Bioorganic & Medicinal Chemistry 22 (2014) 2133–2140

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

N-Acyl-phosphoramidates as potential novel form of gemcitabine prodrugs

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ARTICLE INFO

Article history:
Received 31 October 2013
Revised 13 February 2014
Accepted 18 February 2014
Available online 3 March 2014

Keywords:
Gemcitabine
Prodrug
Cytotoxicity
Anticancer therapy
DNA polymerase inhibitor

ABSTRACT

Gemcitabine (dFdC) is a cytidine analog remarkably active against a wide range of solid tumors. Inside a cell, gemcitabine is phosphorylated by deoxycytidine kinase to yield gemcitabine monophosphate, further converted to gemcitabine di- and triphosphate. The most frequent form of acquired resistance to gemcitabine in vitro is the deoxycytidine kinase deficiency. Thus, proper prodrugs carrying the 5′-pdFdC moiety may help to overcome this problem. A series of new derivatives of gemcitabine possessing *N*-acyl(thio)phosphoramidate moieties were prepared and their cytotoxic properties were determined. *N*-Acyl-phosphoramidate derivatives of gemcitabine have similar cytotoxicity as gemcitabine itself, and have been found accessible to the cellular enzymes. The nicotinic carboxamide derivative of gemcitabine 5′-*O*-phosphorothioate occurred to be the best inhibitor of bacterial DNA polymerase I and human DNA polymerase α.

1. Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC, Fig. 1A) exerts a spectrum of antitumor activity against a wide range of solid tumors, including pancreatic, non-small cell lung, breast and ovarian cancers.[1] Inside a cell, gemcitabine is phosphorylated by deoxycytidine kinase to yield gemcitabine monophosphate (pdFdC), which is, in a stepwise manner, converted to gemcitabine di- and triphosphate (dFdCDP and dFdCTP, respectively). The first phosphorylation, catalyzed by deoxycytidine kinase, is the rate-limiting step. The most frequently described form of acquired resistance to gemcitabine in vitro is deoxycytidine kinase deficiency.[2] Therefore, one of the possible ways to enhance its activity is to deliver appropriately protected gemcitabine 5′-*O*-phosphate to the cell.

There are only a few reports available on pronucleotide derivatives of 5′-pdFdC and processes of their unmasking. *O*-Aryl *N*-alaninyl derivative of pdFdC (Fig. 1B) represents a recent extension of the prodrug strategy that has been extensively investigated for antiviral agents such as 3′-azido-3′-deoxythymidine (AZT).[3] In mouse xenograft models of human prostate and colon cancers the *O*-phenyl-*N*-(benzyloxy-L-alaninyl) derivative of gemcitabine is significantly more potent and less toxic than gemcitabine itself.[4] By analogy to what has been assumed as the metabolic activation process for antiviral activity, pdFdC is thought to be produced by the carboxyesterase-mediated formation of a carboxylate nucleophile that intramolecularly displaces the *O*-aryl substituent.[5] The subsequent P–N bond cleavage may be catalyzed by so far unspecified enzyme with the phosphoramidase activity such as histidine triad nucleotide binding protein (Hint-1).[5] Hint-1 was found ubiquitous in all organisms. It exerts phosphoramidase activity towards several synthetic phosphoramidate substrates, such as AMP-*N*-ε-lysine, AMP-*N*-alanine, AMP-*N*-morpholine and adenosine-5′-phosphoramidate (NH_2-pA).[6] Recently, it has been demonstrated that Hint-1-assisted hydrolysis of the P–N bond in P-chiral nucleoside 5′-*O*-phosphoramidothioate occurs with retention of configuration, what is consistent with the formation of an intermediate covalent enzyme–substrate complex.[7] Hint-1 is suggested to be responsible for activation of nucleotide prodrugs such as mono- and diesters of nucleoside phosphoramidates derived from amino acids.[3,8,9]

An *O*-alkyl-*N*-methyl-*N*-(4-chlorobutyl)phosphoramidate prodrug of gemcitabine (Fig. 1C) has been described by Borch and co-workers.[10] For this prodrug, the metabolic activation involves intracellular generation of an unstable phosphoramidate anionic intermediate, which undergoes spontaneous cyclization accompanied by P–N bond cleavage. However, the P–N bond cleavage by an unspecified phosphoramidase is alternatively considered. The phospholipid gemcitabine conjugate (Fig. 1D) represents a third class of 5′-pdFdC prodrug. This conjugate is thought to undergo the hydrolytic unmasking by phospholipase C.[11] At physiological

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http://dx.doi.org/10.1016/j.bmc.2014.02.034
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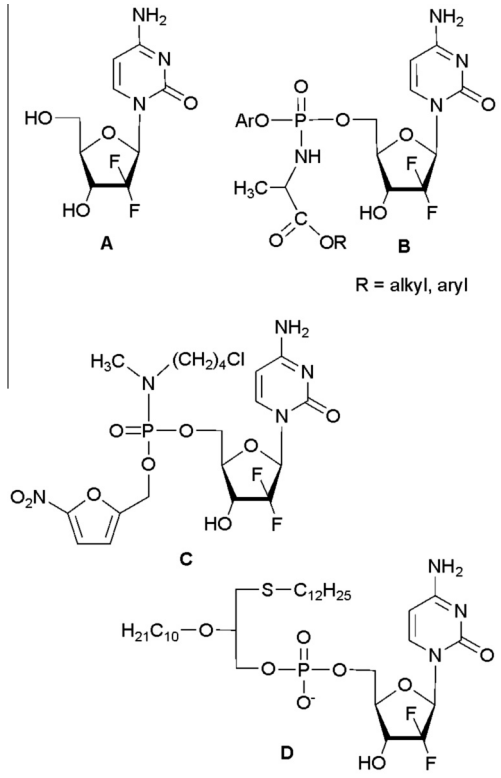


Figure 1. Gemcitabine and its prodrugs.

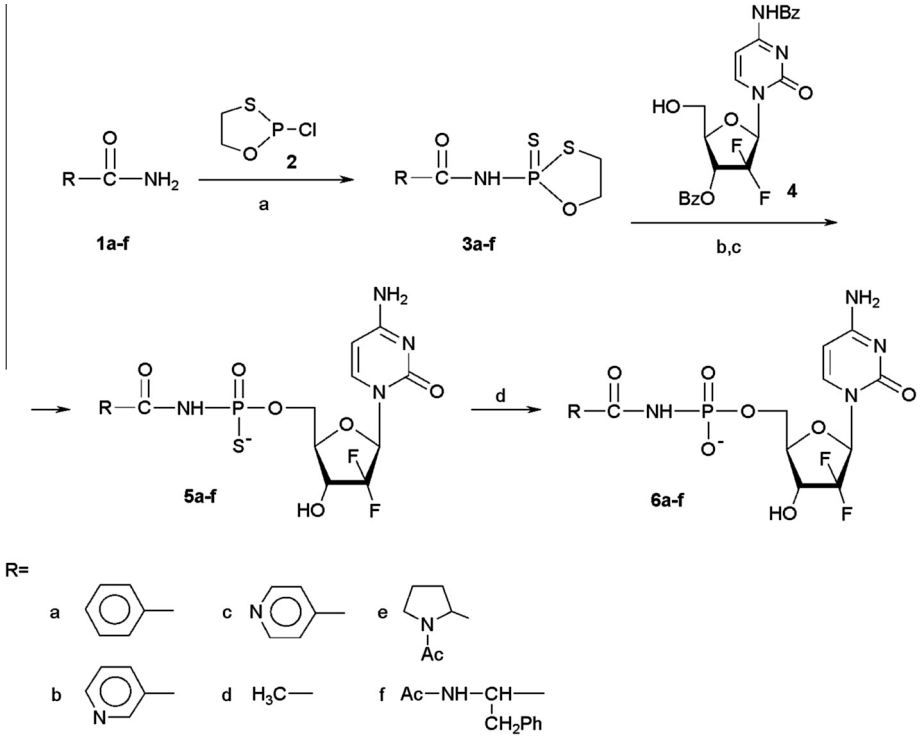
pH, this gemcitabine derivative is a phosphate monoanion, and therefore, differs from the uncharged *O*-ester-*N*-alkylphosphoramidate derivatives shown above. The lipid moiety does not enhance the cellular uptake of such monoanions as evidenced by the uptake of monoanionic amino acid phosphoramidates.[5]

We have recently described a new approach for preparation of *O*-alkyl-*N*-acyl-phosphoramidates and thiophosphoramidates[12] based on the oxathiaphospholane chemistry.[13] To the best of our knowledge, 5′-*O*-(*N*-acyl-phosphoramidate) derivatives of gemcitabine have not been so far tested as potential prodrugs, while relatively low stability of the P–N bond in cellular media makes them interesting in that context. Our goal was to synthesize and evaluate anticancer activity of several conjugates of 5′-*O*-(thio)phosphorylated gemcitabine with representative carboxamides and to check if these compounds are hydrolyzed by the HINT-1 protein.

2. Results and discussion

2.1. Chemistry

We have prepared twelve new derivatives of gemcitabine, consisting of conjugates of 5′-*O*-thiophosphorylated and 5′-*O*-phosphorylated dFdC with alkyl, aryl or amino acid carboxamides (**5a**–**f** and **6a**–**f**, respectively, Scheme 1). The selected amides included benzamide (**a**), nicotinamide (**b**), isonicotinamide (**c**), acetamide (**d**), *N*-acetylprolylamide (**e**), and *N*-acetylphenylalaninylamide (**f**). The syntheses were performed by condensation of 2-chloro-1,3,2-oxathiaphospholane (**2**) with a carboxamide (**1a**–**f**) and subsequent sulfurization of the resulting phosphoramidite with elemental sulfur. The resulting *N*-(2-thiono-1,3,2-oxathiaphospholane) derivatives **3a**–**f** were obtained in 40–62% yield (Table 1) and their structures were confirmed by ^{31}P NMR and mass spectrometry analysis. These compounds are remarkably stable and can be stored at ambient temperature for a long time (>1 year). Their DBU-assisted nucleophilic ring-opening condensation with *N*-4,*O*-3′-dibenzoylgemcitabine (**4**) furnished phosphorothioate derivatives, further debenzoylated with concentrated ammonia. The resulting derivatives **5a**–**f** were isolated by ion-exchange column chromatography in 27–92% yield, and characterized by ^{31}P NMR and FAB-MS. Since compounds **5** were



Scheme 1. Synthesis of *N*-acyl-thiophosphoramidate (**5**) and *N*-acyl-phosphoramidate (**6**) derivatives of gemcitabine. Reagents: (a) S_8, pyridine; (b) DBU, CH_3CN; (c) NH_4OH; (d) $PhIO_2$.

Table 1
The physico-chemical data and yields of isolated compounds **3a–f**, **5a–f** and **6a–f**

	3			5			6		
	Yield(%)	^{31}P NMR (δ, ppm)[a]	FAB-MS (M−1) (m/z)	Yield (%)	^{31}P NMR (δ, ppm) [b]	FAB-MS (M−1) (m/z)	Yield (%)	^{31}P NMR (δ, ppm) [b]	FAB-MS (M−1) (m/z)
a	62	88.78	258	88	48.42, 47.90	461	52	−4.21	445
b	42	89.99	259	80	48.51, 48.26	462	29	−4.65	446
c	46	89.93	259	27	47.97, 47.15	462	21	−4.90	446
d	48	88.46	196	92	47.39, 47.17	399	79	−4.96	383
e	51	88.63, 88.45	293	40	47.96, 47.20	496	35	−5.12	480
f	58	88.57, 88.59	343	42	47.86, 47.14	546	12	−5.26	530

[a] In CD_3OD.
[b] In D_2O.

obtained as mixtures of diastereomers, their ^{31}P NMR spectra contained two resonance signals representing both diastereomers. Compounds **5a–f** were oxidized with iodoxybenzene to yield **6a–f**. The desired compounds **6a–f** were isolated by ion-exchange chromatography (12–79% yield) and their structures were confirmed by ^{31}P NMR and FAB-MS analysis (Table 1). *N*-acyl-phosphoramidates **5a**,**b**,**d**,**e** and *N*-acyl-thiophosphoramidates **6a–f** of gemcitabine were used for further studies.

2.2. Cytotoxicity studies

To check the prodrug properties of *N*-acyl-phosphoramidate and *N*-acyl-thiophosphoramidate derivatives of gemcitabine, the cytotoxicity of **5a**,**b**,**d**,**e** and **6a–f** towards three cancer cell lines—human cervix carcinoma (HeLa), chronic myelogenous leukaemia (K562) and human pancreatic adenocarcinoma (CFPAC) cell lines was determined. The CFPAC cell line was selected because the cells are in vitro sensitive to gemcitabine, and patients with pancreatic cancer are successfully cured with this drug.[14] Although gemcitabine is a standard chemotherapeutic agent for pancreatic cancer, recent data demonstrate (as examined under in vivo conditions) that cells of a side population of human pancreatic ductal adenocarcinoma are more resistant to gemcitabine than other tumor cells.[15] Human umbilical vein endothelial cells (HUVEC, isolated from freshly collected umbilical cords) were used as control, non-cancerous cells. The cells were exposed to the test compounds for 24, 48, 72 and 96 hours, and the cytotoxicity was determined by the MTT assay as described previously.[16] The viability of untreated cells was taken as 100%. The toxicity of the test compounds was compared to the toxicity of gemcitabine, determined in the same MTT test. The IC_{50} values (IC_{50} is a concentration of a test compound required to reduce the cell survival fraction by 50%) were calculated from the corresponding dose-response curves.

At first, we screened the toxicity of all test compounds towards four cell lines at shorter incubation times (24 and 48 h). Under these conditions, the gemcitabine *N*-acyl-thiophosphoramidates **5a**,**b**,**d**,**e** have shown no cytotoxic effect. These PS-compounds occurred to be either non-toxic or their toxicity was rather poor (IC_{50} > 500 μM) (Table 2 for K562 and HUVEC, Figure S1 for CFPAC, data not shown for HeLa). Moreover, the HeLa and CFPAC cell lines were insensitive to gemcitabine *N*-acyl-phosphoramidates **6a–f** (IC_{50} > 600 μM), except for **6c**, for which IC_{50} in HeLa cells was 10 μM. In contrast, derivatives **6a**, **6c** and **6d** were toxic to the K562 cells in the short incubation time experiments (IC_{50} < 0.5 μM, Table 2). Interestingly, in HUVEC cells screened, these compounds were much less toxic compared to gemcitabine alone. For example, **6a** and **6c** were 17 times less toxic than gemcitabine, while **6d**—30 times.

Next, we checked the effects of longer exposure (72 and 96 h) on the survival of CFPAC and HeLa cells upon treatment with the compounds screened. We found that extended treatment of CFPAC cells with phosphoramidates **6a–f** resulted in strong toxic effects (Fig. S1, a lower panel), with the IC_{50} values similar to that for gemcitabine (IC_{50} ca. 0.5 μM, Table 2). In contrast, thiophosphoramidates **5a–b**, **5d–e** occurred much less toxic, except for **5b**, for which IC_{50} values of 77.5 and 62.5 μM were determined after 72- and 96 h-treatment, respectively. The remaining thiophosphoramidates **5a**, **5d** and **5e** showed modest toxicity towards CFPAC cells and only at high concentrations (IC_{50} > 310 μM). The 96 h-exposure of HeLa cells revealed that **6a–e** exert cytotoxicity comparable to that of gemcitabine, while thiophosphoramidates **5a–b**, **5d–e** were non-toxic (IC_{50} > 450 μM). Thus, one may conclude that in the cancer (K562, HeLa, CFPAC) and normal (HUVEC) cells all four screened thiophosphoramidates **5** do not serve as gemcitabine prodrugs. In contrast, the toxicity of gemcitabine phosphoramidates **6a–f** (similar to that of gemcitabine) supports the prodrug pathway of their action, at least in CFPAC cells. The time profiles of toxicity of **6** (Fig. S1, lower panels) suggest that these compounds require rather long time for 'unmasking', but afterwards their activities are virtually identical.

Table 2
The toxicity of compounds **5a**,**b**,**d**,**e** and **6a–f** (IC_{50})[#] towards chronic myelogenous leukaemia (K562) cells, non-cancerous human umbilical vein endothelial (HUVEC) cells, human cervix carcinoma (HeLa) cells and human pancreatic adenocarcinoma (CFPAC) cells, determined by the MTT assay

Compound	IC_{50} (μM)					
	K562	HUVEC	HeLa		CFPAC	
	48 h	48 h	72 h	96 h	72 h	96 h
Gemcitabine	0.05	0.003	10	0.9	0.47	0.35
5a	800	>1000	900	650	425	315
5b	>1000	>1000	>1000	500	77.5	62.5
5d	500	>1000	>1000	700	525	475
5e	>1000	800	500	450	350	310
6a	0.07	0.05	>1000	2	0.47	0.32
6b	>1000	50	10	1.4	0.42	0.40
6c	0.1	0.05	10	0.9	0.62	0.45
6d	0.5	0.09	15	1	0.57	0.42
6e	30	10	80	0.8	0.62	0.55
6f	80	200	>1000	45	0.42	0.45

[#] The IC_{50} values correspond to the concentration (in μM) of the tested compounds required to reduce the cell survival by 50%. Untreated cells were used as a control. The IC_{50} values were calculated from the dose-response curves (mean values from 2 independent experiments performed in triplicate).

2.3. Influence of tested compounds on the activity of selected DNA polymerases

The antitumor effect of gemcitabine may result from inhibition of ribonucleotide reductase by gemcitabine diphosphate. Such inhibition may reduce the level of deoxyribonucleoside triphosphates required for DNA synthesis. Alternatively, gemcitabine triphosphate may inhibit DNA polymerase and, in that way, may

slow down DNA synthesis and repair. Incorporation of the gemcitabine unit into a DNA chain results in inhibition of DNA synthesis. We analyzed the influence of compounds **5a**,**b**,**d**,**e** and **6a**–**f** on the DNA elongation reaction catalyzed by the Klenow fragment (exo+/−) of *Escherichia coli* DNA polymerase I, and by the genetically engineered *E. coli* DNA polymerase variant D141A/E143A/A485L (Therminator™). The latter enzyme is known for enhanced ability to incorporate modified substrates, such as dideoxynucleotides, ribonucleotides and acyclonucleotides, into the growing DNA chain.[17,18] In addition, human DNA polymerase α (hPol α) was also tested.[19]

2.3.1. Inhibitory activity

In one set of experiments, a single base extension reaction (SBE) was catalyzed either by the Klenow fragment of DNA polymerase I (with or without 3′ → 5′ exonucleolytic activity, KF^+ or KF^-, respectively), by the Therminator™ polymerase or by hPol α. In this assay, the compounds **5a**,**b**,**d**,**e** and **6a**–**f** were used as the competitors to 2′-deoxycitidine 5′-triphosphate (dCTP). In a typical procedure, the tested compound and dCTP were added simultaneously to the mixture of the template oligonucleotide (TG) 5′-terminated with a guanosine unit, and a complementary primer oligonucleotide (P) 5′-labeled with a radioactive ^{32}P-phosphate group. For KF^+, KF^- and Therminator™ polymerases, the tested compounds were used at 2.0 mM concentration, i.e. at 5-fold molar excess over 0.4 mM dCTP. The same ratio was maintained in the experiments with hPol α (0.5 mM concentration of the inhibitors and 0.1 mM dCTP). The reaction mixtures were analyzed by PAGE (Fig. S2). The relative activities of the KF^+, KF^-, Therminator™ and hPol α polymerases were described as a fraction of the obtained elongation product. None of the screened gemcitabine derivatives inhibited the Therminator™ polymerase (100% of the expected single-base extended product was seen). In the experiments with $KF^+/^-$ polymerases, **5d**, **6a**, **6c** and **6f** did not show any inhibitory activity. Only **5a**, **5b**, **6b**, **6d** and **6e** stopped the polymerization reactions. Compound **5e** to a certain extent slowed down the KF^- polymerase, while did not inhibit the two other bacterial enzymes. In experiments with hPol α, compounds **5a**, **5b**, **6b**, **6d**, **6e** also demonstrated inhibitory properties while **6a**, **6c**, **5e**, **6f** did not. Thus, the results obtained for bacterial Klenow and human DNA polymerases are very similar, contrary to the base-modified gemcitabine triphosphate analogs, which were good inhibitors only towards hPol α.[19]

For the compounds exhibiting inhibitory activity against the KF^+/KF^- polymerases and hPol α in the SBE reaction, the dose-response curves were produced (Fig. 2). In a typical procedure, **5a**, **5b**, **6b**, **6d** and **6e** were used in 1- to 5-fold molar excess over dCTP (from 0.4 to 2.0 mM for KF^+/KF^- and from 0.1 to 0.5 mM for hPol α). The products were then analyzed by PAGE (20%/7 M urea) and the amount of the n+1 product was quantified. The data indicate (Fig. 2) that among the tested compounds the thiophosphoramidate derivative **5b** is the most potent competitive inhibitor in experiments with KF^+, KF^- enzymes and hPol α. Besides, at ca. 2:1 ratio over dCTP compounds **6b** and **6d** alter significantly the activity of the two bacterial polymerases. In experiments with hPol α, also **6d** showed inhibitory activity as good as **5b** (EC_{50} < 0.1 mM, Table 3). It might be plausible that the derivatives **6** (the PO-series) under cellular conditions act as prodrugs of gemcitabine being unmasked and phosphorylated to dFdCTP, and do not inhibit nuclear human DNA polymerase α. Since **5b** is a nicotinic amide derivative of gemcitabine 5′-*O*-phosphorothioate, we checked an influence of gemcitabine 5′-triphosphate (dFdCTP) and the conjugate of nicotinic amide with 5′-dAMP (NA-pdA) on the progress of the SBE reaction. dFdCTP occurred to be a good substrate for the tested DNA polymerases, while NA-pdA exhibited inhibitory activity towards the KF^+ and KF^- polymerases at

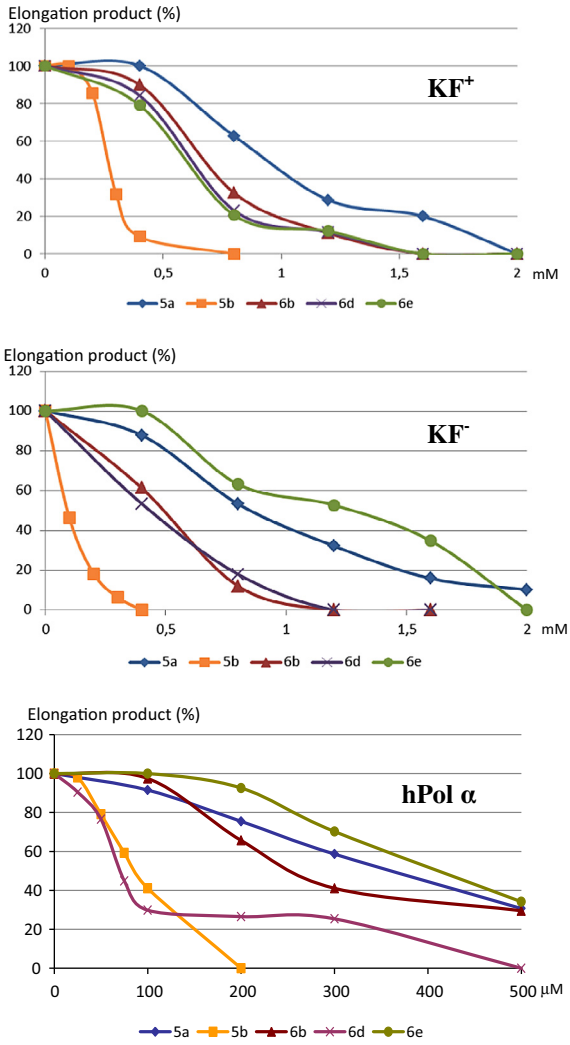


Figure 2. Dose-response inhibition of the elongation activity of the Klenow fragment of DNA polymerase I from *E. coli* with (KF^+) and without 3′ → 5′ exonucleolytic properties (KF^-), and human DNA polymerase α, by **5a**, **5b**, **6b**, **6d** and **6e**. The X axis—a molar concentration of the test compound (up to 2.0 mM); the Y axis—the amount of the obtained elongation product (%). The SBE reaction with dCTP and without any inhibitor was used as a control (100% yield).

Table 3
Inhibitory activity of **5a**,**b** and **6b**,**d**,**e** derivatives of gemcitabine towards the Klenow fragment of DNA polymerase I from *E. coli* with (KF^+) and without 3′ → 5′ exonucleolytic properties (KF^-), and human DNA polymerase α (hPol α) in the single base extension reaction

Entry	Compound	KF^+	KF^-	hPol α
		EC_{50} (mM)		
1	NA-pdA	0.54	0.49	—
2	**5a**	1.03	0.99	0.35
3	**5b**	0.28	0.13	0.08
4	**6b**	0.75	0.52	0.25
5	**6d**	0.71	0.51	0.07
6	**6e**	0.68	1.16	0.41

The dCTP substrate was used at 0.4 mM concentration for experiments with KF^+ and KF^- and at 0.1 mM with hPol α.

effective concentration (EC_{50}, a concentration that causes 50% inhibition) of 0.54 and 0.49 mM, respectively (Table 3).

As mentioned before, **5b** exhibits the lowest EC_{50}, which is two times smaller than for the NA-pdA/KF^+ system, and ca. 4-times lower for NA-pdA/KF^-. Stronger inhibitory activity of **5b** in

comparison to **5a** allows to hypothesize that, both, nicotinic amide and phosphorothioate function, play important roles in the interactions of **5b** with the Klenow (exo+/−) fragments and hPol α. This idea is supported by slightly reduced activity of a gemcitabine derivative with nicotinic carboxamidophosphate residue (**6b**), which differs from **5b** by the absence of the sulfur ligand at the phosphate moiety. Notably, the lack of a nitrogen atom in the aromatic ring of the carboxamide substituent (as in **6a**), or its changed position (as in the isonicotinic residue of **6c**), make these derivatives completely inactive towards the screened polymerases.

For the compounds other than **5a**, **5b**, **6b**, **6d** and **6e** the EC_{50} values were assessed as >2 mM for bacterial polymerases and >0.5 mM for hPol α, and are not listed.

The observed in vitro good inhibitory properties of **5b** towards bacterial polymerases and human DNA polymerase α do not bring any significant effect in human cells, as seen in cytotoxicity studies (Table 2). Thus, cytotoxicity of **5b** may be attributed to the action as hPol α inhibitor (in the intact form), while the compounds of the series **5** do not work as prodrug equivalents of gemcitabine.

2.3.2. Substrate activity

In 2007, Herdewijn et al. reported that conjugates of pdA with selected natural amino acids mimic the natural nucleoside triphosphates and are accepted by certain DNA polymerases.[20] In this case, the amino acid moiety linked to nucleoside 5′-phosphate through the P–N bond serves as a leaving group in the nucleotidyl transfer reaction. Especially, the 5′-aspartyl- and 5′-histidyl-phosphoramidates of 2′-deoxyadenosine were efficient substrates for elongation of the DNA chain by reverse transcriptase of the HIV-1 and the Therminator™ DNA polymerase. Therefore, having the carboxamide conjugates **5a**,**b**,**d**,**e** and **6a**–**f**, a series of the SBE assays was performed as described above, yet without dCTP added. The experiments have shown that none of the tested compounds served as a substrate for Therminator™ neither for KF^+/KF^- polymerases (no n+1 products were obtained) (see Fig. 3 for KF^+ polymerase). Interestingly, **5b**, **6d** and **6e** inhibited (to a certain extent) the 3′ → 5′ exonucleolytic activity of the KF^+ polymerase (the lanes, in which the shorter products are not present, marked with the arrows). This result is compatible with the inhibitory activity of **5b** towards the Klenow fragments KF^+ and KF^-. Thus, among the tested compounds, the thiophosphoramidate derivative **5b** is the most potent inhibitor of the KF^+ and KF^- enzymes, and it blocks efficiently the 3′ → 5′ exonucleolytic activity of KF^+. This activity might be responsible for higher cytotoxicity of the nicotinic carboxamide derivative of 5′-*O*-phosphorothioate of gemcitabine (**5b**) compared to other thiophosphoramidate derivatives **5** screened in the SBE assay.

2.4. Stability of prodrugs in aqueous solutions, pH 3-11

The stability of representative compounds **5b**, **5d**, **6b** and **6d** (20 mM) was checked in 0.1 M ammonium acetate buffered solutions (pH 3–11), at room temperature, over 2, 8 and 24 h. The stability was monitored by RP-HPLC on a C18 column. All screened compounds were found to be stable, even over the prolonged (24 h) incubation time (data not shown).

2.5. Stability of test compounds in the presence of rHint-1 enzyme or lysates of the HeLa and K562 cells

To check whether **5a**,**b**,**d**,**e** and **6a**–**f** are substrates for HINT-1 phosphoramidase, the compounds were treated with either recombinant Hint-1 protein (rHint-1) or with lysates of the HINT-1 expressing HeLa and K562 cells. The resultant mixtures were analyzed by RP-HPLC, and the content of the intact substrates was determined at 260 nm (Table 4).

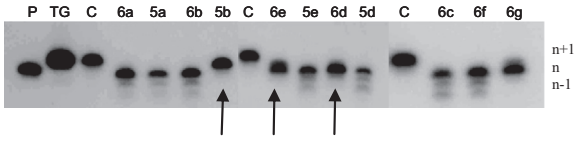


Figure 3. The electrophoretic analysis of the single base extension reaction of the 5′-^{32}P-labeled primer P carried out with **5a**,**b**, **5d**,**e** and **6a**–**f** and the KF^+ enzyme. Reactions were carried on using the TG template, 5′-terminated with the guanosine unit. The dCTP substrate was used as a control (lane C). The products n+1 were expected, while the products n−1, n−2 etc. resulted from the 3′ → 5′ exonucleolytic activity of KF^+. The arrows point to the lines where no shorter products were found.

Despite of the large excess of the rHint-1 enzyme and long incubation time, all tested thiophosphoramidates **5** and phosphoramidates **6** remained unhydrolyzed. Also compounds **5a**,**b**,**d**,**e** remained unchanged by the HeLa lysates. In contrast, the compounds **6a**–**f** (the phosphoramidate series) were partially digested by the HeLa cellular enzymes. After 22 h of incubation, the best substrate **6f** was degraded in 29.0%, while **6b** in 4.5% only (Table 4). Interestingly, in the presence of the K562 cell lysate all tested compounds were partially degraded (93.4–67.44% of intact substrates) with the exception for *N*-acetylphenylalanyl-phosphoramidate derivative **6f**, which was digested in more that 50% (45.9% remaining).

In the screened reaction mixtures, the intact substrates were accompanied by several digestion products. Three of them, i.e. gemcitabine (dFdC, Rt = 11.8 min), gemcitabine 5′-*O*-phosphate (pdFdC, 11.1 min) and 5′-*O*-phosphoramidate (NH_2-pdFdC, 10.6 min) were identified by co-injection with original samples. As an example, an HPLC profile of the products of digestion of **6f** with the K562 lysate is shown in Figure S3.

Unexpectedly, the addition of a phosphatase inhibitor to the reaction mixture of **6f** and K562 lysate resulted in a more complex mixture of products. However, the amount of gemcitabine in the reaction mixture was smaller than the amount remaining in the reaction carried out without the inhibitor (19.6% vs 48.5%) (Fig. S4). This result indicates that gemcitabine 5′-*O*-phosphate is the primary product of the degradation of the prodrugs. This conclusion is further supported by an observation that the treatment of gemcitabine 5′-phosphate with K562 lysate resulted in its partial dephosphorylation (Fig. S5). Similarly, digestion of gemcitabine 5′-phosphoramidate furnished the dephosphorylated product (gemcitabine) and gemcitabine 5′-monophosphate (Fig. S6). It is likely that hydrolysis of NH_2-pdFdC to pdFdC in the lysate is catalyzed by the HINT-1 enzyme, especially that such the transformation was observed in vitro upon action of the recombinant HINT-1 protein. The hydrolytic activity of rHint-1 towards NH_2-pdFdC was assigned as 1.88 ± 0.17 nmol min^{-1} μg^{-1} for 1 mM substrate, or 0.88 ± 0.08 nmol min^{-1} μg^{-1} for 0.2 mM substrate (Fig. S7), which

Table 4
Digestion of **5a**,**b**,**d**,**e** and **6a**–**f** with proteins present in the HeLa and K562 cell lysates

Compound (1 mM)	% of intact substrate	
	HeLa protein extract (40 μg, 22 h)	K562 protein extract (40 μg, 22 h)
5a	100.0	91.5 ± 0.3
5b	100.0	93.4 ± 0.1
5d	100.0	88.4 ± 0.3
5e	100.0	85.3 ± 0.2
6a	78.7 ± 2.2	67.4 ± 1.7
6b	95.5 ± 0.7	86.8 ± 0.5
6c	76.7 ± 2.9	82.8 ± 1.2
6d	94.1 ± 2.9	85.1 ± 1.4
6e	89.6 ± 2.3	80.2 ± 2.4
6f	71.0 ± 3.8	45.9 ± 1.8

is a value comparable to the rHint-1 hydrolytic activity towards NH_2-pA (0.2 mM substrate, 1.71 ± 0.25 nmol min^{-1} μg^{-1}).[6]

Although we assumed that the activation of acylphosphoramidates is done by Hint-1 phosphoramidase (the cleavage of the P–N bond), stability of **5** and **6** towards that enzyme suggests that the unmasking pathway of these prodrugs is different. Since cytotoxicity tests showed that the tested compounds reduced the viability of the cells, one can conclude that they undergo some intracellular enzymatic transformations. It is plausible that intracellular proteases (e.g. numerous cathepsins present mainly in lysosomes), cleave the C(O)–N bond to yield gemcitabine 5′-*O*-phosphoramidate, which is subsequently converted by Hint-1 to gemcitabine 5′-phosphate, the desired active intermediate. Although in cellular extracts we observed further conversion of pdFdC into dFdC, this process may be less efficient inside the cell, because phosphatases localized in different cell compartments may have limited access to the drugs in a cytoplasm. Moreover, the HeLa and K562 cell extracts used in the stability tests contained the protease inhibitor to prevent the total degradation of cellular enzymes. If this inhibitor also slowed down the cleavage of the C(O)–N bond in the prodrugs, the efficiency of the activation of the screened compounds measured in the extracts might actually be underestimated.

3. Conclusions

The experiments on cytotoxicity of gemcitabine *N*-acyl-thiophosphoramidates **5** and *N*-acyl-phosphoramidates **6** indicate that the compounds of PO-series (derivatives **6**) are metabolized by the cellular enzymes to yield active intermediates and exert similar cytotoxicity as gemcitabine itself. Thus, these derivatives can be considered good candidates for gemcitabine prodrugs. Their most appreciated property is lower cytotoxicity towards non-cancerous HUVEC cells, than that of gemcitabine. In contrast, the compounds of PS-series (the derivatives **5**), are more resistant to unmasking and to enzymatic conversion to the active form (dFdCTP), and their cellular toxicity is lower. The only interesting property is shown for thiophosphoramidate **5b**, which in vitro (in the intact form) exerts good inhibitory activity towards bacterial DNA polymerase I and human DNA polymerase α. This property, however, if translated into cellular system (as intracellular inhibition of human DNA polymerases), results in only slight toxic effect for cancer cells. Therefore, we conclude that *N*-acyl-thiophosphoramidates **5** rather do not serve as gemcitabine prodrugs, while may exert some inhibitory properties towards enzymes vital for the cells. Described here gemcitabine *N*-acyl-phosphoramidates **6** are suitable models for further development of better gemcitabine prodrugs.

4. Experimental

4.1. General experimental methods (chemistry)

4.1.1. General procedure for the synthesis of N-(2-thiono-1,3,2-oxathiaphospholanyl) carboxamides (3a–f)

To a solution of carboxamide **1a**–**f** (1 mmol) in dry pyridine (8 mL) containing suspended elemental sulfur (1.5 mmol), neat 2-chloro-1,3,2-oxathiaphospholane (**2**)[21] (1.1 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 12 h (for carboxamide derivatives) or 3 h (for amino acid carboxamide derivatives). Then the solvent was removed under reduced pressure. To the residue, acetonitrile (10 mL) was added and excess sulfur was filtered off. The solvent was evaporated under reduced pressure, the residue was dissolved in chloroform (2–3 mL), and the sample was applied on a silica gel column (2.5 × 18 cm). The column was eluted with a gradient of methanol in chloroform (0 → 1%). Appropriate fractions were combined and evaporated under reduced pressure. The yields of **3a**–**f**, their chemical shift values (^{31}P NMR), and FAB-MS data are presented in Table 1.

4.1.2. General procedure for the synthesis of gemcitabine *N*-acyl-thiophosphoramidates (5a–f, Scheme 1)

Into a solution of starting **3a**–**f** (1 mmol) dissolved in dry acetonitrile (8 mL), a solution of *N*-4,*O*-3′-dibenzoyl-gemcytabine (**4**, dFdC-2Bz, 1 mmol) in acetonitrile (6 mL) was added, followed by DBU (1.2 mmol, 184 μL). The mixture was stirred for 24 h at room temperature for the amino acid carboxamide derivatives, or for 48 h at 40 °C for the other carboxamide derivatives. Then, the solvent was removed under reduced pressure and the residue was treated with concentrated aq. ammonia (15 mL) to remove the benzoyl protecting groups. The reaction mixture was stirred for 24 h at room temperature, concentrated in vacuo and compounds **5a**–**f** were isolated using ion-exchange column chromatography [DEAE-Sephadex A-25; TEAB (0.0 → 0.1 M; pH 7.5) as the eluent].

4.1.3. General procedure for the synthesis of gemcitabine N-acyl-phosphoramidates (6a-f)

To a solution of **5a**–**f** (0.1 mmol) in water (1 mL), $PhIO_2$ (0.2 mmol) was added. The suspension was stirred at room temperature until got brown (15–120 min. depending on a compound) and then was concentrated under reduced pressure. The residue was dissolved in H_2O (5 mL) and washed with chloroform (5 mL). The aqueous layer was concentrated and the products **6a**–**f** were isolated using ion-exchange column chromatography [DEAE-Sephadex A-25; TEAB (0.0 → 0.1 M, pH 7.5) as the eluent].

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(benzoyl)thiophosphoramidate (**5a**):* Yield 88%. ^{31}P NMR (D_2O) δ: 48.42, 47.90. ^{1}H NMR (D_2O) δ: 7.62 (d, 1H, H-6), 7.45 (m, 5H, H-Ar), 6.05 (t, 1H, H-1′), 5.71 (d, 1H, H-5), 4.35 (m, 4H, H-3′, H-4′, 2xH-5′). FAB-MS *m*/*z*: (M−1) 461.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(3-carbonyl-pyridine)]thiophosphoramidate (**5b**):* Yield 80%. ^{31}P NMR (D_2O) δ: 48.51, 48.26. ^{1}H NMR (D_2O) δ: 8.70 (d, 1H, H-6), 8.49 (d, 1H, H-Ar), 8.01 (m, 1H, H-Ar), 7.65 (m, 1H, H-Ar), 7.39 (m, 1H, H-Ar), 6.18 (t, 1H, H-1′), 5.89 (d, 1H, H-5), 4.52 (m, 1H, H-3′), 4.11 (m, 1H, H-4′), 3.71 (m, 2H, H-5′). FAB-MS *m*/*z*: (M−1) 462.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(4-carbonyl-pyridine)] thiophosphoramidate (**5c**):* Yield 27%. ^{31}P NMR (D_2O) δ: 47.97, 47.15. ^{1}H NMR (D_2O) δ: 8.50 (d, 1H, H-6), 7.60 (m, 2H, H-Ar), 7.54 (d, 2H, H-Ar), 6.15 (t, 1H, H-1′), 5.70 (d, 1H, H-5), 4.18 (m, 4H, H-3′, H-4′, 2xH-5′). FAB-MS *m*/*z*: (M−1) 462.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(acetyl)]thiophosphoramidate (**5d**):* Yield 92%. ^{31}P NMR (D_2O) δ: 47.39, 47.17. ^{1}H NMR (D_2O) δ: 7.80 (m, 1H, H-6), 6.15 (t, 1H, H-1′), 6.03 (d, 1H, H-5), 4.38 (m, 4H, H-3′, H-4′, 2xH-5′), 1.98 (s, 3H, CH_3). FAB-MS *m*/*z*: (M−1) 399.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(α-N-acetylprolyl)] thiophosphoramidate (**5e**):* Yield 40%. ^{31}P NMR (D_2O) δ: 47.96, 47.20. ^{1}H NMR (D_2O) δ: 7.87 (m, 1H, H-6), 6.18 (m, 1H, H-1′), 6.03 (m, 1H, H-5), 4.32 (m, 2H, H-3′, H-4′), 4.11 (m, 2H, H-5′), 3.88 (m, 2H, CH_2), 3.53 (m, 2H, CH_2), 2.04 (s, 3H, CH_3), 1.89 (m, 2H, CH_2). FAB-MS *m*/*z*: (M−1) 496.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(α-N-acetylphenyl alanyl)]thiophosphoramidate (**5f**):* Yield 42%. ^{31}P NMR (D_2O) δ: 47.86, 47.14. ^{1}H NMR (D_2O) δ: 7.73 (d, 1H, H-6), 7.20 (m, 5H, H-Ar), 6.18 (t, 1H, H-1′), 6.13 (d, 1H, H-5), 4.30 (m, 1H, H-3′), 4.17 (m, 1H, H-4′), 4.11 (m, 2H, H-5′), 2.98 (m, 2H, CH_2), 2.76 (m, 1H, CH), 1.81 (s, 3H, CH_3). FAB-MS *m*/*z*: (M−1) 546.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(benzoyl)]phosphoramidate (**6a**):* Yield 52%. ^{31}P NMR (D_2O) δ: −4.21. ^{1}H NMR (D_2O) δ: 7.63 (d, 1H, H-6), 7.40 (m, 5H, H-Ar), 6.06 (t, 1H, H-1′), 5.67 (d, 1H, H-5), 4.41 (m, 4H, H-3′, H-4′, 2xH-5′). FAB-MS *m*/*z*: (M−1) 445.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(3-carbonyl-pyridine)] phosphoramidate (**6b**):* Yield 29%. ^{31}P NMR (D_2O) δ: −4.65. ^{1}H NMR (D_2O) δ: 8.76 (d, 1H, H-6), 8.54 (d, 1H, H-Ar), 8.05 (m, 1H, H-Ar), 7.63 (m, 1H, H-Ar), 7.43 (m, 1H, H-Ar), 6.12 (m, 1H, H-1′), 5.94 (m, 1H, H-5), 4.49 (m, 1H, H-3′), 4.11 (m, 1H, H-4′), 3.78 (m, 2H, H-5′). FAB-MS *m*/*z*: (M−1) 446.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(4-carbonyl-pyridine)]phosphoramidate (**6c**):* Yield 21%. ^{31}P NMR (D_2O) δ: −4.90. ^{1}H NMR (D_2O) δ: 8.53 (m, 2H, H-6, H-Ar), 7.61 (m, 2H, H-Ar), 7.44 (d, 1H, H-Ar), 6.05 (t, 1H, H-1′), 5.71 (d, 1H, H-5), 4.28 (m, 4H, H-3′, H-4′, 2xH-5′). FAB-MS *m*/*z*: (M−1) 446.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(acetyl)]phosphoramidate (**6d**):* Yield 79%. ^{31}P NMR (D_2O) δ: −4.96. ^{1}H NMR (D_2O) δ: 7.72 (d, 1H, H-6), 6.17 (t, 1H, H-1′), 5.99 (d, 1H, H-5), 4.37 (m, 1H, H-3′), 4.27 (m, 1H, H-4′), 4.09 (m, 2H, H-5′), 1.97 (s, 3H, CH_3). FAB-MS *m*/*z*: (M−1) 383.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(α-N-acetyl prolyl)]phosphoramidate (**6e**):* Yield 35%. ^{31}P NMR (D_2O) δ: −5.12. ^{1}H NMR (D_2O) δ: 7.71 (m, 1H, H-6), 6.15 (m, 1H, H-1′), 6.11 (m, 1H, H-5), 4.28 (m, 2H, H-3′, H-4′), 4.01 (m, 2H, 2xH-5′), 3.49 (m, 2H, CH_2), 1.97 (s, 3H, CH_3), 1.84 (m, 4H, 2xCH_2). FAB-MS *m*/*z*: (M−1) 480.

*2′,2′-Difluoro-2′-deoxycytidine-5′-O-[N-(α-N-acetylphenyl alanyl)] phosphoramidate (**6f**):* Yield 12%. ^{31}P NMR (D_2O) δ: −5.26. ^{1}H NMR (D_2O) δ: 7.76 (d, 1H, H-6), 7.12 (m, 5H, H-Ar), 6.18 (t, 1H, H-1′), 6.03 (d, 1H, H-5), 4.37 (m, 1H, H-3′), 4.19 (m, 1H, H-4′), 4.01 (m, 2H, H-5′), 2.88 (m, 2H, CH_2), 2.66 (m, 1H, CH), 1.81 (s, 3H, CH_3). FAB-MS *m*/*z*: (M−1) 530.

4.2. Chemical synthesis of oligonucleotides

The template 5′-GGAGGCCATAGCTGTTCCT-3′ (TG) and the primer oligonucleotide 5′-AGGAACAGCTATGGCCTC-3′ (P) were synthesized on a Gene World synthesizer (K&A, Germany) using commercially available phosphoramidite monomers (Glen Research). The oligonucleotides were deprotected and RP-HPLC purified by a two-step (DMT-on and DMT-off) procedure.[22] Molecular mass of the oligomers was confirmed by MALDI-TOF mass spectrometry (the primer MW 5505, *m*/*z* 5501; the template: MW 5817, *m*/*z* 5819).

4.3. Oligonucleotide labeling

The primer oligonucleotide was labeled at the 5′-end with [γ-^{32}P]ATP (Hartmann Analytic) and T4 polynucleotide kinase (USB). A mixture (20 μL) containing buffer (0.05 M Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 0.55 nmol of oligonucleotide, 0.032 mCi [γ-^{32}P]ATP, and T4 polynucleotide kinase (5U) was incubated for 1 h at 37 °C, then heat denatured and stored at −20 °C.

4.4. Single nucleotide incorporation catalyzed by the Klenow fragment of Pol I from E. coli with and without 3′ → 5′ exonucleolytic activity

A mixture (25 μL) containing 5 mM $MgCl_2$, 1 mM DTT, template and 5′-labeled primer oligonucleotides (4.4 μM each), *O*-alkyl-*N*-acylphosphoramidates of gemcitabine (**5a**–**b**, **5d**–**e** and **6a**-**f**, 400 μM, 10 nmoles) and *E. coli* polymerase $KF^+/^-$ (Fermentas, 1U) in a 0.05 M Tris-HCl buffer (pH 8.0) was incubated for 2 h at 37 °C, and then heat denatured. An analogous reaction was performed with dCTP as a control. The resulting mixtures were analyzed by PAGE (20%/7 M urea). The gels were autoradiographed and the amounts of the products were quantified using a G-box instrument.

4.5. Single nucleotide incorporation catalyzed by Therminator™ polymerase

A mixture (25 μL) containing 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, the template and 5′-labeled primer oligonucleotides (4.4 μM each), *O*-alkyl-*N*-acylphosphor amidates of gemcitabine (**5a**–**b**,**5d**–**e** and **6a**-**f**, 400 μM, 10 nmoles), and 2U of Therminator™ (BioLabs) in a 20 mM Tris-HCl buffer (pH 8.8) was incubated for 1 h at 75 °C, and then deep-frozen in liquid nitrogen to stop the reaction. An analogous reaction was performed with dCTP as a control. The resulting products were analyzed by PAGE (20%/7 M urea). The gels were autoradiographed and the amounts of the products were quantified using a G-box instrument.

4.6. Inhibition of SBE catalyzed by $KF^+/^-$ polymerase

A reaction mixture (25 μL) containing the template and 5′-labeled primer oligonucleotides (4.4 μM each), dCTP (400 μM, 10 nmoles), *O*-alkyl-*N*-acylphosphoramidate of gemcitabine (**5a**–**b**, **5d**–**e**, **6a**–**f**, 0.4, 0.8, 1.2, 1.6 or 2.0 mM) and $KF^+/^-$ (1U) in a 0.05 M Tris–HCl, 5 mM $MgCl_2$, 1 mM DTT buffer (pH 8.0) was incubated for 2 h. The resulting products were analyzed by PAGE (20%/7 M urea). The gels were autoradiographed and the amounts of the products were quantified using a G-box instrument.

4.7. Inhibition of SBE catalyzed by Therminator™ polymerase

A reaction mixture (25 μL) containing the template and 5′-labeled primer oligonucleotides (4.4 μM each), dCTP (400 μM, 10 nmoles), *O*-alkyl-*N*-acylphosphoramidate of gemcitabine (**5a**–**b**, **5d**–**e**, **6a**–**f**, 0.4, 0.8, 1.2, 1.6 or 2.0 mM), and Therminator™ (2U) in a 20 mM Tris-HCl, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$ and 0.1% Triton X-100 buffer (pH 8.8) was incubated for 1 h. The resulting products were analyzed by PAGE (20%/7 M urea). The gels were autoradiographed and the amounts of the products were quantified using a G-box instrument.

4.8. Inhibition of SBE catalyzed by human DNA polymerase α

A reaction mixture (8 μL) containing a template (1.9 μM) and a 5′-labeled primer oligonucleotide (1.2 μM), dCTP (100 μM), an *O*-alkyl-*N*-acylphosphoramidate derivative of gemcitabine (**5a**,**b**, **5d**–**e**, **6a**–**f**, at 100, 200, 300 or 500 μM concentration) and 2U of hPol α (EURx, Gdańsk, Poland) in a buffer (60 mM Tris–HCl (pH 8.0), 5.0 mM magnesium acetate, 0.3 mg/mL BSA, 1 mM DTT and 0.1 mM spermine) was incubated at 37 °C for 2 h. The reaction was stopped by addition of formamide with bromophenol blue, followed by heating at 95 °C for 4 min. The products were analyzed by PAGE (20%/7 M urea). The gels were autoradiographed and the amounts of the products were quantified using a G-box instrument.

4.9. Cells and cytotoxicity assay

Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords as previously described,[23] and cultured in plastic dishes coated with gelatin, in RPMI 1640 medium supplemented with 20% FBS (fetal bovine serum), 90 U/mL heparin, 150 μg/mL ECGF (Endothelial Cell Growth Factor, Roche Diagnostics, Mannheim, Germany) and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin). The HeLa (human cervix carcinoma) and K562 (chronic myelogenous leukaemia) cells were cultured in RPMI 1640 medium (developed by Moore et al., 1976, Roswell Park Memorial Institute) supplemented with antibiotics and 10% fetal calf serum, in a 5% CO_2—95% air atmosphere.

The CFPAC (human pancreatic adenocarcinoma) were cultured in RPMI 1640 medium supplemented with antibiotics (100 μg/mL streptomycin, 100 U/mL penicillin) and 10% fetal calf serum in a 5% CO_2—95% air atmosphere.

The HUVEC, HeLa and K562 cells (in amount 7×10^3) and CFPAC cells (10^4 cells) in 200 μL of the medium were seeded on each well on a 96-well plate (Nunc). After 24 h, the cells were exposed to the tested compounds. The stock solutions of the compounds (100 mM) were prepared in water and added to each well to achieve the required final concentration (1 mM, 1×10^{-1} mM, 1×10^{-2} mM, 1×10^{-3} mM, 1×10^{-4} mM, 1×10^{-5} mM and 1×10^{-6} mM) in the cell cultures.

All cells cultures were incubated for another 24 or 48 h except for the CFPAC cells, which were incubated additionally up to 72 and 96 h. The toxicity of all compounds was determined by the MTT assay which employs 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO). Briefly, after incubation with drugs, the cells were treated with the MTT reagent for 2 h. MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH 4.7, and the absorbance was measured at 570 and 650 nm on an ELISA-PLATE READER (FLUOstar Omega). To determine the viability of the cells, untreated cells were used as a control (100% viability). The IC_{50} values (the concentration of the test compound required to reduce the cell survival fraction by 50% of the control) were calculated from the dose-response curves.

4.10. Stability of prodrugs in aqueous solutions pH 3-11

Stability of representative compounds **5b**, **5d**, **6b** and **6d** in the form of 20 mM solutions in aqueous 0.1 M ammonium acetate (pH 3–11, room temperature) was checked after 2, 8 and 24 h incubation. The stability was monitored by RP-HPLC (a C18 column) in 0.1 M TEAB/acetonitrile solvent system.

4.11. Stability of prodrugs in the presence of rHint-1, or HeLa or K562 cells lysates

4.11.1. The protein and cells lysate preparation

The rabbit Hint1 protein was expressed from a plasmid pSGA02-HINT[24] in *E. coli* (the BL 21*strain) and purified using AMP-agarose (Sigma) affinity chromatography, according to a published procedure.[6] The homogenous enzyme preparation was dialyzed against a buffer containing 20 mM Tris (pH 7.5) and 150 mM NaCl, then concentrated and stored at −80 °C.

Total protein lysates from the HeLa and K562 cells (cellular extracts) were prepared from cell cultures (75 mL flasks) with cells grown to confluence. Cells were scraped, sonicated in a PBS buffer containing a mixture of protease inhibitors (Complete®, Roche, Penzberg, Germany). The samples were centrifuged and the molecules of molecular weight smaller than 1800 Da (e.g. nucleosides, nucleoside mono-, di- and triphosphates) were removed from the supernatant using Protein Desalting Spin Columns (Pierce). The eluates were used as the protein lysates. The total protein amount was measured by a spectrophotometric method.

4.11.2. Enzymatic assays

For the enzymatic digestion, 1 mM solutions of the substrates **5** or **6** were prepared in 20 mM Na-HEPES buffer (pH 7.2, 20 μL) containing 0.5 mM $MgCl_2$, and either rHint-1 (14 μg) or protein lysate from HeLa or K562 cells (40 μg). The samples were incubated for 22 h at 37 °C. The reactions were quenched by cooling on ice, then the samples were heat denatured and centrifuged. The supernatants were analyzed by RP-HPLC on a BDS Hypersil C18 column (5 μm, 250 × 4.6 mm; Thermo Electron Corporation) with a gradient of acetonitrile in 0.1 M triethylammonium bicarbonate (pH 7.4, 0% → 8% over 20 min at a flow rate 1 mL/min. Quantification was performed by electronic integration of the areas of the peaks of interest. Each experimental point represents the mean ± SE from measurements performed in at least duplicate.

Acknowledgements

The cytotoxicity studies were done in Screening Laboratory of Department of Bioorganic Chemistry. The authors thank Professor Wojciech J. Stec for valuable comments and stimulating discussions. Financial support from Ministry of Science and Higher Education (through the statutory funds of CMMS PAS, Lodz, Poland) is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.034.

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