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Synthesis and Biological Evaluation of Purine 2′-Fluoro-2′-deoxyriboside ProTides as Anti-influenza Virus Agents

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2′-Fluoro-2′-deoxyguanosine has been reported to have potent anti-influenza virus activity in vitro and in vivo. Herein we describe the synthesis and biological evaluation of 6-modified 2′-fluoro-2′-deoxyguanosine analogues and their corresponding phosphoramidate ProTides as potential anti-influenza virus agents. Whereas the parent nucleosides were devoid of antiviral activity in two different cellular assays, the 5′-*O*-naphthyl-(methoxy-L-alaninyl) ProTide derivatives of 6-*O*-methyl-2′-fluoro-2′-deoxyguanosine, 6-*O*-ethyl-2′-fluoro-2′-deoxyguanosine, and 2′-deoxy-2′-fluoro-6-chloroguanosine, and the 5′-*O*-naphthyl(ethoxy-L-alaninyl) ProTide of 6-*O*-ethyl-2′-fluoro-2′-deoxyguanosine displayed antiviral EC_{99} values of ~12 μM. The antiviral results are supported by metabolism studies. Rapid conversion into the L-alaninyl metabolite and then 6-modified 2′-fluoro-2′-deoxyguanosine 5′-monophosphate was observed in enzymatic assays with yeast carboxypeptidase Y or crude cell lysate. Evidence for efficient removal of the 6-substituent on the guanine part was provided by enzymatic studies with adenosine deaminase, and by molecular modeling of the nucleoside 5′-monophosphates in the catalytic site of a model of ADAL1, thus indicating the utility of the double prodrug concept.

Introduction

Influenza viruses are responsible for annual epidemics and occasional pandemics and are the cause of significant morbidity and mortality, especially among young, aged, and chronically ill individuals.[1] Annual vaccination is the primary approach to prevent influenza virus infections, but provides only partial protection in some target populations. Currently available anti-influenza virus drugs fall into two major classes:[2,3] M2 ion channel blockers (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir). However, the worldwide spread of amantadine- and oseltamivir-resistant influenza viruses, even among untreated patients, underscores the urgent need for novel antiviral drugs with an original mode of action.[4] The conserved nature of the influenza virus polymerase and the critical role of this enzyme complex in virus replication make it an attractive target for antiviral therapy.[5]

Nucleoside analogues are the first-line therapeutics for the treatment of HIV, herpes virus, and hepatitis B virus infections.[6] The development of nucleoside inhibitors against an RNA virus such as influenza is complicated by the fact that their activation and inhibitory effect on the viral polymerase must be achieved in the presence of high intracellular levels of the natural ribose nucleoside and nucleotide substrates. The viability of the approach is, however, proven by the recent advances of nucleoside inhibitors for HCV therapy.[7] Another issue relates to the absolute dependence on intracellular bioactivation to the active nucleoside 5′-triphosphate form. Because the first phosphorylation is often the rate-limiting step, intracellular delivery of a nucleoside 5′-monophosphate through a prodrug approach improves the therapeutic activity of many nucleoside analogues.[8] Among several technologies, our versatile phosphoramidate ProTide approach has shown to be effective for antiviral (as well as anticancer) applications, by delivering the 5′-monophosphate forms of nucleoside analogues directly into intact cells.[9] The intracellular activation pathway of ProTides consists of the following steps:[10–12] 1) removal of the carboxyl ester moiety (e.g., the methyl group in compound **9b**; Figure 3 below) by human cathepsin A (which is structurally homologous to the yeast enzyme carboxypeptidase Y) and/or a carboxylesterase; 2) nucleophilic attack of the free carboxyl group on the phosphate, resulting in spontaneous elimination of the aromatic substituent and formation of an amino acyl phosphate intermediate (metabolite **II** in Figure 3); 3) cleavage of the phosphoramidate bond (see Figure 4 below: from metabolite **II** to **III**), possibly by a histidine triad nucleotide-binding protein (Hint) enzyme; and 4) further phosphorylation of the nucleoside 5′-monophosphate by cellular kinases.

In 1993, Tuttle et al. reported the anti-influenza virus activity of several purine 2′-fluoro-2′-deoxyribosides in Madin–Darby canine kidney (MDCK) cells, including 2′-fluoro-2′-deoxyguanosine (**1**; 2′-FdG) and some 6-modified 2′-FdG derivatives.[13] In an enzymatic assay, 2′-fluoro-2′-deoxy-GTP was shown to inhib-

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it RNA transcription by the influenza virus polymerase, by acting as a GTP competitor and RNA chain terminator.[14] These investigators also studied the potential activation pathway of 2′-FdG to its active 5′-triphosphate, and showed that the cellular deoxycytidine kinase (which also recognizes 2′-deoxyguanosine) is capable of phosphorylating 2′-FdG to its monophosphate with moderate efficiency. Relative to 2′-deoxyguanosine, the V_{max} of 2′-FdG was fourfold lower, and its catalytic efficiency (V_{max}/K_M) was 20-fold lower.[15] The second phosphorylation (to 2′-FdG diphosphate) can be catalyzed by GMP kinase.[13] Intriguingly, the anti-influenza virus activity of 2′-FdG appears to be markedly cell-type dependent, as its antiviral effective concentrations (EC_{50} or EC_{90}) were reported as 18 or 42 μM in MDCK cells,[13,16] 0.55 μM in chicken embryo fibroblast (CEF) cells,[15] and <0.03 μM in primary human respiratory epithelium cells.[16] In the latter case, 2′-FdG was highly selective (ratio of cytotoxic to antiviral concentration >1000),[16] whereas its reported selectivity index in MDCK or CEF cells was ~25 or <5.[15,16] In influenza-virus-infected mice, 2′-FdG proved superior to amantadine and ribavirin, as assessed from mouse survival data and virus lung titers at 24 h after infection.[15] Because the inefficiency of the first phosphorylation may be the reason for the relatively low antiviral potency of 2′-FdG, at least in some cell lines such as MDCK, our ProTide strategy to directly deliver its 5′-monophosphate appeared a logical strategy to further improve its antiviral effect.

In this work, we first performed enzymatic studies to evaluate a selection of base- and/or sugar-modified GTP derivatives for their inhibitory activity against the influenza virus RNA polymerase. 7-Deaza-GTP and 2′-fluoro-2′-deoxy-GTP were shown to inhibit the RNA incorporation of GTP, and their IC_{50} values were lower than that observed with the obligate chain terminator 3′-deoxy-GTP. Next, we synthesized four 6-modified guanosine analogues (compounds **2**, **3**, **4**, and **5**), which were intended to act as prodrugs of 2′-FdG (**1**, Figure 1), and applied the ProTide approach to all of them. We selected L-alanine as the amino acid motif and focused on modifications in the aromatic moiety (either phenyl or 1-naphthyl) and the amino acid ester moiety (benzyl, methyl, ethyl, and cyclohexyl). A detailed investigation of their metabolism and anti-influenza virus activity and selectivity in cell culture was performed.

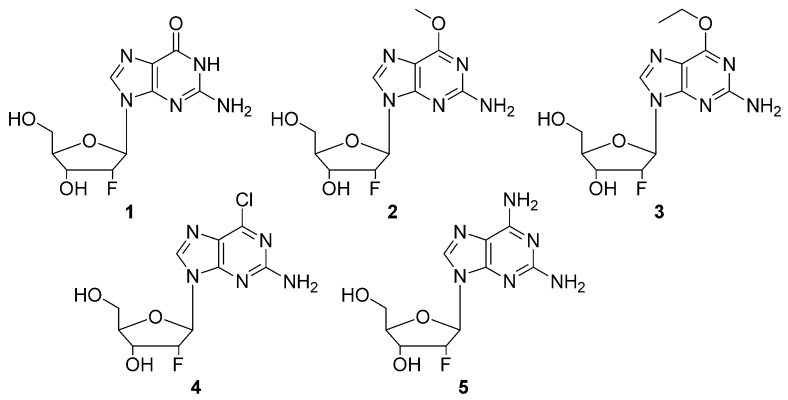
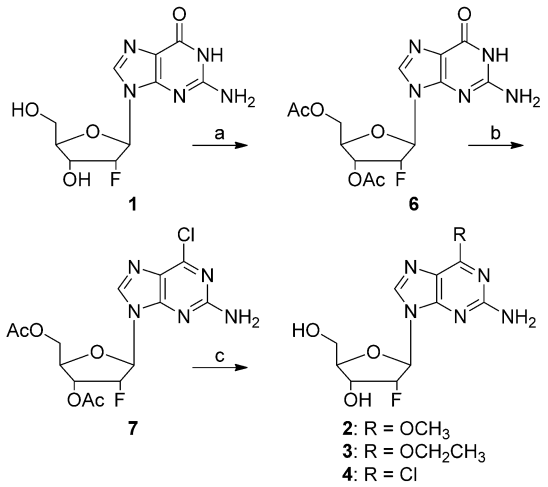


Figure 1. Structures of 2′-fluoro-2′-deoxyguanosine (**1**) and its 6-modified derivatives **2**, **3**, **4**, and **5** considered for this study.

Results and Discussion

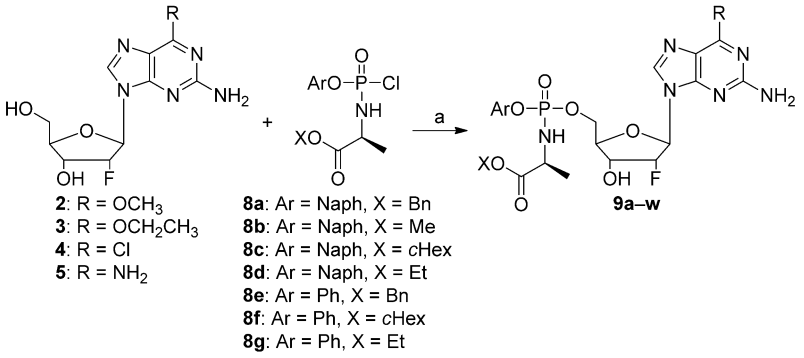
Chemistry

The 6-modified 2′-FdG analogues **2**, **3**, and **4** were synthesized in three steps, starting from compound **1**, whereas compound **5** was commercially available (Scheme 1). In the first step, protection of the 3′- and 5′-OH groups in the sugar was performed with acetic anhydride, triethylamine (TEA) and 4-dimethylaminopyridine (DMAP) to obtain compound **6**, with a yield of 78%. The second step involved chlorination in the 6-position using phosphoryl chloride, benzyl triethylammonium chloride (BTEA-Cl) and *N*,*N*-dimethylaniline (DMA) to yield compound **7** (75% yield). For the last step, various procedures were adopted depending on the final product required. Simultaneous deprotection of the sugar and substitution of the chlorine with a methoxy or ethoxy group at the 6-position yielded compounds **2** and **3** (92 and 74% respective yields), whereas deprotection in methanolic ammonia at room temperature led to the formation of compound **4** (87% yield).



Scheme 1. *Reagents and conditions:* a) acetic anhydride, TEA, DMAP, CH_3CN, RT, 2 h; b) BTEA-Cl, DMA, $POCl_3$, CH_3CN, 85 °C, 20 min; c) NaOMe, MeOH, RT, overnight, for **2**; NaOEt, EtOH, RT, overnight, for **3**; NH_3/MeOH, RT, overnight, for **4**.

For the synthesis of the 6-modified 2′-FdG ProTides, L-alanine was selected as the amino acid of choice. The synthesis of the first synthon, the requisite phosphorochloridate, was previously reported by us,[17] and it involves phosphorylation of an aromatic alcohol to give the dichlorophosphate intermediate and subsequent coupling with the appropriate amino acid ester salts to give the desired compounds **8a**–**g**.



Scheme 2. *Reagents and conditions:* a) *t*BuMgCl, THF, RT, overnight.

The coupling reactions between the nucleosides **2**, **3**, **4**, and **5** and the appropriate phosphorochloridates **8a–g** were performed with *tert*-butylmagnesium chloride as a hydroxy group activator, using a published procedure (Scheme 2).[18] A summary of all the ProTides synthesized in this first series is reported in Table 1; the variation of the ester moiety in combination with the aromatic moiety led to a clog*P* range of ~1.1–3.6. All the compounds were obtained as a mixture of two diastereomers, as confirmed by the presence of two peaks in their ^{31}P NMR spectra. The optimal clog*P* value for cell membrane permeation lies between 2 and 4, and most of our ProTide compounds meet this criterion.

Table 1. Chemical characteristics of 6-modified 2′-FdG ProTides.

Compd	Nuc[a]	Ar	X	clog*P*[b]	^{31}P NMR
9a	2	Naph	Bn	3.6	4.25, 4.07
9b	2	Naph	Me	1.9	4.22, 4.13
9c	2	Naph	*c*Hex	3.9	4.22, 4.18
9d	2	Naph	Et	2.4	4.23, 4.16
9e	2	Ph	Bn	2.4	3.98, 3.68
9f	2	Ph	*c*Hex	2.7	3.94, 3.78
9g	2	Ph	Et	1.2	3.87, 3.70
9h	3	Naph	Bn	4.1	4.26, 4.07
9i	3	Naph	Me	2.4	4.23, 4.14
9j	3	Naph	Et	2.9	4.23, 4.16
9k	3	Ph	Bn	2.9	3.97, 3.71
9l	3	Ph	Et	1.8	3.92, 3.76
9m	4	Naph	Bn	3.3	4.24, 4.06
9n	4	Naph	Me	1.6	4.21, 4.12
9o	4	Naph	Et	2.2	4.23, 4.15
9p	4	Ph	Bn	2.2	3.95, 3.67
9q	4	Ph	Et	1.0	3.93, 3.74
9r	5	Naph	Bn	2.7	4.29, 4.09
9s	5	Naph	*c*Hex	3.0	4.28, 4.21
9t	5	Naph	Et	1.5	4.21, 4.10
9u	5	Ph	Bn	1.5	3.93, 3.68
9v	5	Ph	*c*Hex	1.8	3.98, 3.79
9w	5	Ph	Et	0.3	3.91, 3.74

[a] Nucleoside. [b] ChemDraw 12.0 prediction.

Next, we decided to investigate how the change of fluorine stereochemistry at the 2′-position of the sugar may influence the antiviral activity of these nucleoside analogues and their ProTides. Therefore, we synthesized three different nucleosides bearing the fluorine in the 2′-β-position and the 6-modifications in the nucleobase: 6-*O*-methyl-2′-β-fluoro-2′-deoxyguanosine (**10**), 6-*O*-ethyl-2′-β-fluoro-2′-deoxyguanosine (**11**), and 2′-deoxy-2′-β-fluoro-6-chloroguanosine (**12**) (Figure 2). For the synthesis of the 6-modified 2′-β-fluoro-2′-deoxyguanosine nucleosides **10**, **11** and **12**, the commercially available 2-β-fluoro-2-deoxy-1,3,5-tribenzoylribose (**13**) was coupled with nucleobase **14** (Scheme 3). The coupling reaction was carried out with 1,8-diazabicycloundec-7-ene (DBU) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) in acetonitrile.[19] From this TMSOTf-mediated condensation, we observed the formation of different isomers, which needed to be separated. The formation of the undesired N^7 isomer was avoided by controlling the temperature of the reaction (65 °C). In addition, the formation of the α-anomer was observed during the reaction, together with the required β-anomer. To isolate the pure β-anomer from the mixture, crystallization and sometimes column chromatographic purifications were required, depending on the scale. For the small-scale reaction, the mixture was stirred for 6 h at reflux (65 °C), and purification by chromatography was carried out to sepa-

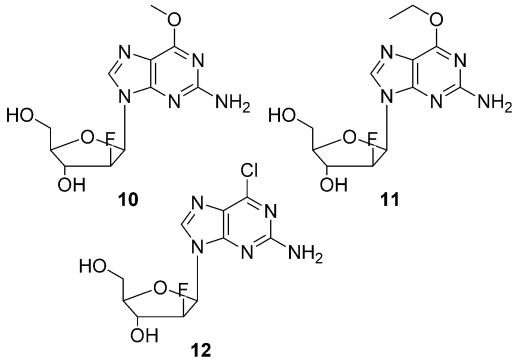
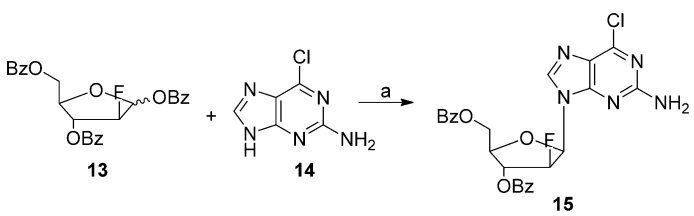
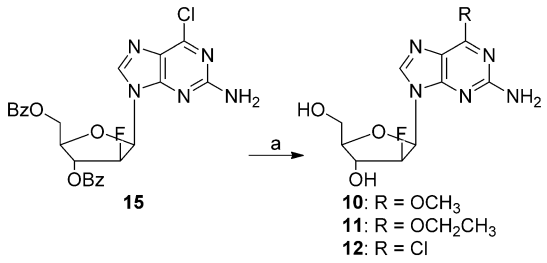


Figure 2. Structures of 6-modified 2′-β-fluoro-2′-deoxyguanosine nucleosides **10**, **11** and **12**.



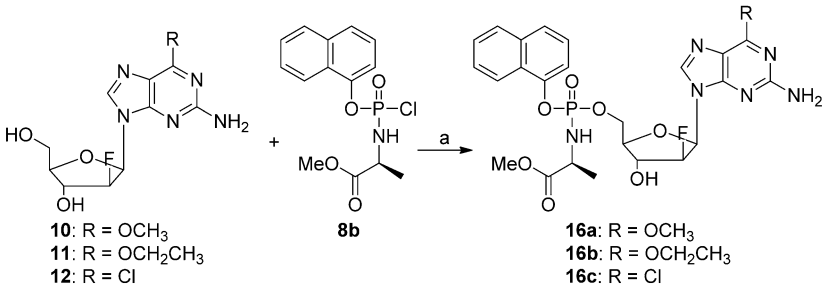
Scheme 3. *Reagents and conditions:* a) DBU, TMSOTf, CH_3CN, 65 °C, 6–21 h.

rate the β-anomer from the α-anomer (yield: 14%). The large-scale reaction was stirred for 21 h at reflux, and compound **15** was crystallized from methanol (17–20% yield). The α- and β-anomers were identified by 2D NOESY NMR and by comparing the coupling constant (*J*) values between H1′-F and H3′-F in the two molecules. The synthesis of 6-modified 2′-β-fluoro-2′-deoxyguanosine analogues **10**, **11**, and **12** followed the same procedures as described above (Scheme 4). One ProTide for



Scheme 4. *Reagents and conditions:* a) NaOMe, MeOH, RT, overnight, for **10**; NaOEt, EtOH, RT, overnight, for **11**; NH_3/MeOH, RT, overnight, for **12**.

each of the 2′-β-fluoro nucleosides was synthesized and subjected to biological evaluation. The coupling reactions were initially performed by using *t*BuMgCl in THF, but the low solubility of the nucleosides in THF caused the reaction to fail. The second attempt was executed with *N*-methylimidazole (NMI) in THF/Py to successfully obtain the desired compounds **16a–c** (Scheme 5). For this small family of compounds we chose to synthesize and investigate only the L-alanine methyl ester derivatives bearing naphthyl as the aromatic moiety. All compounds were characterized and isolated as roughly equimolar mixtures of phosphorus diastereomers, as determined by ^{31}P NMR spectroscopy.



Scheme 5. *Reagents and conditions:* a) NMI, THF/Py, RT, overnight.

Biological results

Inhibitory effect of GTP analogues on influenza virus polymerase activity

A selection of commercially available base- or sugar-modified GTP analogues was evaluated in an enzymatic assay to determine their inhibitory activity against the influenza virus RNA polymerase. More specifically, we determined their inhibitory effect on the incorporation of [8-^{3}H]-labeled GTP during ApG-primed synthesis of full-length uncapped viral RNA transcripts by using purified vRNP complexes, which contain the viral RNA polymerase complex and the vRNA template (Table 2). The two most potent GTP derivatives were 7-deaza-GTP and 2′-fluoro-2′-deoxy-GTP, displaying IC_{50} values of 4.1 and 3.7 μM, respectively. Our IC_{50} value for 2′-fluoro-2′-deoxy-GTP (3.7 μM) is in the same range as the K_i value of 1.1 μM, published by Tisdale et al.[14] The higher IC_{50} value (93 μM) obtained in their assay is likely related to methodological differences, as these authors used the natural GTP substrate at 500 μM, whereas our inhibition studies were carried out with GTP at 3.7 μM. In our assay, 2′-fluoro-2′-deoxy-GTP proved to be a better inhibitor than the obligate chain terminator 3′-deoxy-GTP (IC_{50}: 37 μM). The data listed in Table 2 indicate that a fluorine substituent at the 2′-position is preferred, as the analogues containing a 2′-azido or 2′-*O*-methyl group were inactive, whereas the 2′-amino derivative had a marginal inhibitory effect on [8-^{3}H]GTP incorporation (IC_{50}: 271 μM). The lack of activity of 6-*O*-methyl-GTP indicates that intracellular cleavage of this substituent is required to achieve antiviral activity with the 6-modified guanosine ProTides. Finally, no inhibition of [8-^{3}H]GTP incorporation was observed with the GTP analogues carrying an N^1-methyl- or 8-oxo-substituted guanine base.

Table 2. Inhibitory effect of base- or sugar-modified GTP analogues on RNA synthesis by influenza virus RNA polymerase.

Compound name	IC_{50} [μM][a]
Base-modified derivatives	
N^1-Methyl-GTP	>400
6-*O*-Methyl-GTP	>400
7-Deaza-GTP	4.1 ± 0.2
8-Oxo-GTP	>400
Sugar-modified derivatives	
2′-Fluoro-2′-deoxy-GTP	3.7 ± 0.4
2′-Azido-2′-deoxy-GTP	>400
2′-Amino-2′-deoxy-GTP	271
2′-*O*-Methyl-GTP	>400
3′-Deoxy-GTP	37 ± 12

[a] Compound concentration required for 50% inhibition of [8-^{3}H]GTP incorporation during influenza virus vRNP-mediated RNA synthesis; values are the mean ± SEM of three to six independent experiments.

Anti-influenza virus activity in MDCK cells

The 2′-FdG derivatives and their ProTides were evaluated for their suppressive effect on influenza virus replication, which is expressed as the EC_{99} value, or compound concentration causing a two-$\log_{10}$-unit decrease in virus yield, as determined by RT-qPCR (Table 3). The reference compound ribavirin had an

Table 3. Inhibitory effect of 6-substituted 2′-FdG ProTides against influenza virus replication in MDCK cells.

Compd	Nuc	Ar	AA	Ester	EC_{99} [μM][a]	MCC [μM][b]
1	–	–	–	–	66 ± 19	
9a	2	Naph	L-Ala	Bn	35 ± 12	>100
9b	2	Naph	L-Ala	Me	15 ± 6	≥100
9c	2	Naph	L-Ala	*c*Hex	≥70	≥100
9d	2	Naph	L-Ala	Et	65	>100
9e	2	Ph	L-Ala	Bn	≥100	>100
9f	2	Ph	L-Ala	*c*Hex	40 ± 13	≥100
9g	2	Ph	L-Ala	Et	>100	>100
2	–	–	–	–	>100	>100
9h	3	Naph	L-Ala	Bn	100	>100
9i	3	Naph	L-Ala	Me	14	>100
9j	3	Naph	L-Ala	Et	12	>100
9k	3	Ph	L-Ala	Bn	>100	>100
9l	3	Ph	L-Ala	Et	36	>100
3	–	–	–	–	>100	>100
9m	4	Naph	L-Ala	Bn	≥45	>100
9n	4	Naph	L-Ala	Me	12 ± 2	>100
9p	4	Ph	L-Ala	Bn	39 ± 16	>100
9q	4	Ph	L-Ala	Et	>100	>100
4	–	–	–	–	>100	>100
9r	5	Naph	L-Ala	Bn	>100	>100
9s	5	Naph	L-Ala	*c*Hex	63 ± 29	>100
9u	5	Ph	L-Ala	Bn	>100	>100
9v	5	Ph	L-Ala	*c*Hex	>100	>100
5	–	–	–	–	>100	>100
Ribavirin	–	–	–	–	8.6 ± 0.8	≥100

[a] Compound concentration required to produce a two-$\log_{10}$-unit decrease in the number of virus particles released from infected MDCK cells at 24 h p.i. [b] Minimum cytotoxic concentration: compound concentration that causes minimal alterations in MDCK cell morphology after incubation for 24 h; values are the mean ± SEM of two independent experiments.

EC_{99} value of 8.6 μM, and unsubstituted 2′-FdG (**1**) displayed an EC_{99} value of 66 μM. The EC_{90} value (concentration producing a single-$\log_{10}$-unit decrease in virus yield) of 2′-FdG was 26 μM (data not shown), which is similar to the value reported earlier (EC_{90} in MDCK cells by virus yield assay: 42 μM).[16] For each subseries of ProTides, the corresponding 6-modified nucleoside analogue (compounds **2**, **3**, **4**, and **5**) was found to be inactive (EC_{99} > 100 μM), whereas their ProTides did have antiviral activity, with the 1-naphthyl derivatives being preferred. In the ProTide series derived from 6-*O*-methyl-2′-fluoro-2′-deoxyguanosine (**2**), compound **9b** displayed an EC_{99} value of 15 μM and no visible cytotoxicity at 100 μM, showing the best profile relative to its analogues (**9a** and **9c–g**). Compound **9b** carries a 1-naphthyl moiety and an L-alanine methyl ester. The other naphthyl derivatives, bearing different esters (**9a**, **c**, and **d**) showed moderate activity, such as **9a** with an EC_{99} value of 35 μM. Within the series of 6-*O*-ethyl-2′-fluoro-2′-deoxyguanosine (**3**) ProTides, the two best compounds were **9i** and **9j**, with EC_{99} values of 14 and 12 μM, respectively, and no visible toxicity at 100 μM. Both are naphthyl derivatives; ProTide **9i** has a methyl ester, whereas **9j** carries an ethyl ester. Within the ProTide series derived from 2′-deoxy-2′-fluoro-6-chloroguanosine (**4**), three compounds showed antiviral activity (**9m**, **n**, and **p**) and no visible cellular toxicity. The most active compound (**9n**), with an EC_{99} value of 12 μM, is again a naphthyl derivative bearing an L-alanine methyl ester, like ProTide **9b** in the 6-*O*-methyl series and ProTide **9i** in the 6-*O*-ethyl series. Within the series of 2-amino-2′-fluoro-2′-deoxyadenosine (**5**) ProTides, only one (**9s**) was moderately active, with an EC_{99} value of 63 μM.

To summarize, whereas the three 6-modified 2′-FdG nucleosides were inactive by themselves, favorable EC_{99} values were noted for their ProTides carrying a naphthyl function and a methyl- or ethyl-esterified L-alanine moiety.

No strict correlation between lipophilicity and activity was observed (**9b**, clog *P* = 1.9; **9n**, clog *P* = 1.6; **9i**, clog *P* = 2.4; and **9j**, clog *P* = 2.9). However, compound **9q**, which has the lowest clog *P* (1.0) and compound **9h**, which has the highest clog *P* (4.1), showed no antiviral activity. We therefore presume that an intermediate clog *P* value (in the range of 2–3) may be favorable. From these antiviral results, the ProTide approach proved to effectively bypass the first phosphorylation step and to convert inactive nucleoside analogues into their antivirally active species. Besides influenza virus, all the compounds were tested against HSV-1, HSV-2, TK-deficient HSV-1, vaccinia virus, and vesicular stomatitis virus, with no relevant antiviral activity (data not shown).

Activity in the cell-based viral polymerase assay

The activity of some of the 2′-FdG ProTides and their parent nucleosides was further investigated in the influenza virus vRNP reconstitution assay, which assesses viral polymerase activity in a cellular context (Table 4). This method is based on reconstitution of the influenza virus vRNP complex in cells transfected with polymerase-expressing plasmids. Firefly luciferase (FLuc) and Renilla luciferase (RLuc) reporter plasmids are co-transfected. FLuc expression relies on the activity of the viral vRNP, whereas expression of RLuc solely depends on the cellular RNA polymerase II. Thus, inhibitors of the viral polymerase within the vRNP complex decrease FLuc activity, whereas inhibition of RLuc activity is indicative of cytotoxicity (i.e., inhibition of cellular RNA polymerase II). By calculating the ratio between the IC_{50} values for RLuc and FLuc, the selectivity index (SI) of the test compounds can be estimated. As shown in Table 4, all ProTides derived from 6-*O*-methyl-, 6-*O*-ethyl- or 6-Cl-modified 2′-FdG were found to suppress viral vRNP activity, whereas the corresponding nucleoside analogues were inactive at 200 μM (the highest concentration tested). This confirms the advantage of our ProTide concept. At higher concentrations, the ProTides also decreased the activity of cellular RNA polymerase II, yielding a selectivity index of ~4 for the three most active compounds (**9b**, **9i**, and **9n**), which all carry the naphthyl and methyl-esterified L-alanine functions. Using this vRNP assay, we also evaluated the nucleoside analogues **10**, **11**, and **12** (which contain the 2′-F substituent in the β configuration) and their corresponding ProTides **16a–c**. Neither of these compounds showed inhibitory activity against influenza virus polymerase, thus confirming the importance of conserving the 2′-α-fluoro-2′-deoxy core in these purine analogues.

Table 4. Inhibitory activity of 6-substituted purine 2′-fluoro-2′-deoxyriboside ProTides in the influenza virus vRNP reconstitution assay.

Compd	Nuc	Ar	Ester	IC_{50} [μM] FLuc[a]	RLuc[b]	SI[c]
9b	2	Naph	Me	34±2	148±13	4.4
9d	2	Naph	Et	56±9	158±34	2.8
9g	2	Ph	Et	65±6	>200	>3
9i	3	Naph	Me	31±6	134±13	4.3
9n	4	Naph	Me	42±5	160±3	3.8
9q	4	Ph	Et	56±2	>200	>3.6
9t	5	Naph	Et	56±13	48±6	0.9
16a	10	Naph	Me	>200	>200	–
16b	11	Naph	Me	>200	>200	–
16c	12	Naph	Me	>200	>200	–
1	–	–	–	>200	>200	–
2	–	-	–	>200	>200	–
3	–	–	–	>200	>200	–
4	–	–	–	>200	>200	–
5	–	–	–	>200	>200	–
10	–	–	–	>200	>200	–
11	–	–	–	>200	>200	–
12	–	–	–	>200	>200	–
Ribavirin	–	–	–	7.4±2.3	>500	>68

[a] Anti-vRNP activity: FLuc IC_{50} represents the compound concentration required to produce a 50% decrease in vRNP activity, estimated from the firefly luciferase reporter. [b] Anti-pol II activity: RLuc IC_{50} represents the compound concentration required to produce a 50% decrease in pol II activity, estimated from the Renilla luciferase reporter. [c] (RLuc IC_{50})/(FLuc IC_{50}) ratio; values are the mean ± SEM of two or three independent experiments.

Metabolism of the ProTides in enzymatic assays

Carboxypeptidase Y assay

An enzymatic study using yeast carboxypeptidase Y was carried out to study the first step of the activation pathway yielding the aminoacyl phosphoramidate metabolite (Figure 3). Yeast carboxypeptidase Y is a structural homologue of human cathepsin A, the lysosomal esterase which has been implicated in cleavage of the ester groups in similar ProTides as ours.[9,17,20] Removal of the ester group (giving the intermediate metabolite **I**) is followed by the spontaneous elimination of the aryl substituent, resulting in the amino acyl phosphoramidate **II**.

For this enzymatic study we selected one of the most active ProTides (**9b**, EC_{99}: 15 μM) and investigated its bioactivation pathway (Figure 3). Compound **9b** was dissolved in [D_6]acetone and Trizma buffer (pH 7.6), and ^{31}P NMR spectra were recorded (blank; δ_P = 4.22, 4.13 ppm). A solution of carboxypeptidase Y in Trizma buffer was then added, and ^{31}P NMR data were recorded every 7 min. The assay showed rapid hydrolysis of **9b** to the first metabolite **I** (δ_P = 5.02, 4.81 ppm) and then to the second metabolite **II** (L-alaninyl phosphoramidate; δ_P = 7.00 ppm). ProTide **9b** was completely metabolized within 26 min, meaning that in this enzymatic assay its half-life was < 5 min.

Cell lysate assay

To investigate the bioactivation process of ProTide **9b** to its monophosphate species (metabolite **III**, Figure 4), an assay with crude cell lysate was performed. Compound **9b** was incubated with the cell lysate (prepared from Huh7 human hepatocytes) in the presence of deuterium oxide at 37 °C, and ^{31}P NMR spectra were recorded every hour. As shown in Figure 4, compound **9b** first appeared as one broad peak due to the initial poor solubility of the sample, whereas after 24 h at 37 °C the two peaks, corresponding to the two phosphorus diastereomers, were clearly visible. Metabolite **II** was detected after 10 h, while conversion into a second metabolite was much slower (24 h). This metabolite ($\delta_P \sim 1$) was tentatively identified as the 5′-monophosphate derivative of the nucleoside **2** (**III**), in accordance with the literature.[21] The starting material **9b** was detected in the spectra throughout the entire experiment. From this assay, we could successfully identify the various metabolites during bioactivation.

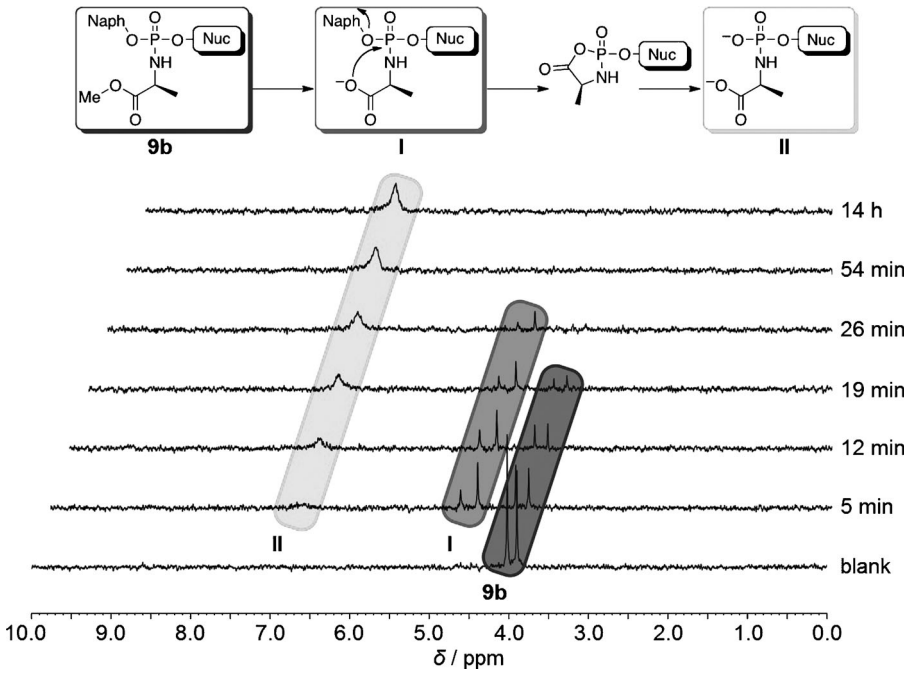


Figure 3. Metabolic conversion of ProTide **9b** when incubated with carboxypeptidase Y, monitored by ^{31}P NMR analysis.

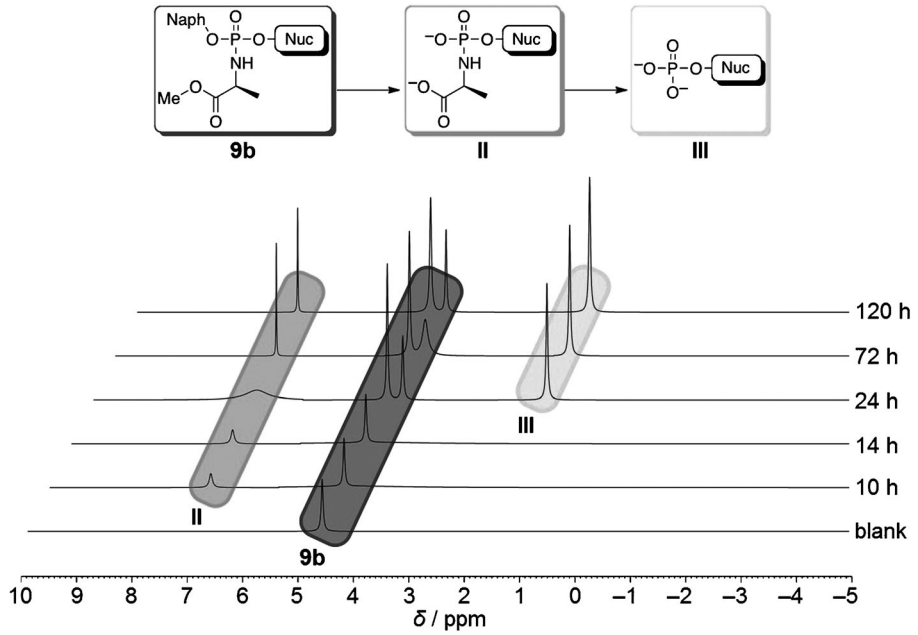


Figure 4. Deconvoluted spectra showing the metabolic conversion of compound **9b** when incubated with crude cell lysate.

Adenosine deaminase assay

An enzymatic assay with adenosine deaminase was performed to study the cleavage of the 6-modified guanosine analogues **2**, **3**, **4**, and **5**. The metabolites were monitored by UV/Vis spectroscopy, with 2′-fluoro-2′-deoxyguanosine (green curve) as a reference. A typical spectrum is shown in Figure 5, illustrating the conversion of nucleoside **4** by adenosine deaminase as a function of time. The red curve in the spectra represents the starting nucleoside **4**, whereas the green curve represents the reference compound 2′-fluoro-2′-deoxyguanosine (**1**). Similar profiles were obtained with the nucleosides **2**, **3**, and **5** (data not shown). All four 6-modified 2′-fluoro-2′-deoxyguanosines showed rapid conversion into **1**; compound **4** was converted within 1 min, whereas compounds **2**, **3**, and **5** were hydrolyzed within 2 min.

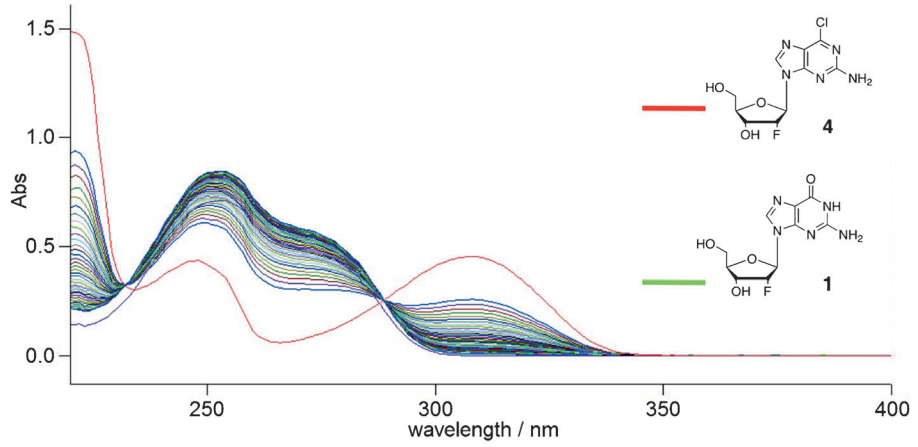


Figure 5. Conversion of nucleoside **4** by adenosine deaminase, monitored by UV/Vis spectroscopy.

Molecular modeling studies

We previously reported that 6-modified 2′-*C*-methylguanosine analogues are processed by adenosine deaminase to form 2′-*C*-methylguanosine, which is then further phosphorylated by host kinases to the active 5′-triphosphate form.[21] Furthermore, recent studies indicated that the 6-*O*-methyl- and 6-*O*-ethyl-substituted forms of 2′-F-2′-*C*-methyl-GMP are readily hydrolyzed at their 6-position by the enzyme adenosine deaminase-like protein isoform 1 (ADAL1), with the 6-*O*-methyl compound being a slightly better substrate than its 6-*O*-ethyl counterpart.[22] Given that this unnatural 2′-*C*-methyl function is not present in our derivatives, it is conceivable that the hydrolysis by ADAL1 may also occur on the 6-modified 2′-FdG 5′-monophosphates formed after removal of the phosphoramidate function in our ProTides.

To investigate this, we performed molecular docking studies of the 6-modified 2′-FdG monophosphates with ADAL1. Although a crystal structure of human ADAL1 is not yet available, it was possible to build a model based on published information.[22] Adenosine 5′-monophosphate was also docked into the model, and the results obtained were used as reference in the analysis of the predicted binding poses of the 6-modified 2′-FdG 5′-monophosphate analogues. Figure 6 shows that 6-*O*-methyl-2′-fluoro-2′-deoxyguanosine 5′-monophosphate overlaps well with the reference AMP, and shares the same interactions within the amino acid residues in the active site. Particular attention is drawn to the interaction between Glu 211 (in the neutral form) and the purine base, which is important for substrate recognition. These data suggest that the modification at the 6-position may be well tolerated in the case of the 2′-FdGMP derivatives, and hydrolysis of the 6-substituent by adenylate deaminase is probable, as observed in the case of 6-modified 2′-*C*-methyl-GMP.[22]

Conclusions

Herein we present the design, synthesis, and biological evaluation of 6-modified analogues of 2′-fluoro-2′-deoxyguanosine and

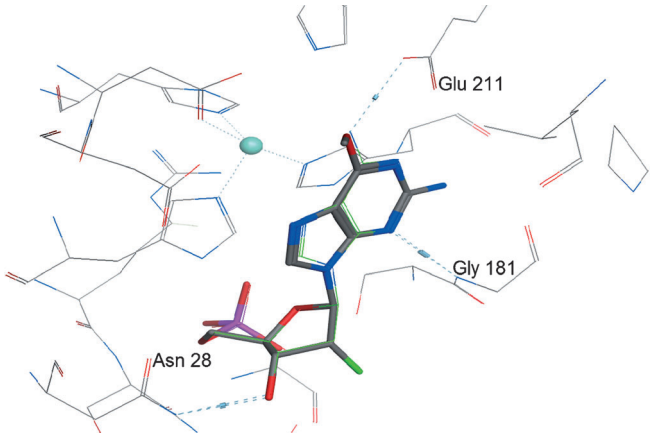


Figure 6. Docking pose of 6-*O*-methyl-2′-fluoro-2′-deoxyguanosine 5′-monophosphate within the active site of the ADAL1 model; AMP is represented in green.

2′-β-fluoro-2′-deoxyguanosine and their corresponding ProTides against influenza virus replication in cell culture. The antiviral data obtained are supported by enzymatic assays with the viral polymerase, the target for antiviral activity, and the cellular enzymes involved in activation of our 2′-FdG ProTides.

First, we confirmed that the 5′-triphosphate form of 2′-FdG is a strong inhibitor of GTP incorporation by the influenza virus polymerase. This compound was equipotent to 7-deaza-GTP, and both were superior to the obligate chain terminator 3′-deoxy-GTP. Metabolism experiments with carboxypeptidase Y and whole-cell lysates demonstrated that the 2′-FdG ProTides are readily cleaved by cellular enzymes to release the 6-modified 2′-FdG 5′-monophosphate. In an enzymatic assay with adenosine deaminase, the 6-modified 2′-FdG analogues showed rapid conversion into 2′-FdG, indicating that the 2′-F substitution does not hinder enzymatic removal of the 6-substituent, which was added to improve cellular uptake of the 2′-FdG ProTides.[23] These combined enzymatic studies indicate that the 2′-FdG ProTides are successful in delivering 2′-FdG 5′-monophosphate, which is then further converted into 2′-FdG 5′-triphosphate, the active metabolite that inhibits the viral polymerase.

The superiority of the 2′-FdG ProTides over the parent nucleoside analogues was demonstrated in both antiviral assay systems, that is, the influenza virus replication (virus yield) assay in MDCK cells, and the vRNP reconstitution assay in HEK-293T cells. Among the various 2′-FdG ProTides synthesized, the 1-naphthyl derivatives were consistently more active than their phenyl counterparts, and a methyl ester attached to the L-alaninyl portion was superior to ethyl, benzyl, or cyclohexyl. With regard to the 6-modification, the 6-*O*-methyl-, 6-*O*-ethyl-, and 6-chloro-modified 2′-FdG ProTides were equipotent (i.e., EC_{99} values of 12–15 μM in MDCK cells), but the 6-amino-substituted analogues proved less active. Although the antiviral concentrations of our 2′-FdG ProTides were quite high (i.e., 12 μM in MDCK cells), their superiority over the parent nucleosides is evident. Biological evaluation of our 2′-FdG ProTides in a more relevant cell culture system is now warranted, particularly primary human airway epithelial cells, in which the anti-influenza virus activity and selectivity of 2′-FdG were reported to be far superior than in MDCK cells.[16] Another avenue is the possibility of synthesizing 2′-FdG ProTides with a 7-deaza modification in the guanine part, as 7-deaza-GTP provided marked inhibition in our influenza virus polymerase assay. This 7-deaza modification was reported to increase the anti-HCV potency of 2′-*C*-methyladenosine by a factor of 20.[24] The preclinical development of nucleoside ProTides for HCV therapy has rapidly progressed in recent years.[7] The results presented herein demonstrate that this class of antiviral compounds should also be pursued for influenza virus therapy. Our 2′-FdG ProTides represent the first step in this challenging and clinically relevant enterprise.

Experimental Section

Chemistry

All anhydrous solvents were purchased from Aldrich, and all commercially available reagents were used without further purification. Thin-layer chromatography (TLC): pre-coated, aluminum-backed plates (60 F_{254}, 0.2 mm thickness, Merck) were visualized under both short- and long-wave UV light (λ 254 and 366 nm). Preparative TLC plates (20×20 cm, 500–2000 μm) were purchased from Merck. Column chromatography processes were carried out with silica gel supplied by Fisher (35–70 μm). Glass columns were slurry packed using the appropriate eluent, and samples were applied either as a concentrated solution in the same eluent or pre-adsorbed on silica gel. Analytical and semi-preparative HPLC were conducted using a Varian Prostar system, with Galaxie Chromatography Data System software. Only compounds with purity ≥95% were considered in this study. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), ^{31}P NMR (202 MHz), and ^{19}F NMR (470 MHz) spectra were recorded on a Bruker Avance 500 MHz spectrometer at 25 °C. Spectra were calibrated to the residual signal of the deuterated solvent used. Chemical shifts (δ) are given in ppm, and coupling constants (*J*) in Hz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), ddd (doublet of doublet of doublets). Low- and high-resolution electrospray ionization mass spectrometry (ES MS) was performed as a service at the School of Chemistry, Cardiff University (UK).

Standard procedure A: synthesis of phosphorochloridates (8a–g)

Anhydrous TEA (2.00 mol equiv) was added dropwise at −78 °C to a stirred solution of the appropriate phosphorodichloridate (1.00 mol equiv) and the appropriate amino acid ester salt (1.00 mol equiv) in anhydrous CH_2Cl_2 (30–40 mL) under an argon atmosphere. After 1 h the reaction was allowed to slowly warm to room temperature and was stirred for 2–3 h. The formation of the desired compound was monitored by ^{31}P NMR. The solvent was removed under reduced pressure, and the crude residue was purified by flash column chromatography (EtOAc/*n*Hex 1:1) to give the product as an oil.

Standard procedure B: synthesis of phosphoramidates (9a–w)

*t*BuMgCl (1.0 M in THF, 1.20–2.00 mol equiv) was added to a suspension/solution of the appropriate nucleoside (1.00 mol equiv) in anhydrous THF (10 mL) under an argon atmosphere and stirred at room temperature for 30 min. The appropriate phosphorochloridate (2.00 mol equiv) dissolved in anhydrous THF was added dropwise, and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the crude residue was purified by flash column chromatography (gradient elution of CH_2Cl_2/MeOH) to give the desired product.

Standard procedure C: synthesis of phosphoramidates (16a–c)

NMI (5.00–10.00 mol equiv) was added dropwise to a stirring suspension/solution of the appropriate nucleoside (1.00 mol equiv) and phosphorochloridate (3.00 mol equiv) in anhydrous THF (10–20 mL), and the reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the crude residue was purified by flash column chromatography (gradient elution of CH_2Cl_2/MeOH) and in some cases by preparative TLC to give the desired product.

Synthesis of 6-*O*-methyl-2′-fluoro-2′-deoxyguanosine-5′-*O*-naphthyl(methoxy-L-alaninyl)phosphate (9b): Prepared according to standard procedure B, using **2** (0.20 g, 0.67 mmol) in anhydrous THF (10 mL), *t*BuMgCl (1.0 M solution THF, 0.80 mL, 0.80 mmol), and **8b** (0.44 g, 1.34 mmol); the reaction mixture was stirred at room temperature overnight. After this period *t*BuMgCl (1.0 M in THF, 0.80 mL, 0.80 mmol) was added, and the reaction mixture was stirred at room temperature for a further 2 h. The crude was purified by column chromatography (gradient elution of CH_2Cl_2/MeOH 98:2, then 96:4). The product was further purified by preparative TLC (gradient elution of CH_2Cl_2/MeOH 98:2, then 96:4) to give a white solid (2%, 0.01 g); ^{31}P NMR (MeOD, 202 MHz): $\delta = 4.22$, 4.13 ppm; ^{19}F NMR (MeOD, 470 MHz): $\delta = -204.83$, -205.48 ppm; ^{1}H NMR (MeOD, 500 MHz): $\delta = 8.12$–8.06 (1H, m, H-8 Naph), 7.96, 7.94 (1H, 2 s, H-8), 7.88–7.33 (6H, m, *Naph*O), 6.21–6.17 (1H, m, H-1′), 5.54–5.39 (1H, m, H-2′), 4.93–4.82 (1H, m, H-3′), 4.56–4.39 (2H, m, H-5′), 4.26–4.27 (1H, m, H-4′), 4.02, 4.00 (3H, 2 s, OCH_3), 3.99–3.88 (1H, m, $CHCH_3$), 3.57, 3.54 (3H, 2 s, $COOCH_3$), 1.28, 1.22 ppm (3H, 2d, $J = 7.0$ Hz, $CHCH_3$); ^{13}C NMR (MeOD, 126 MHz): $\delta = 20.24$ (d, $J_{C\text{-}P} = 6.8$ Hz, CH_3-Ala), 20.42 (d, $J_{C\text{-}P} = 6.4$ Hz, CH_3-Ala), 51.50, 51.53 (CH-Ala), 52.63, 52.72 ($COOCH_3$), 54.16 (OCH_3), 66.73 (d, $J_{C\text{-}P} = 4.9$ Hz, C-5′), 67.12 (d, $J_{C\text{-}P} = 5.2$ Hz, C-5′), 70.28 (d, $J_{C\text{-}F} = 16.5$ Hz, C-3′), 70.44 (d, $J_{C\text{-}F} = 17.5$ Hz, C-3′), 82.66, 82.72 (C-4′), 88.26 (d, $J_{C\text{-}F} = 33.8$ Hz, C-1′), 88.37 (d, $J_{C\text{-}F} = 34.6$ Hz, C-1′), 94.43 (d, $J_{C\text{-}F} = 187.0$ Hz, C-2′), 94.46 (d, $J_{C\text{-}F} = 186.7$ Hz, C-2′), 115.63 (C-5), 116.08, 116.12, 122.65, 122.69, 125.91, 126.44, 127.43, 127.46, 127.72, 127.84, 128.77, 128.81 (C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.23 (C-4a Naph), 139.65 (C-8), 147.89, 147.84 ("ipso" Naph), 154.40 (C-4), 161.96 (C-2), 162.66 (C-6), 175.28, 174.48 ppm ($COOCH_3$); ES MS: 591.18 $[M+H]^+$, 613.16 $[M+Na]^+$, 629.14 $[M+K]^+$; HRMS ($C_{25}H_{29}N_6O_8PF$) calcd: 591.1796, found: 591.1796; RP-HPLC (flow = 1 mL min^{-1}, $\lambda = 254$ nm), eluting with H_2O/MeOH from 90:100 to 0:100 over 30 min, $t_R = 21.81$, 22.36 min, eluting with H_2O/CH_3CN from 90:100 to 0:100 over 30 min, $t_R = 14.27$, 14.63 min.

Synthesis of 2-amino-6-chloro-9-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)-9*H*-purine (15): TMSOTf (6.24 mL, 34.40 mmol) was added dropwise to a precooled (0 °C) solution of **13** (4.00 g, 8.60 mmol), **14** (1.60 g, 9.46 mmol), and DBU (3.86 mL, 25.80 mmol) in anhydrous CH_3CN (80 mL). The reaction mixture was heated at 65 °C for 21 h, after which it was allowed to cool to room temperature, poured into a saturated solution of $NaHCO_3$ (200 mL), and extracted with CH_2Cl_2 (3×100 mL). The combined organic phase was dried over Na_2SO_4 and concentrated. The residue was crystallized from MeOH to give the pure β-anomer as a white solid (17%, 0.74 g); ^{19}F NMR (DMSO, 470 MHz): $\delta = -187.82$ ppm; ^{1}H NMR (DMSO, 500 MHz): $\delta = 8.72$ (1H, s, H-8), 8.06–7.50 (10H, m, Ph), 6.87–6.85 (1H, m, H-1′), 6.72 (2H, bs, NH_2), 6.07–5.98 (1H, m, H-2′), 5.79–5.75 (1H, m, H-3′), 5.31–5.29 (1H, m, H-4′), 4.68–4.66 ppm (2H, m, H-5′); ^{13}C NMR (DMSO, 126 MHz): $\delta = 63.70$ (C-5′), 76.47 (d, $J_{C\text{-}F} = 30.5$ Hz, C-3′), 83.80 (C-4′), 90.15 (d, $J_{C\text{-}F} = 36.1$ Hz, C-1′), 97.42 (d, $J_{C\text{-}F} = 184.9$ Hz, C-2′), 114.39 (C-5), 128.62, 128.78, 129.23, 129.29, 129.44, 133.59, 133.83 (*Ph*, "ipso" Ph), 142.63 (C-4), 146.41 (C-8), 160.14 (C-6), 164.44 (C-2), 164.69, 165.45 ppm (*C*OPh).

Synthesis of 6-*O*-methyl-2′-β-fluoro-2′-deoxyguanosine-5′-*O*-naphthyl(methoxy-L-alaninyl)phosphate (16a): Prepared according to standard procedure C, using **10** (0.19 g, 0.62 mmol), **8b** (0.61 g, 1.86 mmol), NMI (0.25 mL, 3.10 mmol) in anhydrous THF (10 mL), and anhydrous pyridine (3 mL); the reaction mixture was stirred at room temperature overnight. After this period **8b** (0.31 g, 0.93 mmol) and NMI (0.15 mL, 1.86 mmol) were added, and the reaction mixture was stirred at room temperature overnight. The crude was purified by column chromatography (gradient elution of CH_2Cl_2/MeOH 98:2, then 96:4). The product was further purified by preparative TLC (gradient elution of CH_2Cl_2/MeOH 98:2, then 96:4) to give a white solid (6%, 0.02 g); ^{31}P NMR (MeOD, 202 MHz): $\delta = 4.10$, 3.96 ppm; ^{19}F NMR (MeOD, 470 MHz): $\delta = -192.52$, -192.72 ppm; ^{1}H NMR (MeOD, 500 MHz): $\delta = 8.21$–7.43 (8H, m, H-8, *Naph*O), 6.25–6.20 (1H, m, H-1′), 5.44–5.32 (1H, m, H-2′), 4.57–4.55 (1H, m, H-4′), 4.50–4.43 (1H, m, H-3′), 4.41–4.28 (2H, m, H-5′), 4.13–4.09 (1H, m, $CHCH_3$), 4.08, 4.06 (3H, 2 s, OCH_3), 3.65, 3.63 (3H, 2 s, $COOCH_3$), 1.37–1.34 ppm (3H, m, $CHCH_3$); ^{13}C NMR (MeOD, 126 MHz): $\delta = 20.36$ (d, $J_{C\text{-}P} = 7.3$ Hz, CH_3-Ala), 20.44 (d, $J_{C\text{-}P} = 6.6$ Hz, CH_3-Ala), 51.63, 51.72 (CH-Ala), 52.73, 52.77 ($COOCH_3$), 54.59, 54.82 (OCH_3), 67.12 (d, $J_{C\text{-}P} = 5.4$ Hz, C-5′), 67.29 (d, $J_{C\text{-}P} = 5.4$ Hz, C-5′), 74.90 (d, $J_{C\text{-}F} = 24.5$ Hz, C-3′), 75.00 (d, $J_{C\text{-}F} = 24.5$ Hz, C-3′), 85.82, 85.88, 85.92, 85.96, 85.98, 86.02 (C-4′), 91.13 (d, $J_{C\text{-}F} = 35.4$ Hz, C-1′), 91.22 (d, $J_{C\text{-}F} = 35.3$ Hz, C-1′), 101.00 (d, $J_{C\text{-}F} = 187.2$ Hz, C-2′), 101.02 (d, $J_{C\text{-}F} = 187.2$ Hz, C-2′), 116.30 (d, $J_{C\text{-}P} = 3.4$ Hz, C-2 *Naph*), 116.40 (d, $J_{C\text{-}P} = 3.5$ Hz, C-2 *Naph*), 118.79 (C-5), 122.69, 125.81, 126.01, 126.04, 126.51, 127.41, 127.48, 127.76, 127.81, 127.94, 128.85, 128.87 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.31 (C-4a Naph), 145.16, 145.22 (C-8), 148.00, 148.03, 148.06, 148.08 ('ipso' Naph), 159.13 (C-4), 162.00 (C-2), 164.82 (C-6), 175.30 (d, $J_{C\text{-}P} = 5.2$ Hz, $COOCH_3$), 175.60 ppm (d, $J_{C\text{-}P} = 5.0$ Hz, $COOCH_3$); ES MS: 589.17 $[M-H]^+$, 625.14 $[M+Cl]^-$; RP-HPLC (flow = 1 mL min^{-1}, $\lambda = 254$ nm), eluting with H_2O/MeOH from 90:100 to 0:100 in 30 min, $t_R = 18.76$ min.

Biological experiments

Isolation of viral ribonucleoprotein complexes and RNA elongation assay: The detailed procedure for isolation of the viral vRNP complexes from disrupted virions, which is based on published procedures,[25,26] has been described elsewhere.[20] Briefly, influenza A/X-31 virus was pelleted from an allantoic stock by ultracentrifugation, and, after detergent disruption of the viral pellet, vRNP complexes were purified by gradient ultracentrifugation. Collected fractions were subjected to western blot analysis with an anti-PB2 antibody, and the fractions containing the vRNP complexes were pooled, dialyzed against storage buffer, and stored at −80 °C.

The modified GTP analogues were purchased from TriLink Biotechnologies, whereas [8-^{3}H]GTP (specific activity: 12.8 Ci mmol^{-1}) was from PerkinElmer. The enzyme reaction mixture (final volume: 25 μL) consisted of influenza virus vRNP (2 μL), 50 mM Tris pH 8.0, 100 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.4 U μL^{-1} recombinant RNasin (Promega), 200 μM ApG primer [adenyl(3′→5′)guanosine, obtained from Sigma], 100 μM UTP, 100 μM CTP, 500 μM ATP, 2.1 μM GTP, 1.6 μM [8-^{3}H]GTP, and various concentrations of the test compounds. After 60 min incubation at 30 °C, the enzyme reaction was terminated by the addition of 1.0 mL of a mixture containing 5% trichloroacetic acid (TCA) and 20 mM $Na_4P_2O_4$, and the samples were put on ice for 30 min. The precipitates were spotted onto glass microfiber filters, washed 10 times with 5% TCA, and once with denatured ethanol, and then dried for 2 h. Finally, radioactivity incorporated in the filter discs was quantified by liquid scintillation counting. The IC_{50} values were calculated by extrapolation, and are defined as the compound concentration effecting a 50% decrease in incorporated radioactivity relative to the condition receiving no inhibitor.

Antiviral activity in influenza-virus-infected MDCK cell cultures: Madin–Darby canine kidney (MDCK) cells, seeded in 96-well plates, were infected with influenza A virus (strain A/X-31, multiplicity of infection: 0.0004 PFU per cell) and treated with the test compounds at serial dilutions. After 24 h incubation at 35 °C, the supernatants were collected and stored at −80 °C. The virus particles were lysed, and the number of viral genome copies was determined by a one-step qRT-PCR assay using influenza virus M1-specific primers and probe.[27] The technical details are reported elsewhere.[20] An M1-plasmid standard was included to allow absolute quantitation of vRNA copies. The EC_{99} value was calculated by extrapolation and is defined as the compound concentration causing a two-$\log_{10}$-unit decrease in the amount of vRNA copies relative to untreated virus control. In parallel, compound cytotoxicity was estimated from the changes in MDCK cell morphology observed by microscopy after 72 h incubation with the test compounds.

vRNP reconstitution assay: The bidirectional pHW-2000 derived plasmids for expression of the PB2, PB1, PA, and NP proteins from influenza A/WSN/33 were generously donated by Dr. R. Webster (St. Jude Children's Research Hospital, Memphis, TN, USA).[28] The firefly luciferase reporter plasmid, which contains the firefly luciferase coding sequence flanked by the 5′- and 3′-UTR sequences from the A/Puerto Rico/8/34 NS gene, was kindly provided by Dr. M. Kim (Korea Research Institute of Chemical Technology, Daejeon), while the Renilla luciferase reporter plasmid was the pRL-TK plasmid (containing an HSV TK promoter sequence) from Promega. The procedure for transfection of these plasmids into human embryonic kidney (HEK)-293T cells was derived from the reverse genetics protocol published by Martínez-Sobrido and García-Sastre.[29] Specifically, 1 mL of the transfection mixture contained 2.4×10^6 HEK-293T cells, 5 μL Lipofectamine-2000 (Invitrogen), 0.22 μg of each of the four vRNP-reconstituting plasmids, 0.086 μg of the firefly luciferase plasmid, and 0.020 μg of the Renilla luciferase plasmid. This mixture was transferred to a 96-well plate (50 μL per well) containing 10 μL of the compounds at serial dilutions, and the plate was incubated for 24 h at 37 °C. Luciferase activity was determined using the Dual-Glo assay system from Promega.

Molecular modeling

All molecular modeling studies were performed on a MAC pro 2.66 GHz Quad-Core Intel Xeon, running Ubuntu. The human adenylate deaminase sequence was obtained from GenBank (Q6DHV7), and its structure was built using MOE.[30] The homologous template was downloaded from the RCSB Protein Data Bank (PDB code: 3IAR) and pre-processed using MOE. Alignment was manually adjusted according to reported data.[22] The best-scored model was minimized with the Amber99 force field until an RMSD gradient of 0.1 kcal mol^{-1} Å^{-1} was reached. Docking simulations were performed using PLANTS.[31] Ligand structures were built with MOE and minimized using the MMFF94x force field until an RMSD gradient of 0.05 kcal mol^{-1} Å^{-1} was reached. Docking results were analyzed with MOE.

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Keywords: 2′-fluoro-2′-deoxyguanosine · antiviral agents · influenza · nucleosides · ProTides

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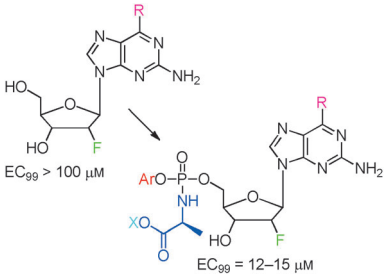
Synthesis and Biological Evaluation of Purine 2′-Fluoro-2′-deoxyriboside ProTides as Anti-influenza Virus Agents



Tidal power: We report the synthesis and biological evaluation of several ProTides of 6-modified analogues of 2′-fluoro-2′-deoxyguanosine as anti-influenza agents. The superiority of the ProTides over the parent nucleosides was demonstrated in both influenza virus replication assays in MDCK cells and vRNP reconstitution assays in HEK-293T cells.