Nucleotide Prodrugs

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Membrane-permeable Triphosphate Prodrugs of Nucleoside Analogues

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In memory of Chris McGuigan (1958–2016)

Abstract: *The metabolic conversion of nucleoside analogues into their triphosphates often proceeds insufficiently. Rate-limitations can be at the mono-, but also at the di- and triphosphorylation steps. We developed a nucleoside triphosphate (NTP) delivery system (TriPPPro-approach). In this approach, NTPs are masked by two bioreversible units at the γ-phosphate. Using a procedure involving H-phosphonate chemistry, a series of derivatives bearing approved, as well as potentially antivirally active, nucleoside analogues was synthesized. The enzyme-triggered delivery of NTPs was demonstrated by pig liver esterase, in human T-lymphocyte cell extracts and by a polymerase chain reaction using a prodrug of thymidine triphosphate. The TriPPPro-compounds of some HIV-inactive nucleoside analogues showed marked anti-HIV activity. For cellular uptake studies, a fluorescent TriPPPro-compound was prepared that delivered the triphosphorylated metabolite to intact CEM cells.*

For many decades, nucleoside analogues have been applied in anticancer and antiviral chemotherapy. They still comprise the frontline of drugs used to combat several virus infections nowadays.[1–4] However, nucleoside analogue drugs have to be metabolized in cells by virus-encoded or, in most cases, by host cell kinases to undergo stepwise addition of phosphate groups to yield the active nucleoside triphosphate analogue.[5,6] Owing to the substrate specificity of the kinases, the activation of nucleoside analogues often proceeds insufficiently. Furthermore, clinical efficacy is hampered by limitations such as low biological half-lives, variable bioavailability after oral administration, or the development of resistant virus strains.[7,8] Within the stepwise biotransformation process, in the case of the anti-HIV drug 3′-deoxy-2′,3′-didehydrothymidine (d4T, **1a**), the first phosphorylation step catalyzed by cytosolic thymidine kinase (TK) has been identified as the metabolism-limiting step.[9,10] However, in the metabolism of 3′-deoxy-3′-azidothymidine (AZT, **1b**), the formation of AZT-diphosphate (AZTDP, **2b**) by thymidylate kinase (TMP-K) is the bottleneck,[11,12] and recently, we showed that d4U- **2e** or ddU-diphosphate **2f** were very poor substrates for nucleoside diphosphate kinase (NDP-K), which showed that the conversion of NDP to NTP can be rate-limiting in the eventual activation of nucleoside analogues as well.[13] To overcome the phosphorylation bottleneck, the design of prodrugs of the monophosphorylated nucleoside analogue metabolite[14–20] was reported, but also a few reports on lipophilic delivery forms of nucleoside di- and triphosphates have been published.[21,22]

To bypass the second phosphorylation step, we reported the Di*PP*ro-approach.[13,23–25] These prodrugs showed very good antiviral activity in HIV-infected thymidine kinase-deficient human T-lymphocyte CEM/TK$^-$ cell cultures. Recently, we disclosed the first delivery of nucleoside triphosphates through a prodrug technology (Tri*PPP*ro-approach).[26] It was demonstrated, with d4T **1a** as a model nucleoside analogue, that the Tri*PPP*ro-derived compounds even retained pronounced anti-HIV activity in CEM/TK$^-$ cell cultures, whereas the parent **1a** was virtually inactive in these cells owing to the inherent lack of cytosolic thymidine kinase. The membrane permeability was achieved by two acceptor-substituted benzyl esters linked to the γ-phosphate group. The intracellular cleavage of these masks and the selective release of d4TTP **3a** from Tri*PPP*ro-compounds **4** is initiated by enzymatic hydrolysis of the phenolic acyl ester followed by spontaneous degradation (hydrolysis pathway; Supporting Information, Figure S1). The stability, hydrolysis, and antiviral activity were significantly influenced by the chain length of the prodrug moieties.[26]

In addition, the masking of nucleotides made these compounds less vulnerable to dephosphorylation by non-specific phosphatases present in the cell culture medium or in the blood.[10,27] In this way, we discovered a unique way to deliver the bioactive triphosphate of nucleosides into cells. In contrast to previous applications of nucleoside analogues, with this approach we are entirely independent of the intracellular phosphorylation process.

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Herein, we report on the use of the Tri*PPP*ro-approach to a variety of approved, as well as so-far non-active, nucleoside analogues to demonstrate the general applicability and potential of the approach (Figure 1). We disclose a robust synthesis approach for this class of compounds, biophysical properties of the Tri*PPP*ro-derivatives, a PCR assay, antiviral activity data, as well as a study to demonstrate the cellular uptake of the compounds and the intracellular delivery of the triphosphate metabolite using a fluorescent nucleoside.

The Tri*PPP*ro-compounds **4b**, **4c**, **4j**, and **4k** were synthesized using the phosphoramidite approach reported earlier.[26] According to this convergent strategy the TriPPPro-nucleotides were synthesized using a 4,5-dicyanoimidazol (DCI)-mediated coupling of the NDP **2** and a phosphoramidite (**5**)-bearing nonanoyloxybenzyl masking group within one minute. The Tri*PPP*ro-NTP derivatives of AZT **4b**, ddT **4c**, and ddBCNA 4j were obtained in yields of 17% to 71%, respectively.

However, the overall yield of the previous route is still limited by the varying yields obtained in the synthesis of NDP **2**. To achieve a more efficient conversion of the parent nucleoside to the Tri*PPP*ro-compounds **4**, an alternative route was developed. The approach is based on a coupling of a NMP **7** and *P*,*P*-bis-(4-nonanoyloxybenzyl)pyrophosphate **8** (Scheme 1). The advantage of this route is that NMPs are generally easier to prepare than NDPs.

First, hydrogen phosphonate **9** was prepared from diphenyl hydrogen phosphonate and 4-(hydroxymethyl)-phenyl-nonanoate **10**. Next, compound **9** was converted into the chlorophosphate using *N*-chlorosuccinimide (NCS)[28] followed by a phosphorylation with tetra-*n*-butylammonium phosphate. Despite its high chemical instability, the crude product was purified by extraction which led to compound **8** in almost quantitative yield.

Nucleoside monophosphates d4TMP **7a**, d4UMP **7e**, ddUMP **7f**, and ABCMP **7l** were synthesized according to a known procedure.[29] However, this method could not be used in the case of acid-labile purine derivatives, for example, ddG **1m**. Therefore, *cyclo*Sal-triesters **6** were used to prepare nucleoside monophosphates, such as ddGMP **7m** and ddIMP

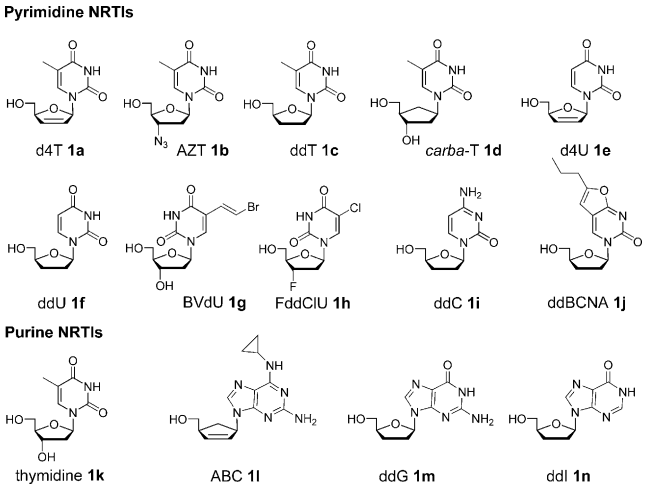
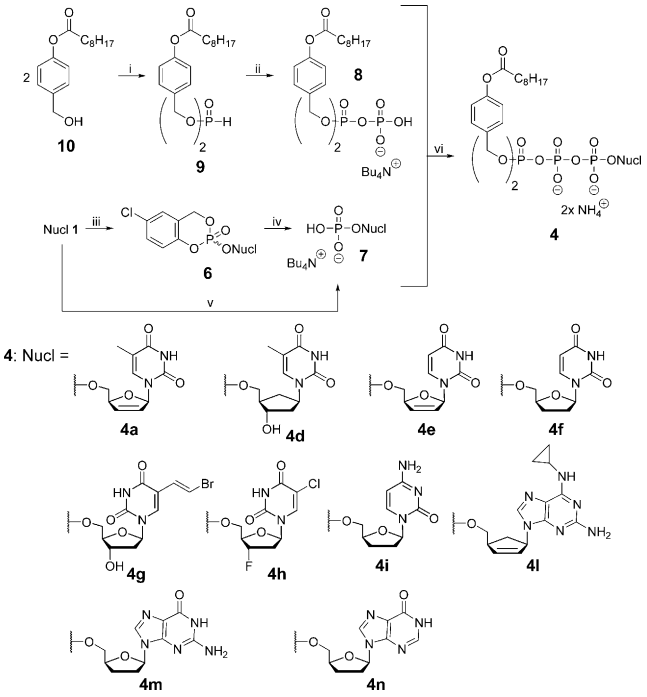


Figure 1. Chemical structures of known nucleoside antivirals as well as potential antiviral agents.



Scheme 1. Reagents and conditions for the synthesis of Tri*PPP*ro-prodrugs **4**. i) diphenyl hydrogen phosphonate, pyridine, 38°C, 2 h; ii) 1. NCS, CH_3CN, RT, 1 h, 2. $(H_2PO_4)Bu_4N$, CH_3CN, RT, 1 h; iii) 5-chlorosaligenylchlorophosphite, *N*,*N*-diisopropylethylamine, CH_3CN, 0°C–RT, 3 h, 2. A) oxone/water, B) *t*-BuOOH/*n*-decane, 0°C–RT, 20 min; iv) basic hydrolysis; v) See Ref. [29]; vi) 1. **8**, TFAA, Et_3N, CH_3CN, 0°C, 10 min, 2. 1-methylimidazole, Et_3N, CH_3CN, 0°C–RT, 10 min, 3. NMP **7**, RT, 1–3 h.

7n, by basic hydrolysis. The final coupling reaction was accomplished by modifying literature methods[30,31] by a stepwise activation of **8** with trifluoroacetic acid anhydride and *N*-methylimidazole, followed by addition of the NMP **7** to give Tri*PPP*ro-nucleotides **4** (non-optimized yields of 7–71%; Table S1). Using this synthetic pathway, the overall yield of the d4T-prodrug **4a** was improved from 15% (three steps)[26] to 25% (two steps), and this method proceeded much faster and gave reliable yields. Moreover, this method is more tolerable to the used solvents; even the addition of some drops of methanol for better solubility of ddGMP **7m** was tolerated. In some cases, such as ddCTP prodrug **7i**, the yield was lower due to difficulties during purification of the prodrug.

The hydrolysis properties of Tri*PPP*ro-compounds **4** were evaluated in different media. The chemical stability was determined in phosphate buffer (PBS, 25 mM, pH 7.3), and the hydrolysis samples were analyzed by means of analytical RP18-HPLC. The calculated half-lives (Table S2) were determined for the cleavage of the first masking unit ($t_{1/2}(1)$) to yield the mono-masked intermediate, and the second hydrolysis step ($t_{1/2}(2)$) corresponds to the formation of the triphosphate (Figure S1). All of the prodrugs were hydrolyzed from the single masked intermediate **11** and then released NTPs **3**, which were identified by HPLC and mass spectrometry. However, for purine derivatives, some non-identified side reactions resulted in lower half-lives. The half-lives of the

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mono-masked intermediates **11** were much higher than those of their precursors **4**. Finally, intermediates **11** delivered the NTPs **3** selectively.

Significant enzymatic cleavage of the masking units was observed during incubations with pig liver esterase (PLE) in phosphate buffer (pH 7.3). The removal of both prodrug groups occurred much faster compared to the chemical hydrolysis. Exceptions were prodrugs **4l** and **4m**, which did not serve as substrates for the enzyme. Because the masking unit was identical in all cases, this difference must be caused by the nucleoside residue. However, as long as the enzymatic cleavage occurred rapidly to yield intermediates **11**, NTPs **3** were formed selectively (Table S2).

To confirm the formation of a biologically active triphosphate metabolite, three thymine-bearing Tri*PPP*ro-compounds **4** were exposed to PLE. After complete consumption of Tri*PPP*ro-prodrugs **4a** (d4T), **4d** (*carba*-T), and **4k** (thymidine),[27] and the corresponding intermediates **11**, the solvents were removed. The products were used in a polymerase chain reaction using FIREPol DNA-polymerase (Figure 2). No amplification occurred from the hydrolysates of compounds **4a** and **4d**. In contrast, formed TTP **3k** from prodrug **4k** led to an amplification of the template.

The incubation of Tri*PPP*ro-NTP prodrugs with human $CD4^+$ T-lymphocyte cell extracts led to a marked acceleration of the NTP formation. The half-lives of prodrugs **4** were in the range of 1–2.5 h independent of the attached nucleoside (Table S2). In addition to intermediates **11** (except for compound **4f**), the corresponding NTPs **3** were detected. However, we often observed NDPs **2** as additional products, which was most likely the result of degradation of NTP **3** by phosphorylases/kinases from the cell extracts.

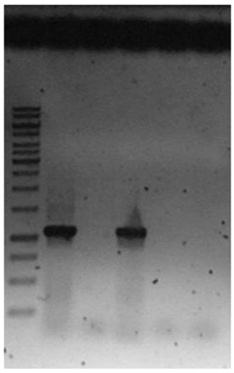

Figure 2. PCR by FIREPol DNA polymerase. Lanes: 1) PCR marker. 2) PCR: dATP, dCTP, dGTP, and TTP. 3) Control reaction conducted with: dATP, dCTP, and dGTP. 4) dATP, dCTP, dGTP, and the PLE-hydrolysate of Tri*PPP*ro-compound **4k**.[26] 5) dATP, dCTP, dGTP, and the PLE-hydrolysate of **4a**. 6) dATP, dCTP, dGTP, and the PLE-hydrolysate of **4d**.

The effectiveness of the Tri*PPP*ro-compounds **4** to act as inhibitors against HIV-1 and HIV-2 was determined in HIV-infected wild-type- (CEM/0) and thymidine-kinase-deficient (CEM/TK^-) cell cultures. The inhibition of the replication of HIV-1 and HIV-2 by prodrugs **4** was much higher, or at least similar, compared to their parent nucleosides (Table S3). The retention of the antiviral activity in the TK-deficient cells strongly indicates cellular uptake of the Tri*PPP*ro-compounds bearing thymidine analogues. In particular, the high potential of the Tri*PPP*ro concept was demonstrated by the prodrugs of *carba*-TTP **4d** and ABCTP **4l** with 15–18-fold higher antiviral activity against HIV-1 compared to their parent nucleosides (Table 1). In addition, the completely inactive FddClU **1h** was converted into a highly potent compound (Tri*PPP*ro-compound **4h**). Surprisingly,

Table 1: Antiviral activity and cytotoxicity of Tri*PPP*ro-nucleoside triphosphates **4** in comparison to their parent nucleosides **1**.

	EC_{50} [μM][a]			CC_{50} [μM][b]
	CEM/0		CEM/TK^-	CEM/0
Comp.	HIV-1	HIV-2	HIV-2	
carba-T **1d**	36 ± 2	12 ± 3.5	8.0 ± 1.7	> 250
4d	2.4 ± 1.5	2.7 ± 0.57	2.8 ± 0.8	> 10
FddClU **1h**	> 250	> 250	> 250	188 ± 88
4h	3.4 ± 2.5	> 10	> 10	42 ± 7
ddC **1i**	0.07 ± 0.03	0.13 ± 0.02	0.10 ± 0.02	2.6 ± 0.2
4i	0.11 ± 0.01	0.19 ± 0.02	0.15 ± 0.04	3.7 ± 0.6
ABC **1l**	18 ± 4.1	34 ± 3.3	14 ± 1.1	119 ± 7
4l	0.99 ± 0.23	5.3 ± 2.0	2.1 ± 1.2	26 ± 2
ddI **1n**	16 ± 3.1	30 ± 12	9.6 ± 7.6	> 250
4n	2.7 ± 0.78	6.3 ± 1.3	2.8 ± 1.1	56 ± 10

[a] Antiviral activity in $CD4^+$ T-lymphocytes: 50% effective concentration; values are the mean ± SD of $n = 2$–3 independent experiments. [b] Cytotoxicity: 50% cytostatic concentration or compound concentration required to inhibit $CD4^+$ T-cell (CEM) proliferation by 50%; values are the mean ± SD of $n = 2$–3 independent experiments.

the Tri*PPP*ro-compounds of d4U **4e** and ddU **4f** showed no significant antiviral activity. Incubations of ddUTP **3f** in cell extracts showed an extremely fast dephosphorylation ($t_{1/2} <$ 1 min) of the triphosphate to yield ddUDP and later ddUMP. Therefore, we speculate that NTPs **3e**,**f** (although released from Tri*PPP*ro-compounds **4e**,**f** as proven by hydrolysis in PLE) were not retained in sufficient concentrations in cells to exhibit antiviral activity. As a result, it can be concluded from the data obtained here that the uridine analogues d4U **1e** and ddU **1f** seem to be inappropriate for antiviral therapy, although the triphosphates were found to be very potent inhibitors against isolated RT. In contrast, other nucleoside analogues were converted into potent inhibitors against HIV by application of the Tri*PPP*ro approach. Interestingly, ddITP **3n** and ABCTP **3l**, released from **4n** and **4l**, are to be considered as novel metabolites that are usually not formed by the corresponding parent nucleoside analogues ddI **1n** and ABC **1l**. Indeed, the eventual triphosphate metabolite intracellularly formed from ddI **1n** is ddATP (not ddITP **3n**, owing to prior metabolic amination of ddIMP **7n** to ddAMP), and from ABC **1l** is carbovir-TP (not ABCTP **3l**, owing to prior deamination of ABCMP **7l** to carbovir-MP).[32,33] Thus, the Tri*PPP*ro technology also enables delivery and discovery of antiviral compounds that otherwise cannot be formed by the parent (or any other) nucleoside analogue.

To study the cell uptake of Tri*PPP*ro-compounds **4**, and to confirm again that Tri*PPP*ro-compounds intracellularly delivered the NTP metabolite, the fluorescent 2′,3′-dideoxy-bicyclic nucleoside analogue triphosphate (ddBCNATP)-prodrug **4j** was incubated in T-lymphocytes for 60 min or 180 min at 37 °C, respectively. After incubation, the cells were washed, centrifuged, and ultrasonicated. The fluorescent ddBCNA **1j** was used because the analysis of the cell extract samples could be performed by fluorescence detection on the HPLC, which greatly increased the sensitivity and avoided concomitant detection of any interfering cellular components. This study confirmed that prodrug **4j** penetrated the cell membrane and delivered ddBCNATP **3j** intracellularly within 1 h as the main metabolite present in the extracts

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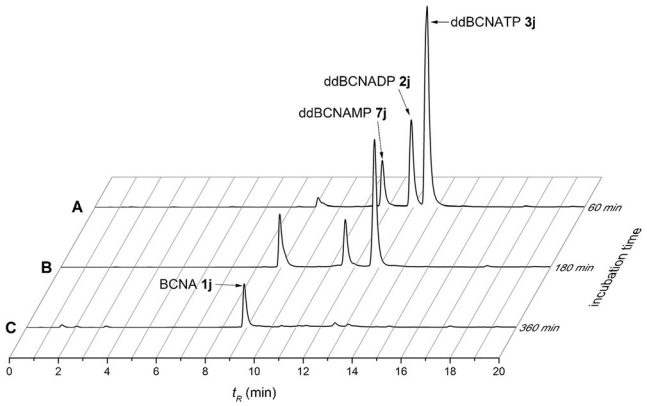

Figure 3. Incubation of Tri*PPP*ro-ddBCNATP prodrug **4j** (A + B) and the parent ddBCNA **1j** (C) in CEM/O cells.

(Figure 3). As expected, ddBCNATP **3j** was also dephosphorylated by cellular hydrolases. After 180 min, no triphosphate **3j** was detected in the extracts but high amounts of ddBCNADP **2j** and small amounts of ddBCNAMP **7j** and ddBCNA **1j** were detected instead. This dephosphorylation process was also detected in hydrolysis studies in CEM cell extracts. As a control, studies with ddBCNA **1j** revealed that the nucleoside was taken-up by cells in relatively small amounts, and it was not phosphorylated to yield one of the three metabolites. Therefore, ddBCNATP **3j** observed in the former experiment was formed by hydrolysis of the prodrug **4j**.

In summary, a series of NTP-prodrugs **4** bearing different nucleoside analogues has been synthesized proving the general applicability of the Tri*PPP*ro-approach. Furthermore, we confirmed the successful formation of the bioactive triphosphate inside the cell through a cell uptake study. The results reported here clearly show that the Tri*PPP*ro-approach allowed the intracellular delivery of nucleoside triphosphates, and the approach could be used to convert inactive nucleoside analogues into powerful biologically active metabolites. Thus, this strategy offers high potential to be used in antiviral and antitumor therapies. In addition, this approach might be also suitable to address chemical biology questions by delivering triphosphorylated nucleoside probes into living cells. Work along this line is currently underway in our laboratories.

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