**, *48*, 3504−3515.
(22) Vernachio, J. H.; Bleiman, B.; Bryant, K. D.; Chamberlain, S.; Hunley, D.; Hutchins, J.; Ames, B.; Gorovits, E.; Ganguly, B.; Hall, A.; Kolykhalov, A.; Liu, Y.; Muhammad, J.; Raja, N.; Walters, C. R.; Wang, J.; Williams, K.; Patti, J. M.; Henson, G.; Madela, K.; Aljarah, M.; Gilles, A.; McGuigan, C. INX-08189, a phosphoramidate prodrug of 6-O-methyl-2′-C-methyl guanosine, is a potent inhibitor of hepatitis C virus replication with excellent pharmacokinetic and pharmacodynamic properties. *Antimicrob. Agents Chemother.* **2011**, *55*, 1843−1851.
(23) Walsby, E.; Congiatu, C.; Schwappach, A.; Walsh, V.; Burnett, A. K.; McGuigan, C.; Mills, K. I. Nucleoside analogues of cladribine produce enhanced responses in cell lines. *Blood* **2005**, *106*, 941A−942A (Abstract 3369)..
(24) McGuigan, C.; Congiatu, C.; Mills, K. Phosphoramidate derivatives of nucleoside compounds for use in the treatment of cancer. EP1866324A1, WO 2006100439 A1.
(25) (a) Vande Voorde, J.; Liekens, S.; McGuigan, C.; Murziani, P. G. S; Slusarczyk, M.; Balzarini, J. The cytostatic activity of NUC-3073, a phosphoramidate prodrug of 5-fluoro-2′-deoxyuridine, is independent of activation by thymidine kinase and insensitive to degradation by phosphorolytic enzymes. *Biochem. Pharmacol.* **2011**, *82*, 441−452. (b) McGuigan, C.; Murziani, P. G. S.; Slusarczyk, M.; Gonczy, B.; Vande Voorde, J.; Liekens, S.; Balzarini, J. Phosphoramidate ProTides of the anticancer agent FUDR successfully deliver the preformed bioactive monophosphate in cells and confer advantage over the parent nucleoside. *J. Med. Chem.* **2011**, *54*, 7247−7258.
(26) (a) McGuigan, C. Preparation of amino acid-containing nucleotide phosphoramidates as antitumor agents. *PCT. Int. Appl.* 2005 WO 2005012327 A2 20050210. (b) McGuigan, C. Nucleotide phosphoramidates as anticancer agents. US 7951787 B2, 2005.
(27) Ghazaly E. A; Simon J.; Gribben, J. G.; Mohammad, T.; Oluwadunni E.; Stavraka, Ch.; Hopkins, T.; Gabra, H.; Harpreet, W.; Habib, N. A.; Leonard, R. C. F.; McGuigan, C.; Slusarczyk, M.; Blagden, S. P. ProGem1: Phase 1 first-in-human study of the novel nucleotide analogue NUC-1031 in adult patients with advanced solid tumors. 2013 ASCO Annual Meeting *J. Clin. Oncol.* **2013**, *31* (suppl; Abstract 2576).
(28) Guo, Z.; Gallo, J. M. Selective protection of 2′,2′-difluorodeoxycytidine (Gemcitabine). *J. Org. Chem.* **1999**, *64*, 8319−8322.
(29) McGuigan, C.; Pathirana, R. N.; Balzarini, J.; De Clercq, E. Intracellular delivery of bioactive AZT nucleotides by aryl phosphate derivatives of AZT. *J. Med. Chem.* **1993**, *36*, 1048−1052.
(30) Greene, T. W.; Wuts, P. G. M. Protective groups, in *Organic Chemistry*, 2nd ed.; Wiley: New York, 1991.
(31) Curley, D.; McGuigan, C.; Devine, K. G.; O′Connor, T. J.; Jeffries, D. J.; Kinchington, D. Synthesis and anti-HIV evaluation of some phosphoramidate derivatives of AZT: studies on the effect of chain elongation on biological activity. *Antiviral Res.* **1990**, *14*, 345−356.
(32) McGuigan, C.; Devine, K. G.; O′Connor, T. J.; Kinchington, D. Synthesis and anti-HIV activity of some haloalkyl phosphoramidate derivatives of 3′-azido-3′-deoxythymidine (AZT): potent activity of the trichloroethyl methoxyalaninyl compound. *Antiviral Res.* **1991**, *15*, 255−263.
(33) Saboulard, D.; Naesens, L.; Cahard, D.; Salgado, A.; Pathirana, R.; Velazquez, S.; McGuigan, C.; De Clercq, E.; Balzarini, J. Characterization of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. *Mol. Pharmacol.* **1999**, *56*, 693−704.
(34) Birkus, G.; Wang, R.; Liu, X.; Kutty, N.; MacArthur, H.; Cihlar, T.; Gibbs, C.; Swaminathan, S.; Lee, W.; McDermott, M. Cathepsin A

is the major hydrolase catalyzing the intracellular hydrolysis of the antiretroviral nucleotide phosphonoamidate prodrugs GS-7340 and GS-9131. *Antimicrob. Agents Chemother.* **2007**, *51*, 543−550.
(35) Bengala, C.; Guarneri, V.; Giovannetti, E.; Lencioni, M.; Fontana, E.; Mey, V.; Fontana, A.; Boggi, U.; Del Chiaro, M.; Danesi, R.; Ricci, S.; Mosca, F.; Del Tacca, M.; Conte, P. F. Prolonged fixed dose rate infusion of gemcitabine with autologous haemopoietic support in advanced pancreatic adenocarcinoma. *Brit. J. Cancer.* **2005**, *93*, 35−40.
(36) Camiener, G. W. Studies of the enzymatic deamination of cytosine arabinoside. 3. Substrate requirements and inhibitors of the deaminase of human liver. *Biochem. Pharmacol.* **1967**, *16*, 1691−1702.
(37) Song, X.; Lorenzi, P. L.; Landowski, C. P.; Vig, B. S.; Hilfinger, J. M.; Amidon, G. L. Amino acid ester prodrugs of the anticancer agents gemcitabine: synthesis, bioconversion, metabolic bioevasion, and hPEPT1-mediated transport. *Mol. Pharm.* **2005**, *2*, 157−167.
(38) Li, A. P.; Lu, C.; Brent, J. A.; Pham, C.; Fackett, A.; Ruegg, C. E.; Silber, P. M. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem. Biol. Interact.* **1999**, *121*, 17−35.
(39) Kuhnz, W.; Gieschen, H. Predicting the oral bioavailability of 19-nortestosterone progestins *in vivo* from their metabolic stability in human liver microsomal preparations *in vitro*. *Drug Metab. Dispos.* **1998**, *26*, 1120−1127.
(40) (a) Murakami, E.; Niu, C.; Bao, H.; Micolochick Steuer, H. M.; Whitaker, T.; Nachman, T.; Sofia, M. A.; Wang, P.; Otto, M. J.; Furman, P. A. The mechanism of action of ß-D-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine involves a second metabolic pathway leading to ß-D-2′-deoxy-2′-fluoro-2′-*C*-methyluridine 5′-triphosphate, a potent inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. *Antimicrob. Agents Chemeother.* **2008**, *52*, 458−464. (b) CDA Activity Assay, Material Safety Datasheets available at www.acris-antibodies.com.