Influence of 4'-Substitution on the Activity of Gemcitabine and Its ProTide Against VZV and SARS-CoV-2

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ABSTRACT: In addition to its therapeutic value as a chemotherapy drug, gemcitabine is of ongoing interest to the scientific community for its broad-spectrum antiviral activity. Herein the synthesis of 4'-methoxy- and 4'-fluorosubstituted gemcitabine analogues along with their phosphoramidate prodrugs is described. Among these derivatives, 4'-fluorogemcitabine proved to be active against varicella zoster virus (VZV, TK+ strain) with an EC50 of 0.042 μM and produced significant cytotoxicity (CC50 = 0.11 μM). Upon derivatization of this trifluoronoucleoside as its prodrug, decreased anti-VZV activity was observed, but with a concomitantly improved selectivity index (SI = 36). When this prodrug was tested against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), its antiviral activity (EC50 = 0.73 μM) was comparable to or slightly lower than its cytotoxic concentration in measurements of cell growth and cell morphology, respectively.

KEYWORDS: Gemcitabine analogues, phosphoramidate prodrug, varicella zoster virus, severe acute respiratory syndrome coronavirus 2, antiviral activity

Gemcitabine (2'-deoxy-2',2'-difluorocytidine, dFdC; Figure 1) is a pyrimidine nucleoside analogue that is in clinical use for the treatment of various solid tumors, including non-small cell lung, pancreatic, bladder, and breast cancer.1 By acting as a potent antimetabolite, it inhibits two cellular processes that are both required for DNA biosynthesis, i.e., nucleotide reduction and replicative DNA chain elongation. Upon phosphorylation in cells, it is converted to its biologically active metabolites, namely, gemcitabine diphosphate (dFdCDP) and triphosphate (dFdCTP).2 While dFdCDP acts as an inhibitor of ribonucleotide reductase (RNR), thus preventing the biosynthesis of deoxynucleotide building blocks that are required for DNA synthesis, dFdCTP is competitively incorporated into DNA in place of natural 2'-deoxycytosine-5'-triphosphate (dCTP).3

Furthermore, gemcitabine was also demonstrated to exert broad-spectrum in vitro activity against a range of RNA viruses. In particular, its antiviral inhibitory effect was assessed against Zika virus (ZIKV) (EC50 = 0.01 μM),4 hepatitis C virus (HCV) (EC50 = 12 nM),5 human immunodeficiency virus 1 (HIV-1) (EC50 = 16.3 nM),6 and poliovirus (IC50 = 0.3 μM)7 as well as respiratory viruses such as influenza A virus (IAV) (EC50 = 0.068 μM),8 human rhinovirus (HRV) (EC50 = 0.81–1.92 μM),9 Middle East respiratory syndrome coronavirus (MERS-CoV) (EC50 = 1.22 μM),10 and severe acute respiratory syndrome coronavirus (SARS-CoV) (EC50 = 4.95 μM).10 In addition, a recent report described the activity of this modified nucleoside against the emerging coronavirus SARS-CoV-2 (EC50 = 1.24 μM) that is responsible for the current worldwide viral pneumonia outbreak.11

The modification of gemcitabine as a means to convert a life-saving antitumoral drug into an active compound for the treatment of severe viral infections represents an attractive research endeavor. In this context, a variety of gemcitabine analogues have been synthesized to increase its selectivity as...
antiviral agent. The rationale behind this strategy aims at reducing the inhibitory activity of dFdCDP and dFdCTP against the cellular RNR and polymerase, respectively, while maintaining the viral polymerase inhibitory capacity of the dFdCTP metabolite.

It is well-known that subtle structural modifications of nucleosides can have a profound impact on their biological profiles. In this study, we selected a methoxy group as an electron-withdrawing substituent via the inductive effect and a fluorine atom as a strongly inductive electron-withdrawing substituent to modify the 4'-position of gemcitabine in order to identify compounds that could lead to reduced cell toxicity while retaining activity against selected viruses. Sterically, both substituents are small, as larger groups might be detrimental for an effective interaction with the target enzymes. In addition, the presence of substituents with different electronic properties might influence the reactivity of the primary hydroxyl group, which needs to undergo intracellular phosphorylation to deliver the active metabolite. The synthesis of 4'-methoxy- and 4'-fluorgemcitabine analogues (1a and 1b, respectively; Figure 1) was complemented by that of the corresponding prodrugs (2a and 2b; Figure 1). A comparison of the antiviral activity of a nucleoside itself (which still needs to undergo three consecutive phosphorylations) and its prodrug (bypassing the first phosphorylation to the nucleoside monophosphate) was expected to provide useful information for the design of new gemcitabine analogues.

Herein, varicella zoster virus (VZV) and human cytomegalovirus (HCMV) were chosen as the two most important herpes viruses. Although there are anti-VZV and anti-HCMV drugs on the market, toxicity is an issue for some of these drugs, and the emergence of drug resistance has been described for all of them among immunocompromised patients. Novel nontoxic antiviral chemotherapeutics that are more potent and effective than the currently available drugs are therefore required for the treatment of these viral infections in at-risk populations. At the same time, SARS-CoV-2 was privileged among RNA viruses because of the extremely urgent need to develop new antiviral treatments for this infection. As shown in Scheme 1, commercially available gemcitabine served as a convenient starting point for the introduction of fluoro and methoxy substituents at the 4'-position.

First, uridine congener 5 was obtained in an overall yield of 71% via a three-step sequence including complete functional group protection, nucleobase deamination, and O-debenzylation using standard literature procedures. Next, the primary hydroxyl group of compound 5 was substituted with an iodine atom upon treatment with iodine and triphenylphosphine to yield 6 in good yield (84%). When 6 was subjected to an elimination reaction under strongly basic conditions, methylene derivative 7 was obtained in 65% yield and served as a key...
Scheme 2. Conversion of Gemcitabine and Its 4'-Substituted Analogues to Their Phosphoramidate Prodrugs

\[ \text{HO}_2^\text{C} \rightarrow \text{N}^2 \rightarrow \text{N}^3 \rightarrow \text{OH} \]

Reagents and conditions: (a) DBDC, Na$_2$CO$_3$, dioxane/H$_2$O, 35% for 13a, 23% for 13b, and 59% for 13c; (b) phenyl dichlorophosphate, triethylamine, CH$_2$Cl$_2$, –78 °C, quantitative yield; (c) tert-butylimagnesium chloride, anhydrous THF; (d) trifluoroacetic acid/CH$_2$Cl$_2$, 0 °C, 49% for 16a, 54% for 16b, and 32% for 16c.

| compound | TK$^+$ strain | TK$^-$ strain | AD-169 strain | Davis strain | VZV SI$^a$ |
|----------|----------------|----------------|---------------|-------------|-----------|
| gemcitabine | 0.028 ± 0.020 | 0.054 ± 0.012 | 0.074 ± 0.004 | 0.053 ± 0.049 | ND |
| 1a | >100 | >100 | >100 | >100 | ND |
| 1b | 0.042 ± 0.014 | 0.166 ± 0.13 | 0.815 ± 0.64 | 0.93 ± 0.75 | 2 |
| 12a | >100 | >100 | >100 | >100 | ND |
| 12b | >100 | >100 | >100 | >100 | ND |
| 2a | >100 | >100 | >100 | >100 | ND |
| 2b | 2.32 ± 0.43 | 2.84 ± 0.021 | 15.47 ± 6.40 | 4.00 ± 0.00 | 36 |
| 2c | 0.074 ± 0.006 | 0.089 ± 0.011 | 0.066 ± 0.007 | 0.032 ± 0.00 | 4 |
| acyclovir | 1.6 | 22.15 ± 11.6 | ND | ND | 265 |
| brivudin | 0.036 ± 0.004 | 6.04 | ND | ND | >8333 |
| ganciclovir | ND$^d$ | ND$^d$ | 2.77 ± 0.53 | 1.67 ± 0.48 | ND |
| cidofovir | ND$^d$ | ND$^d$ | 0.65 ± 0.38 | 0.34 ± 0.056 | 300 |

Table 1. Antiviral Activities and Cytotoxicities of 4'-Substituted Gemcitabine Analogues and Their Prodrugs against VZV and HCMV

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The arylox triester phosphoramidate prodrug or ProTide technology is a strategy that has proven to be very effective in bypassing the first rate-limiting phosphorylation step in the nucleoside activation cascade to their 5'-triphosphate forms and facilitate intracellular delivery. For instance, the application of this approach to gemcitabine led to enhanced biological activities and decreased drug resistance. Herein, phosphoramidate prodrugs of compounds 1a and 1b along with that of gemcitabine were synthesized as shown in Scheme 2. First, 1a, 1b, and gemcitabine were selectively protected at the 3'-position by treatment with di-tert-butyl dicarbonate (DBDC) and sodium carbonate in a 4:1 mixture of dioxane and water as the solvent to afford compounds 13a-c, respectively. In parallel, l-alanine benzyl ester hydrochloride (14) was converted to its phenyl aminoacyl phosphorochloridate 15 upon reaction with phenyl phosphorochloridate. Then compounds 13a-c were reacted with compound 15 to furnish prodrugs 16a-c in moderate yields. Finally, the Boc protecting group was cleaved under acidic conditions to afford the desired compounds 2a-c. All of the novel compounds (1a, 1b, and 2a-c) were evaluated for their antiviral activity (expressed as EC$_{50}$) against VZV (strains TK$,^+$ thymidine kinase-competent, and TK$,^-$ thymidine kinase-deficient) and HCMV (strains AD-169 and HCMV).
Davis), with concomitant determination of cytotoxicity and cytostatic effects (CC_{50}) (Table 1). Gemcitabine was included as a reference compound, and acyclovir, brivudine, ganciclovir, and cidofovir were included as positive controls.

The potent cytostatic activity of gemcitabine in human embryonic lung (HEL) fibroblasts (CC_{50} = 0.0036 μM) leads to a complete absence of selective antiviral activity. The phosphoramidate prodrug of gemcitabine (compound 1b) was 100-fold less inhibitory toward HEL cell growth (CC_{50} = 0.34 μM), which is in agreement with what was previously observed for other ProTides of gemcitabine. The introduction of a fluorine at the 4′-position of gemcitabine yielded compound 1b, which was in general less active against VZV and HCMV. Remarkably, an EC_{50} of 0.042 μM was observed against TK+ strain of VZV, making it only 2-fold less active than the parent nucleoside 1b.

Interestingly, phosphoramidate prodrug 2b is less active than the parent nucleoside 1b, showing moderate activity against the TK- and TK+ strains of VZV with EC_{50} values in the 2–3 μM range. However, a 700-fold lower cytostatic activity was observed for ProTide 2b in comparison with the parent compound 1b. Consequently, compound 2b showed a selectivity index (SI) of 36, which was higher than those of 1b (SI = 2) and 2c (SI = 4). On the other hand, the 4′-methoxy gemcitabine analogue 1a and the 4′-substituted uridine analogues 12a and 12b were completely devoid of activity against VZV and HCMV.

In a second screening assay, the cytosine-containing compounds were tested against two different clinical isolates of SARS-CoV-2 in Vero cells (Table 2). Remdesivir and hydroxychloroquine were included as positive controls. As expected, the high cytotoxicity of gemcitabine in Vero cells precluded selective antiviral activity. The application of the ProTide technology to gemcitabine (affording compound 2c) led to decreased cytotoxicity but also lack of activity against SARS-CoV-2. The introduction of a 4′-methoxy group afforded compound 1a and the corresponding prodrug 2a, both of which were devoid of antiviral activity.

Interestingly, in this cellular test system, compounds 1b and 2b were 20 and 10 times more active than remdesivir, respectively.

In summary, the synthesis of 4′-methoxy- and 4′-flourogemcitabine analogues and their phosphoramidate prodrugs and an evaluation of their antiviral activities against VZV, HCMV, and SARS-CoV-2 have been described. The introduction of a fluorine atom at the 4′-position led to a trifluorinated gemcitabine analogue that exhibited potent antiviral activity but no selectivity against these three viruses. A phosphoramidate prodrug of the 4′-fluorocongener displayed reduced activity against VZV but less pronounced cytotoxicity with an enhanced selectivity index. A similar although somewhat reduced effect was observed against HCMV. Unfortunately, this effect was not determined upon testing against SARS-CoV-2.

### ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00485.

Experimental details and characterization data for the reported compounds, NMR spectra, and biological assays (PDF)

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Table 2. Antiviral Activities and Cytotoxicities of 4′-Substituted Gemcitabine Analogues and Their Prodrugs against SARS-CoV-2 in Vero Cells

| compound | antiviral activity, EC_{50} (μM) | cytotoxicity (μM) |
|----------|---------------------------------|------------------|
|          | UC-1074 strain | UC-1075 strain | MCC | CC_{50} |
| gemcitabine | >0.0032 ± 0 | >0.0016 ± 0 | 0.008 | 0.0043 ± 0.0008 |
| 1a | ND | ≥86.2 ± 20.4 | >100 | >100 ± 0 |
| 1b | 0.36 | 0.096 ± 0.034 | 0.16 | 0.26 ± 0.13 |
| 2a | ≥100 | ≥55.1 ± 42.8 | ≥100 | ≥29.6 ± 11.1 |
| 2b | ≥2.4 ± 2.3 | 0.73 ± 0.15 | 4 ± 0 | 1.44 ± 0.62 |
| 2c | >0.032 ± 0 | >0.0128 ± 0 | 0.048 ± 0.018 | 0.020 ± 0.009 |
| remdesivir | 5.8 ± 3.1 | 1.52 ± 1.60 | >40 | >40 ± 0 |
| hydroxychloroquine | 8.1 ± 2.4 | 1.74 ± 0.68 | 100 | 36.9 ± 7.5 |

*Effective concentration required to reduce virus plaque formation by 50%. Virus input was 100 PFU. Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology. Cytotoxic concentration required to reduce cell growth by 50%.

GC (SI = 2) and GC (SI = 4). On the other hand, the 4′-methoxy gemcitabine analogue 1a and the 4′-substituted uridine analogues 12a and 12b were completely devoid of activity against VZV and HCMV.

In a second screening assay, the cytosine-containing compounds were tested against two different clinical isolates of SARS-CoV-2 in Vero cells (Table 2). Remdesivir and hydroxychloroquine were included as positive controls. As expected, the high cytotoxicity of gemcitabine in Vero cells precluded selective antiviral activity. The application of the ProTide technology to gemcitabine (affording compound 2c) led to decreased cytotoxicity but also lack of activity against SARS-CoV-2. The introduction of a 4′-methoxy group afforded compound 1a and the corresponding prodrug 2a, both of which were devoid of antiviral activity.

The presence of a 4′-fluorine substituent (compound 1b) reduced the cytostatic activity of gemcitabine, although no selective antiviral activity was observed. The 4′-fluorophosphoramidate prodrug 2b was less cytostatic (CC_{50} = 1.44 μM) than the parent nucleoside 1b and displayed minimal antiviral activity.

Interestingly, in this cellular test system, compounds 1b and 2b were 20 and 10 times more active than remdesivir, respectively.

In summary, the synthesis of 4′-methoxy- and 4′-fluorogemcitabine analogues and their phosphoramidate prodrugs and an evaluation of their antiviral activities against VZV, HCMV, and SARS-CoV-2 have been described. The introduction of a fluorine atom at the 4′-position led to a trifluorinated gemcitabine analogue that exhibited potent antiviral activity but no selectivity against these three viruses. A phosphoramidate prodrug of the 4′-fluorocongener displayed reduced activity against VZV but less pronounced cytotoxicity with an enhanced selectivity index. A similar although somewhat reduced effect was observed against HCMV. Unfortunately, this effect was not determined upon testing against SARS-CoV-2.
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HCMV, human cytomegalovirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VZV, varicella zoster virus

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Complete contact information is available at: https://pubs.acs.org/10.1021/acsmedchemlett.0c00485

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Z.Z. thanks the China Scholarship Council (CSC) for funding (Grant 201704910837). The authors are grateful to Mr. Brecht Dirix for excellent technical assistance in the antiviral assays.

ABBREVIATIONS

HCMV, human cytomegalovirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VZV, varicella zoster virus