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Triphosphate and diphosphate nucleoside prodrugs: broadening the therapeutic potential of nucleoside-based treatments

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Multiple prodrug strategies have been developed to deliver nucleoside monophosphates (NMPs) and thereby overcome the initial kinase-mediated intracellular phosphorylation of nucleoside analogues (NAs), a major bottleneck in their activation. However, some NAs are limited by a second and/or third slow and inefficient phosphorylation step, which can lead to accumulation of mono- and diphosphate intermediates with associated toxicity. In addition, the metabolic pathways leading to the formation of the active nucleoside triphosphates remain poorly characterised for many reported analogues. Consequently, di- and triphosphate prodrug strategies for NAs have gained increasing attention in recent years. Here, we provide a comprehensive overview of the current approaches for development of these higher phosphorylated nucleotide analogues, including their activation pathways in different media, biological activity, synthetic methodologies, and their applicability across a broad range of NAs. Taken together, this review highlights both the potential of NDP and NTP prodrug strategies and the challenges associated with their development, providing a framework to guide future advances in their rational design and broader application.

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1. Introduction

Prodrug is a strategy by which a pharmacologically active molecule is masked with one or more cleavable groups (promoiety) that under specific conditions in the human body is cleaved, releasing the active species. Prodrug design aims to optimize the physicochemical and pharmacological properties of drugs.¹ A key class of biological substrates subjected to prodrug approaches is nucleoside analogues (NAs), which resemble naturally occurring nucleosides and are an important class of antiviral and anticancer drugs,² with some also showing antibacterial and anti-biofilm activity.³ Many NAs require intracellular phosphorylation to the corresponding nucleoside triphosphate (NTP), *via* mono- (NMP) and diphosphate (NDP) intermediates, to exert their biological activity. However, this activation pathway is not universal, and some NAs act through alternative mechanisms.

NTPs act by interfering with viral or human enzymes as competitive inhibitors of their natural substrates or by being incorporated into newly synthesized viral DNA and RNA strands. Unfortunately, NAs suffer from many drawbacks such as poor cellular uptake because of insufficient expression of membrane transporters, premature breakdown, and slow conversion to the triphosphate form due to rate-limiting phosphorylation steps. To overcome these limitations, the design of NA prodrugs carrying either one masked phosphate group or an isosteric and isoelectronic phosphonate moiety has been investigated and extensively reported.⁴ Among these prodrug strategies, the ProTide approach⁵ (phosphate masked with an amino acid ester and an aryl group) proved the most successful, leading to three FDA-approved antiviral medicines^{6–8} and several clinical candidates in the oncology setting.^{9,10} However, NMP still requires two additional intracellular phosphorylation steps to reach the active NTP responsible for therapeutic activity. As not only the first phosphorylation but sometimes the second and third phosphorylations^{11–16} can be inefficient and rate-limiting, recent efforts have increasingly focused on the development of di- and triphosphate prodrugs, which aim to

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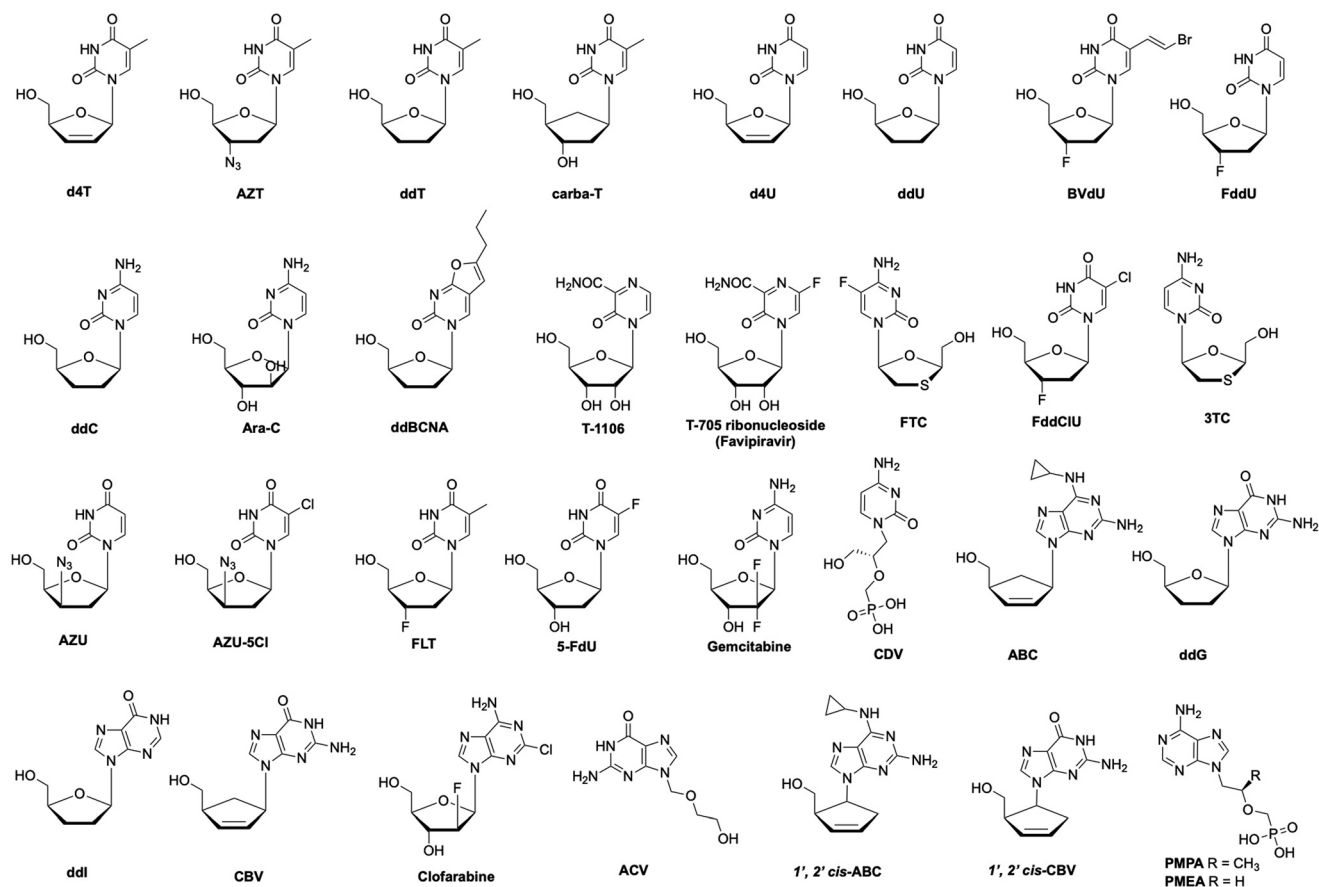


Fig. 1 Structures of NAs included in this review.

bypass additional phosphorylation steps, thereby enhancing cellular activation and improving efficacy.^{17–20} This review summarizes the recent advances in prodrug strategies for the direct delivery of di- and triphosphate NAs as potential drug candidates, including their activation mechanisms in different media, biological activities, and synthetic approaches. Fig. 1 shows the structures of all NAs discussed in this review for which NDP and NTP prodrug approaches have been reported.

2. Nucleoside diphosphate prodrugs with two bio-reversible masking groups

The design of the early examples of NDP prodrugs was inspired by natural liponucleotides, in which a nucleotide is covalently linked to a lipophilic moiety, typically a lipid such as diacylglycerol, *via* a phosphate bond. These endogenous liponucleotides play essential roles in phospholipid biosynthesis,²¹ participating in the *de novo* formation of several major classes of glycerophospholipids. Their properties motivated the development of synthetic liponucleotide prodrugs (Fig. 2A) to address limitations of conventional NAs, such as poor uptake, instability, and low bioavailability. Although not

originally designed to deliver NDPs, these systems provided a foundation for improving intracellular delivery of NAs. Related lipid-based strategies include also alkylalkoxyalkyl phosphates of acyclic nucleoside phosphonates (ANPs; Fig. 2B), which are a class of broad-spectrum antivirals developed in the 1980s, containing a stable P–C bond that resists enzymatic cleavage.²²

More recent efforts have focused on prodrugs that directly deliver NDPs. These investigations led to the development of an enzymatically triggered pronucleotide system incorporating two masking groups at the β -phosphate, a strategy that was later termed the diphosphate pronucleotide (DiPPro) approach (Fig. 2C and D).¹⁷

2.1. Liponucleotide prodrugs

The first liponucleotide prodrug example was reported in 1978, when researchers at Argonne National Laboratory synthesized AraC-DP-DL-dipalmitin, a phospholipid derivative of 1- β -D-arabinofuranosyl cytosine (AraC) (Fig. 2A).²³ This work was based on earlier findings demonstrating that lipophilic ester derivatives of AraC exhibited enhanced anticancer activity²⁴ and AraC-diphosphate (DP) dipalmitin resembled a natural ribonucleotide metabolite. Intrigued by these findings,²⁵ the Argonne group prepared the pure diastereoisomer Ara-CDP-L-dipalmitin for its evaluation on L1210 cell line (mouse-derived

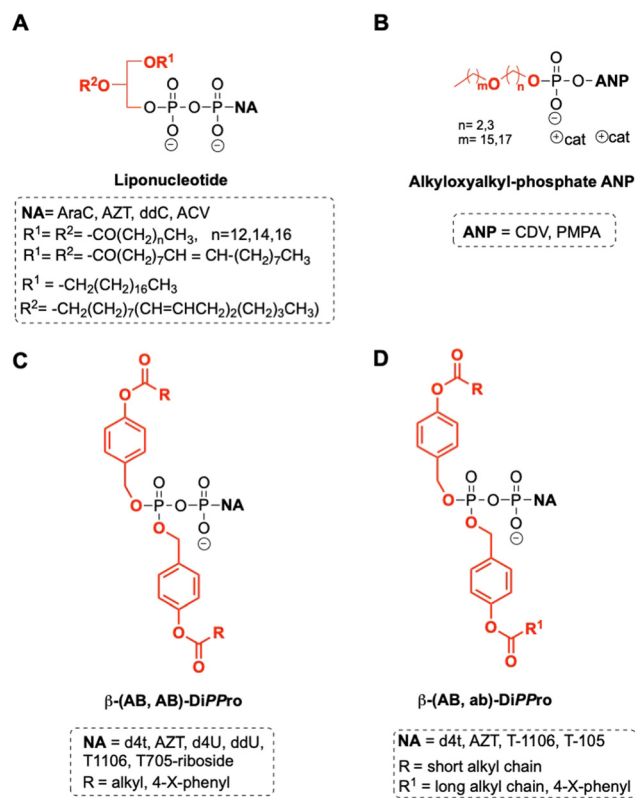


Fig. 2 General structure of a synthetic liponucleotide (A), alkoxyalkyl phosphate of ANP (B), symmetric (C) and non-symmetric-DiPPro (D).

lymphocytic leukaemia). Due to poor solubility, a sonication-based method was developed to form micellar systems, enabling *in vitro* testing. The prodrug showed moderate activity (50% growth inhibition at 0.062 μ M after 50 h) against MPC-11 mouse myeloma cell line but was less potent than AraC, likely due to slow drug release from micelles. No activation mechanism or specific released species was identified. A subsequent study²⁶ evaluated Ara-CDP-L-dipalmitin and other AraC liponucleotides for antitumor activity in animal models. Notably, the Ara-CDP-diacylglycerol in which the diacyl species (R^1 and R^2) are a mixed multispecies (as the acyl chains found in egg lecithin) exhibited greater *in vivo* antitumor efficacy than the parent nucleoside AraC when tested in mouse models of leukaemia (L5178Y) and colon carcinoma (HCT-15). The authors suggested that liponucleotides could be converted enzymatically to NMP and phosphatidylinositol (PI) *in vivo* (Fig. 3A). To further explore this class, a series of liponucleotide derivatives (*e.g.*, Ara CDP L distearin, dimyristin, and diolein) were evaluated to assess the impact of fatty acid side-chain modifications.²⁷ Preliminary studies showed that antiproliferative activity against MPC-11 was strongly dependent on side-chain structure, with fully saturated fatty acids exhibiting reduced potency as chain length increased. All derivatives demonstrated greater efficacy than the parent nucleoside *in vivo*, although increased toxicity was observed, particularly for the dimyristin analogue.

Subsequently, researchers developed liponucleotide derivatives of dideoxynucleosides such as 3'-azido-3'-deoxythymidine (AZT),

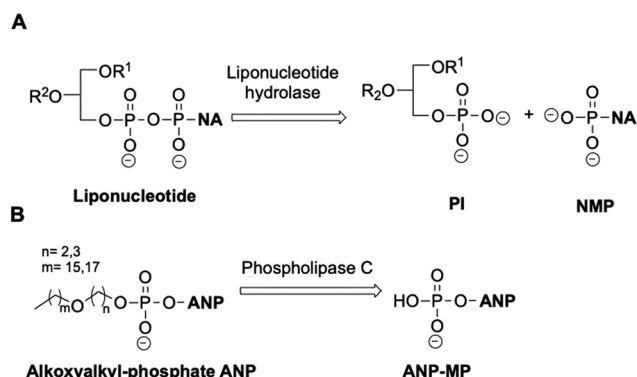
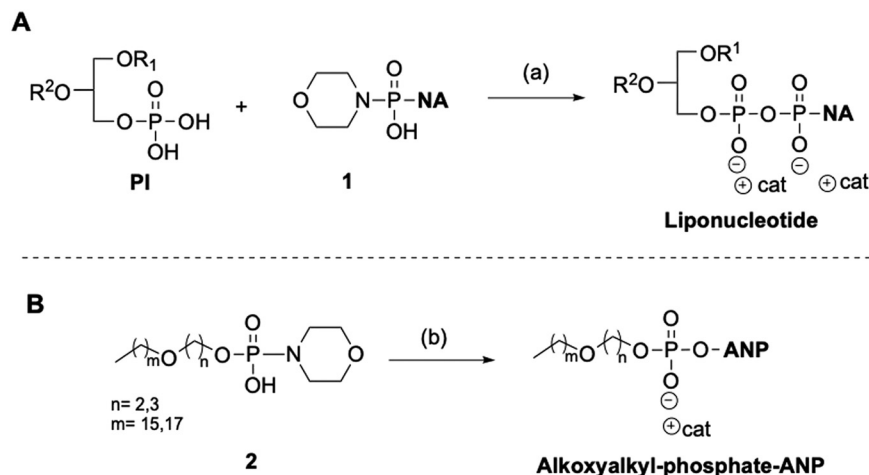


Fig. 3 Enzymatic activation pathway of liponucleotides (A); suggested activation pathway for alkoxy-phosphate of ANPs (B).

2',3'-dideoxythymidine (ddT), and 2',3'-dideoxycytidine (ddC) to improve the delivery of these antiretroviral NAs to macrophages, the major reservoir of HIV in the human body.^{28,29} This approach aimed at overcoming resistance to dideoxynucleosides in HIV strains with low nucleoside kinase activity required for drug activation. These synthetic liponucleotides, formulated as liposomes, inhibit the HIV antigen p24 in infected U937 ($IC_{50} < 0.7 \mu$ M) and wild-type CEM (CEM/0) cell lines ($IC_{50} = 1.7\text{--}22 \mu$ M). Further studies showed that treatment of these prodrugs with rat liver mitochondrial enzymes led to the release of free nucleosides and NMPs, likely *via* pyrophosphate hydrolase activity, generating PI and NMP as previously suggested (Fig. 3A).³⁰

To restore the effectiveness of acyclovir (ACV) against thymidine kinase deficient (TK⁻) herpes virus (HSV) mutants, a dimyristoylglycerol diphosphate prodrug of ACV was developed.³¹ This liponucleotide exhibited strong antiviral activity in WI-38 human lung fibroblasts infected with the ACV-resistant TK⁻ HSV DM21 strain ($IC_{50} = 0.25 \mu$ M), whereas ACV and its monophosphate were inactive ($IC_{50} > 100 \mu$ M). Comparable activity was observed in both TK-altered and TK⁻ strains of HSV-1 and in ACV-resistant isolates of HSV-2 obtained from two AIDS patients. The phospholipid prodrug operates through TK-independent pathways, producing a 56-fold increase in intracellular ACV-MP levels relative to ACV alone. In contrast, ACV-MP did not overcome resistance, likely due to extracellular dephosphorylation and poor permeability.

To improve delivery of ANPs, several prodrug strategies have been developed, leading to FDA-approved drugs, such as bis-POM and bis-POC derivatives of adefovir (PMEA) and tenofovir (PMPA)³² as well as tenofovir alafenamide (TAF), a phosphonamidate prodrug approved for HIV and hepatitis B.³³ In parallel, Hostetler and co-workers developed alkoxyester prodrugs of ANPs, leading to brincidofovir (CMX001), a hexadecyloxypropyl (HDP) prodrug of CDV, which was approved in 2021 against smallpox.³⁴ As the first phosphorylation of CDV is rate-limiting, alkoxyalkyl phosphate conjugates of CDV and adefovir (Fig. 2B) were also explored, including HDP-monophosphate and octadecyloxyethyl (ODE) monophosphate derivatives.³⁵ These compounds showed enhanced antiviral activity, with HDP-MP-CDV and ODE-MP-CDV being 4.6–40



Scheme 1 General synthesis of liponucleotide (A) and alkoxyalkyl phosphate conjugate prodrugs of ANPs (B). Reagents and conditions: (a) pyridine, rt; (b) ANP, pyridine, cat. acetic acid.

times more active against HCMV and 7–30 times more active against cowpox and vaccinia viruses, although generally less potent than the corresponding alkoxyesters. Although no activation studies were carried out, the authors suggested that these prodrugs can be activated *via* alkoxyester hydrolysis operated by phospholipase C (Fig. 3B).

Synthesis. Liponucleotide prodrugs are typically synthesised by activating the nucleoside 5'-monophosphate as a phosphomorpholidate (1), followed by displacement with L- α -phosphatidic acid (PI) (Scheme 1A).^{23,27,28} The nucleoside phosphonate-phosphate conjugates were prepared instead by coupling the alkoxyalkyl-phosphomorpholidates (2) to the ANPs (Scheme 1B).³⁵

2.2. Diphosphate pronucleotide prodrugs (DiPPro)

Among the various NA monophosphate prodrug strategies, Meier's group developed the *cycloSal* technology,³⁶ in which the phosphate group is masked with a cyclic salicylic alcohol, which intracellularly undergoes pH-driven chemical activation. Building on this concept, they applied the *cycloSal* approach to AZT-DP.³⁷ However, incubation of the *cycloSal* AZT-DP prodrug in PBS at pH 7.3 predominantly produced AZT-MP with only trace amounts of AZT-DP. This outcome resulted from undesired pyrophosphate cleavage *via* nucleophilic attack at the β -phosphate, demonstrating that *cycloSal*-NDPs are ineffective for delivering intact NDPs. This prompted Meier's group to devise another strategy, named "DiPPro"¹⁷ which employs two identical (symmetric) or different (non-symmetrical) bis(acyloxybenzyl) (AB) masking groups on the β -phosphate of an NDP (Fig. 2C and D).

2.2.1. Symmetric DiPPro. In 2008, Meier *et al.* reported the first examples of DiPPro (β -(AB,AB)-NDP), in which the β -phosphate of either 2',3'-dideoxy-2',3'-dideoxythymidine diphosphate (d4T-DP) or AZT-DP was masked with two identical AB groups.³⁸ The α -phosphate was left unmasked because a negative charge in this position is needed to

stabilize the pyrophosphate bond. The general structure of this class of prodrug is reported in Fig. 2C. The acyl group (R) can be a linear or branched alkyl chain or a *para*-substituted phenyl group. These compounds were the first NDP prodrugs showing high chemical stability ($t_{1/2}$ = 10–82 h in PBS at pH 7.3) while undergoing rapid and highly selective enzymatic cleavage in cell extracts ($t_{1/2}$ = 0.02–7 h) to release the corresponding NDPs. Intracellular release of NDP is driven chemically or by esterase-mediated cleavage of the acyl ester bonds on the DiPPro masking groups. This initial hydrolysis transforms the ester substituents into electron-donating hydroxyl groups, triggering a spontaneous cascade of β -elimination to first release the mono-masked intermediate (β -(AB)-DiPPro), followed by the corresponding NDP (Fig. 4A). When tested for their ability to inhibit the replication of HIV in T-lymphocyte cells, all d4T-DiPPro showed antiviral activity with EC₅₀ in the range of 0.5–1 μ M in HIV-1 and 0.3–1.5 μ M for HIV-2 infected CEM/0, comparable to d4T (HIV-1 EC₅₀ = 0.4 μ M and HIV-2 EC₅₀ = 0.3 μ M), without displaying marked cytotoxicity (CC₅₀ >36 μ M). However, similarly to the parent nucleoside (HIV-2 EC₅₀ = 173 μ M), short or branched acyl substituents (R) failed to show antiviral activity in CEM/TK⁻ cells. As a plausible explanation, the authors suggested that when the alkyl chain (R) is short, the residual polarity arising from the negatively charged α -phosphate remains too high, preventing efficient cellular uptake, and it is thus degraded to the parent nucleoside before entering the cell.

To address this limitation and to increase the chemical stability but still ensuring enzymatic cleavage, longer acyl chains were investigated.³⁹ As expected, the chemical stability of the long-chain d4T-DiPPro prodrugs increased with the length of the acyl chains (R = C1 $t_{1/2}$ = 10 h; R = C15 $t_{1/2}$ = 258 h) in PBS (pH 7.3). In all cases, prodrugs bearing linear alkyl residues produced substantially more d4T-DP than d4T-MP. Cleavage was then examined in CEM cell extracts, where a predominant formation of d4T-DP was again observed. The

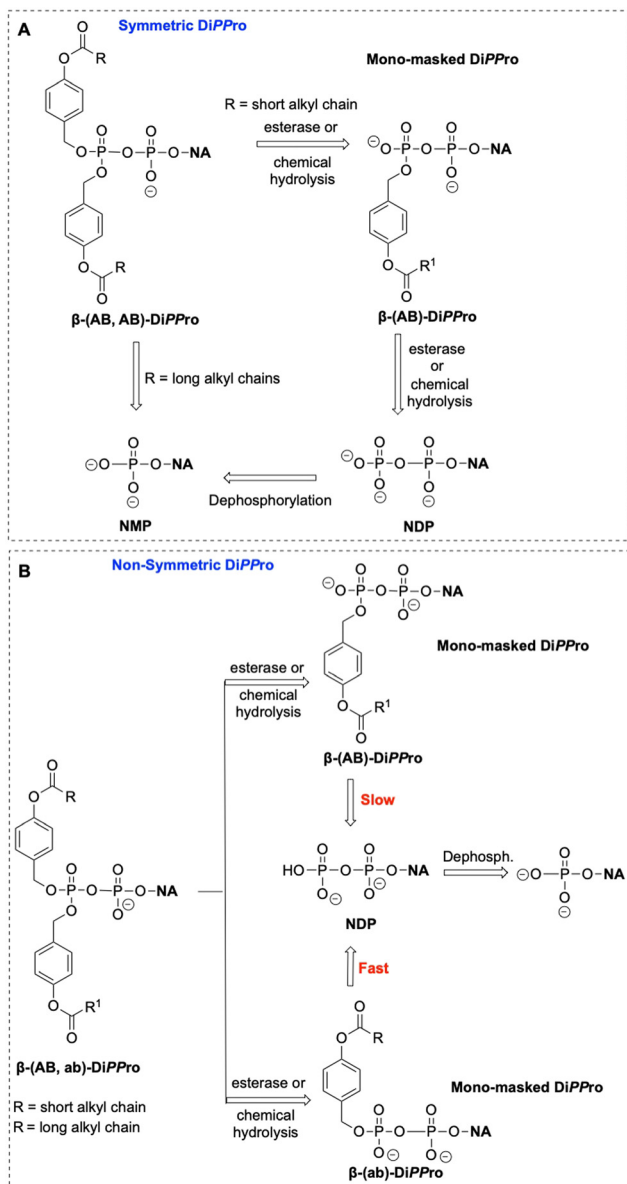


Fig. 4 Enzymatic activation of $\beta\text{-(AB,AB)-DiPPro}$ (A) and $\beta\text{-(AB,ab)-DiPPro}$ (B).

ratio of d4T-DP/d4T-MP correlated with the stability of the compounds ($R = \text{C1}$, $t_{1/2} = 0.05$ h; $R = \text{C15}$, $t_{1/2} = 21$ h): the longer the half-life for removal of the first AB masking group, the greater the proportion of d4T-MP formed (Fig. 4A). The best ratio d4T-DP/d4T-MP was obtained with $R = \text{C6}$. These studies demonstrated that increasing the chemical stability of the first cleavage step, by introducing branching in the acyl moiety, was detrimental, as rapid removal of the masking groups is required in biological media; otherwise, pyrophosphate hydrolysis leading to NMP becomes a significant competing pathway (Fig. 4A). Interestingly, the mono-masked intermediates $\beta\text{-(AB)-DiPPro}$ did not release d4T-MP at all but exclusively d4T-DP. The appearance of an additional negative charge at the β -phosphate after the first deprotection evidently suppresses nucleophilic attack at this

position. In *in vitro* anti-HIV assays, all prodrugs showed antiviral activity against HIV-1 and HIV-2 infected CEM/0 cells, comparable to the parent nucleoside. Only compounds with acyl residues $R > \text{C6}$ displayed high activity in CEM/TK⁻ cells (HIV-2 $\text{EC}_{50} = 0.11\text{--}2.6$ μM), reiterating the importance of increased lipophilicity for cellular uptake, as previously observed in the initial studies of d4T-DiPPro analogues. No significant cytotoxicity was observed, even with these prodrugs.

This DiPPro strategy was subsequently extended to 2',3'-dideoxyuridine (ddU) and 2',3'-dideoxy-2',3'-didehydrouridine (d4U), which despite their structural similarity to AZT and d4T are inactive against HIV.⁴⁰ As their corresponding triphosphate forms inhibit HIV reverse transcriptase, the DiPPro approach was expected to bypass inefficient phosphorylation. Hydrolysis studies in PBS and CEM cell extracts showed comparable stability to d4T- and AZT-DiPPro analogues and confirmed NDP release. However, ddU- and d4U-DiPPro compounds showed little antiviral activity against HIV in CEM/0 cells. Nucleoside diphosphate kinase (NDPK) studies revealed the cause: unlike UDP, neither d4U-DP nor ddU-DP was phosphorylated, preventing formation of the active triphosphates.

When the symmetric DiPPro approach was applied to the ribonucleoside T-1106 (derived from the pyrazinecarboxamide nucleobase T-1105), a hydrolysis pathway similar to that of d4T-DiPPro was observed in PBS and Madin-Darby canine kidney (MDCK) cell extracts (Fig. 4A).³⁷ This involved the formation of the mono-masked T-1106-DP intermediate ($\beta\text{-(AB)-DiPPro}$), followed by the corresponding T1106-DP and minor T1106-MP. T1106-MP arises from dephosphorylation of the NDP and hydrolysis of the prodrug anhydride bond. Biologically, the T-1106-DiPPro prodrug showed markedly improved anti-influenza activity, with up to 44-fold higher potency than the parent nucleoside and ~ 5 -fold higher activity than the nucleobase. Importantly, it retained activity in 6-thioguanine-resistant (TG^{res}) cells lacking functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The HGPRT enzyme normally converts the nucleobase T-1105 into the ribonucleoside monophosphate (T-1106-MP). These results indicate that the prodrug bypasses HGPRT- and kinase-dependent activation, whereas the parent nucleoside is significantly less active and the nucleobase is inactive.

In comparison to alkanoyl analogues ($R = \text{alkyl}$), benzoyloxybenzyl ($R = \text{Ph}$) masking groups enable fine-tuning of DiPPro hydrolysis *via* substitution on the benzoyl ring without significantly impacting lipophilicity. Based on this rationale, a series of DiPPro of AZT and d4T, bearing two identical lipophilic benzoyloxybenzyl moieties substituted at the 4-position of the benzoyl moiety with different donor and acceptor groups, was investigated.⁴¹ Chemical hydrolysis studies in PBS demonstrated that most compounds were converted into the corresponding NDP. This was also observed in CEM cell extracts, although prodrug stability was reduced ($t_{1/2} = 0.5\text{--}9$ h) compared to PBS ($t_{1/2} = 4\text{--}480$ h). Stability and product distribution were strongly substituent-dependent: electron-

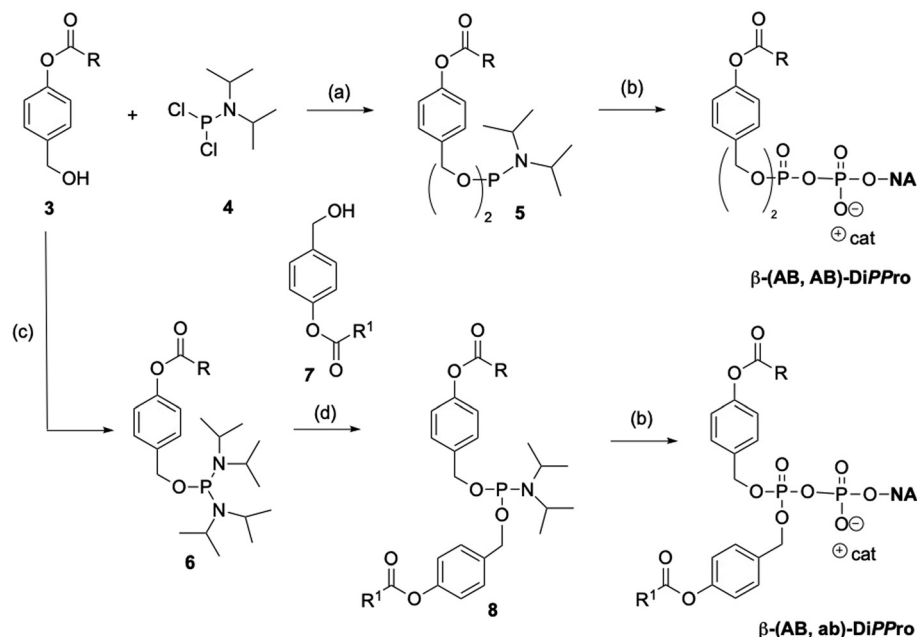
donating groups (e.g. $X = -OCH_3$) increased stability ($t_{1/2} = 480$ h; CEM extract $t_{1/2} = 9$ h) and favoured NMP formation, whereas electron-withdrawing groups (e.g. $X = -CF_3$, $-CN$, $-NO_2$) decreased stability ($t_{1/2} = 4.5$ h; CEM extract $t_{1/2} = 1.5$ h) and promoted selective NDP release. All these DiPPro compounds exhibited antiviral activity against HIV-1 and HIV-2 in CEM/0 cells in the same range as the parent nucleoside, with the electron donor substituted-benzoyl DiPPro derivatives showing up to a 60-fold increase in activity against HIV-2 in CEM/TK⁻ cells (e.g. NA = d4T $X = Cl$, $EC_{50} = 2.9$ μM) compared with the parent nucleoside (d4T $EC_{50} = 173$ μM). In contrast, most electron acceptor-substituted DiPPro analogues displayed a marked loss of antiviral activity in the CEM/TK⁻ cell line likely due to their rapid hydrolysis, limiting cellular uptake. The most active prodrugs exhibited slightly increased cytotoxicity ($CC_{50} = 36$ – 51 μM) relative to the parent nucleosides ($CC_{50} > 250$ μM).

2.2.2. Non-symmetric DiPPro. Symmetric DiPPro compounds exhibit an undesired tendency to generate the corresponding nucleoside monophosphate *via* nucleophilic attack of water at the β -phosphorus. This side reaction becomes more pronounced as the stability of the ester linkage increases ($R =$ long chain), since formation of the mono-masked intermediate is slowed. In contrast, with less stable ester linkages ($R =$ short chain), rapid conversion to the mono-masked intermediate suppresses NMP formation. However, these compounds are inactive in TK⁻ cells due to excessively rapid cleavage and insufficient cellular uptake. These limitations prompted the development of a second generation of non-symmetric DiPPro prodrugs ($R^1 \neq R^2$) (β -(AB,ab)-DiPPro, Fig. 2D), designed to better control activation and lipophilicity. In this approach, one short chain (AB) is intended to enable rapid chemical or enzymatic cleavage, while a long-chain or substituted aromatic ester (ab) serves to enhance lipophilicity and promotes cellular uptake. However, when the non-symmetric DiPPro derivatives of AZT and d4T were evaluated for chemical stability in PBS, both masking groups were unexpectedly cleaved at similar rates (Fig. 4B).⁴² Moreover, the non-symmetric DiPPro compounds exhibited substantially longer half-lives ($t_{1/2} = 15$ – 63 h) than their symmetric counterparts. The same alkyl ester ($R = CH_3$) that was rapidly cleaved in the symmetric system ($t_{1/2} = 10$ h), hydrolyses much more slowly in the non-symmetric analogue ($t_{1/2} = 40$ h). The authors attributed this increased stability to steric hindrance or aggregation effects arising from the second more lipophilic masking group. In contrast, once the mono-masked intermediate was formed, a clear difference in hydrolysis rates emerged: short-chain acyl esters were cleaved significantly faster than the long-chain alkyl or aromatic moieties. As seen before for the symmetric DiPPro, some NMP was formed probably because of their unexpected high hydrolytic stability. However, the NDP/NMP ratio was in general higher when compared to the symmetric DiPPro. Enzymatic hydrolysis studies with pig liver esterase (PLE) showed that non-symmetric DiPPro prodrugs rapidly produced the corresponding NDPs within minutes, while NDPs formed over 2–7 h alongside some NMP in cell extracts.

Most non-symmetric d4T-DiPPro derivatives displayed equal or superior antiviral activity relative to their parent nucleosides in CEM/0 cells. In addition, the lipophilicity-dependent activity of the d4T-DiPPro series translated into high potency against HIV-2 in mutant CEM/TK⁻ cell. However, d4T-DiPPro bearing benzoyl ester functions ($R = 4-X-Ph$) in the masking groups proved to be less potent (HIV-1 $EC_{50} = 0.3$ – 0.4 μM ; HIV-2 $EC_{50} = 0.72$ – 1.15 μM) as compared to the alkanoyl ($R =$ alkyl) counterparts (HIV-1 $EC_{50} = 0.1$ – 0.7 μM ; HIV-2 $EC_{50} = 0.28$ – 1.47 μM) and less active compared to d4T (HIV-1 $EC_{50} = 0.52$ μM ; HIV-2 $EC_{50} = 2.23$ μM) and lost all antiviral activity in the CEM/TK⁻ cell cultures (HIV-1 $EC_{50} > 10$ μM). Among the AZT series, there was no marked difference in the antiviral activity between the prodrugs with a benzoyl ester ($R = 4-X-Ph$) and those with an alkanoyl group ($R =$ alkyl). All the prodrugs were significantly more potent (HIV-1 $EC_{50} = 0.011$ – 0.042 μM ; HIV-2 $EC_{50} = 0.06$ – 0.16 μM) than AZT (HIV-1 $EC_{50} = 0.44$ μM ; HIV-2 $EC_{50} = 0.49$ μM). Unexpectedly, the strong antiviral activity of the AZT-DiPPro observed in wild-type cells was only weakly retained in the TK⁻ cell line. The authors attributed this to slow prodrug uptake, which may permit decomposition of AZT-DP to AZT-MP before cellular entry, and to an additional bottleneck in the conversion of AZT-DP to the active triphosphate metabolite.

When this approach was extended to T-1106 and its fluoro analogue T-705, the T-705 DiPPro rapidly decomposed in PBS without forming the diphosphate likely due to nucleophilic displacement of fluorine.^{37,43} In contrast, T-1106 showed a hydrolysis pathway as d4T-DiPPro (Fig. 4B), with the NDP released slower than the symmetric prodrugs in cell extract. When evaluated for their anti-influenza activity, the non-symmetric T-705-DiPPro was around 3-fold more active than the nucleobase, while T-1106-DiPPro derivatives showed 35–42-fold higher activity than the nucleoside and 4–5-fold higher than the nucleobase. Both retained activity in TG^{res} cells, whereas the nucleoside was much less active and the nucleobase inactive.

Synthesis. The symmetric^{38–41} and non-symmetric⁴² prodrugs were synthesized by dicyanoimidazole-mediated coupling of para-acyloxybenzylphosphoramidites (5 or 8) with the bis(tetra-*n*-butylammonium) salt of NMP using dicyanoimidazole (DCI), and subsequent oxidation to P(V) with *tert*-butyl hydroperoxide (Scheme 2). Preparation of the phosphoramidites 5 (symmetric) and 8, (non-symmetric) was accomplished with a different methodology. For the symmetric, two equivalents of 4-acyloxybenzyl alcohol (3) were reacted with dichloroisopropylphosphine (4) while for the non-symmetric the alcohol 3 was first reacted with phosphorus trichloride, followed by addition of *N,N*-diisopropylamine (DIPA) affording the corresponding bis(diisopropylamino)phosphoramidites (6). Subsequently, one diisopropylamino group was selectively displaced by a second 4-acyloxybenzyl alcohol (7) after activation with DCI, providing the non-symmetric phosphoramidites (8). These activated phosphoramidites were then subjected to a



Scheme 2 General synthesis of β -(AB,AB)-DiPPro and β -(AB,ab)-DiPPro using phosphoramidite chemistry. Reagents and conditions: (a) Et₃N, THF, 0 °C–rt; (b) (i) [N(nBu₄)₂NMP, DCl-activator solution (0.25 M in CH₃CN), CH₃CN, rt, 30 min; (ii) 5.5 M *t*-BuOOH in *n*-nonane, –25 °C to rt; (c) (i) PCl₃, pyridine, –78 °C to rt, 24 h, THF; (ii) *N,N*-diisopropylamine, –10 °C to rt, 24–48 h, THF; (d) DCl-activator solution (0.25 M in CH₃CN), 4 °C to rt, 30–60 min, CH₃CN.

second acid-promoted coupling with the appropriate NMP, furnishing the desired non-symmetric DiPPro.

3. Nucleoside triphosphate prodrugs with two bio-cleavable masking groups

In 2015, Meier and co-workers introduced the first prodrug-based system for intracellular delivery of NTPs, termed the triphosphate pronucleotide (TriPPPro) strategy.¹⁸ In this design, the γ -phosphate of the NTP is masked with two biolabile protecting groups, enabling efficient cellular uptake. Once inside the cell, chemical or enzymatic hydrolysis

releases the active NTP. Since its introduction, the TriPPPro concept has been expanded using diverse masking groups, broadening its applicability for delivering various modified NTP analogues. As seen for the DiPPro compounds, the last phosphate of NTP can be masked with two identical (symmetric) (Fig. 5A and B) or different (non-symmetric) masking groups (Fig. 5C–E). An alternative NTP prodrug design was recently reported by Tisnerat *et al.*, in which the γ -phosphate is masked with an aryloxy group and an amino acid ester (γ -ProTriP) (Fig. 5F).⁴⁴ Although this approach also employs two biocleavable masking groups, it differs fundamentally from the TriPPPro strategy of Meier and co-workers in the nature of the masking units and the proposed activation mechanism.

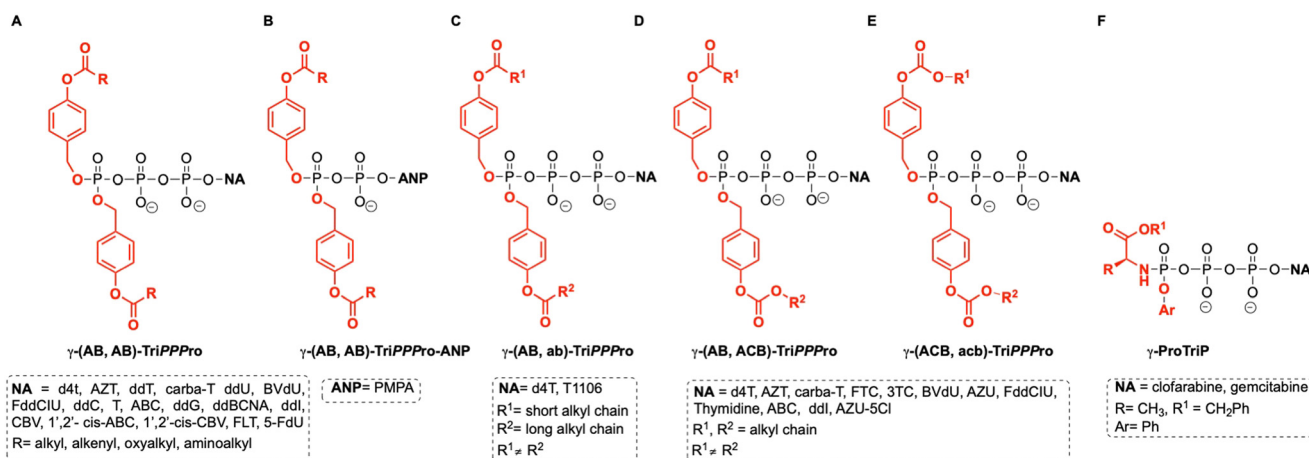


Fig. 5 General structure of symmetric TriPPPro (A and B), non-symmetric TriPPPro (C–E), and γ -ProTriP (F).

3.1. Triphosphate pronucleotide (TriPPPPro) prodrugs

TriPPPPro prodrugs can be classified into two categories: symmetric and non-symmetric derivatives. Symmetric TriPPPPro systems contain two identical biocleavable masking groups, whereas non-symmetric derivatives bear two different masking groups on the triphosphate moiety, allowing modulation of their activation and biological properties.

3.1.1. Symmetric TriPPPPro. Fig. 5A illustrates the general structure of symmetric TriPPPPro, in which the γ -phosphate is masked with two identical acyloxybenzyl (AB) groups. These masking groups include 4-alkanoyloxybenzyl, 4-alkoxycarbonyloxybenzyl, and 4-aminocarbonyloxybenzyl substituents. The first TriPPPPro example was demonstrated with d4T,⁴⁵ and subsequent studies showed that this strategy is broadly applicable to a wide range of purine and pyrimidine NAs.⁴⁶ Like the DiPPPPro system, the TriPPPPro concept was designed to be activated by (carboxy)esterase-mediated cleavage. In PBS, the γ -(AB,AB)-TriPPPPro d4T compounds first generated the mono-masked intermediate, which subsequently hydrolysed more slowly to the corresponding d4T-TP. Alongside the d4T-TP, small amounts of the d4T-MP and d4T-DP were detected, most likely arising

from nucleophilic attack at the α - and β -phosphate positions of the original prodrug. The chemical stability of these TriPPPPro prodrugs increased with longer alkyl chain (R) lengths ($t_{1/2}$ = 17–99 h first step; $t_{1/2}$ = 65–590 h second step). In PLE studies, enzymatic removal of both masking groups occurred much more rapidly ($t_{1/2}$ = 0.013–44 h first step; $t_{1/2}$ = 1.6–177 h second step) than chemical hydrolysis, and the d4T-TP was formed selectively as the predominant products. Hydrolysis of the d4T-TriPPPPro compounds was also examined in CEM cell extracts. Again, the half-lives correlated with alkyl chain length and were significantly shorter ($t_{1/2}$ = 0.50–5.3 h first step) than in PBS, indicating that enzymatic cleavage occurred similarly to the PLE experiments. In contrast to the PLE studies, however, both d4T-TP and d4T-DP were detected as major products in the CEM extracts, most likely due to the presence of additional hydrolytic enzymes, such as phosphatases and esterases (Fig. 6A). By fine-tuning the lipophilicity using different acyl esters, the authors showed that the chemical stability of the prodrugs could be modulated, allowing efficient compensation for the polarity associated with the remaining charges at the α - and β -phosphates. Among the tested substituents, the C8-alkyl chain provided the optimal balance.

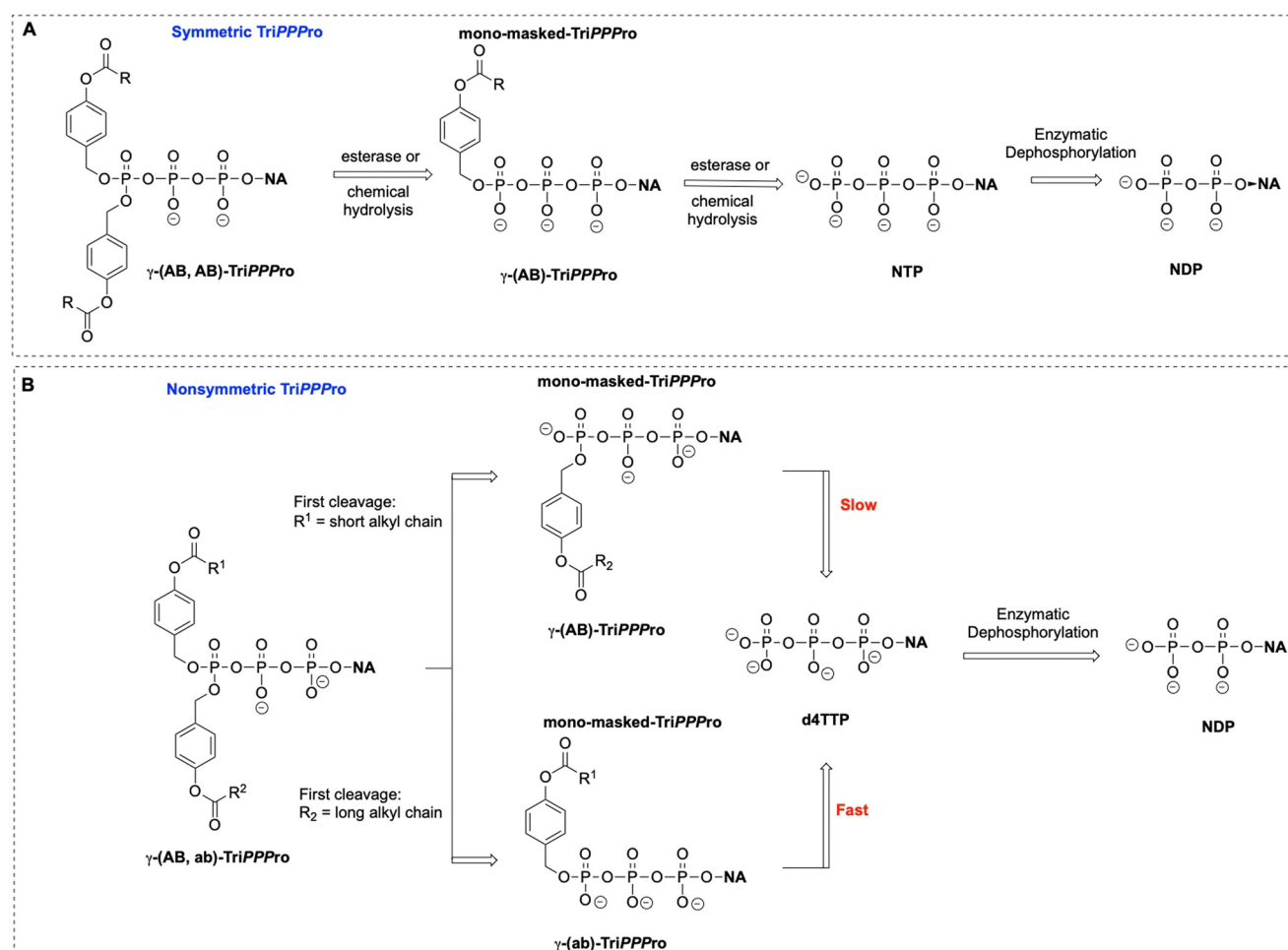


Fig. 6 Enzymatic activation pathway of γ -(AB,AB)-TriPPPPro (A) and γ -(AB,ab)-TriPPPPro (B).

In terms of antiviral activity, all symmetric d4T-TriPPPPro showed similar potency against HIV-1 and HIV-2 (HIV-1 EC_{50} = 0.17–0.62 μ M; HIV-1 EC_{50} = 0.27–1.16 μ M) compared with the parent nucleoside (HIV-1 EC_{50} = 0.33 μ M; HIV-1 EC_{50} = 0.89 μ M). An increase in antiviral activity with increasing lipophilicity was observed, likely reflecting improved cell permeability. Notably, all prodrugs with $R \geq C8$ were also highly active in CEM/TK[−] cells (EC_{50} = 2.26 μ M), whereas d4T itself was inactive (EC_{50} = 150 μ M). The retention of antiviral activity in TK[−] cells strongly indicates that γ -(AB,AB)-TriPPPPro-d4T analogues do not depend on thymidine kinase activity and are consistent with the direct intracellular release of phosphorylated species. In subsequent studies,⁴⁶ Meier and co-workers applied this approach, using the C8-alkyl chain (R) to a broad range of approved and experimental NAs. As previously observed for d4T-TriPPPPro in PBS, all prodrugs were first hydrolysed to the single-masked intermediate, followed by conversion to the corresponding NTPs. However, purine TriPPPPro derivatives, due to unidentified side reactions, resulted in shorter half-lives ($t_{1/2}$ = 12–44 h first step; 188–544 h second step) than pyrimidine analogues ($t_{1/2}$ = 18–96 h first step; $t_{1/2}$ = 215–539 h second step). In the presence of PLE and in CEM cell extracts, the enzymatic cleavage of the masking groups proceeded much faster than chemical hydrolysis. However, in CEM cell extract, both NTPs and NDPs were observed as major products, most likely due to dephosphorylation of NTP by phosphatases and kinases present in extract as seen before for the d4T-TriPPPPro (Fig. 6A). In the same studies, the successful intracellular formation of NTP was demonstrated using a fluorescent NA in an uptake assay.⁴⁶

When the γ -(AB,AB)-TriPPPPro strategy was applied to abacavir (ABC), carbavir (CBV) and their 1',2'-*cis* isomers,⁴⁷ the enzymatic cleavage by PLE predominantly yielded the corresponding NTPs, as observed for d4T-TriPPPPro. In contrast, chemical hydrolysis produced markedly different NTP/NDP ratios around 1:1 for ABC and 1:2 for CBV, compared with 3:1 for d4T. The authors attributed this difference to the exocyclic amine, which may activate the β - and γ -phosphorus anhydride bonds, facilitating dephosphorylation. In chemical hydrolysis studies, CBV-TriPPPPro has significantly higher half-lives ($t_{1/2}$ = 29.2 h) than ABC ($t_{1/2}$ = 8.5 h) and their respective 1',2'-*cis* derivatives ($t_{1/2}$ = 8.5–12.4 h). For all analogues, only trace amounts of the NTPs were detected in CEM cell extracts. The NDPs instead formed rapidly, and within 48 h, the corresponding NMPs and even the parent nucleosides appeared, indicating extensive enzymatic degradation. Similar results were obtained with γ -(AB,AB)-TriPPPPro of T-1106, likely due to fast dephosphorylation of the triphosphate species after its initial formation.^{37,43}

The γ -(AB,AB)-TriPPPPro approach was also applied to 3'-deoxy-3'-fluorothymidine (FLT), an antiviral nucleoside, whose clinical trials were stopped in phase II due to hematologic and bone marrow toxicity. The hydrolysis of γ -(AB,AB)-TriPPPPro-FLT was evaluated in different media, showing a hydrolysis pathway and half-lives like the d4T-TriPPPPro series.

Across the broader panel of antiviral NAs, the γ -(AB,AB)-TriPPPPro prodrugs inhibited HIV-1 and HIV-2 replication to a degree that was significantly higher or at least comparable to their respective parent nucleosides. Remarkably, the otherwise inactive nucleoside 5-chloro-2',3'-dideoxy-3'-fluorouridine (FddClU; HIV-1 EC_{50} >250 μ M) was converted into a highly potent TriPPPPro compound (HIV-1 EC_{50} = 3.4 μ M). In contrast, the γ -(AB,AB)-TriPPPPro derivatives of d4U and ddU showed no significant antiviral activity, likely due to extremely rapid dephosphorylation of their triphosphate forms. This suggests that uridine analogues are unsuitable for antiviral therapy, even though their isolated triphosphate forms are potent RT inhibitors in biochemical assays. The γ -(AB,AB)-TriPPPPro-CBV and -ABC derivatives exhibited 3.5-fold and 4.5-fold increases in activity, respectively, against HIV-1 in CEM/0 and CEM/TK[−] compared with their parent nucleosides. Unfortunately, the 1',2'-*cis* derivatives did not show any antiviral activity in their nucleoside or TriPPPPro forms. Similarly, the γ -(AB,AB)-TriPPPPro-FLT was found to be 2.3- and 1.3-fold less active against HIV-1 and HIV-2, respectively, than FLT.⁴⁸ On the contrary, the anti-influenza activity of γ -(AB,AB)-TriPPPPro of T-1106 in MDCK and MDCK-TG^{res} cells was 4–22-fold higher than the parent nucleoside but 1.5–10-fold lower as compared to the corresponding T-1106-DiPPPPro. Their antiviral activity was retained in MDCK-TG^{res} cells.^{37,43}

In a more recent study, Meier *et al.*⁴⁹ generated γ -(AB,AB)-TriPPPPro of 5-fluorodeoxyuridine triphosphate (FdUTP) and evaluated them for their antiparasitic activity in *Plasmodium falciparum*-infected human red blood cells. These prodrugs rapidly and potently inhibited parasite proliferation, arresting development at the trophozoite-to-schizont transition by stalling DNA replication. However, they also impaired the growth of human cells. Supplementation with thymidine or cell-permeable dTTP (cpdTTP) selectively rescued human cells while maintaining parasite inhibition, demonstrating that modulation of intracellular nucleotide pools can create a selective therapeutic window.

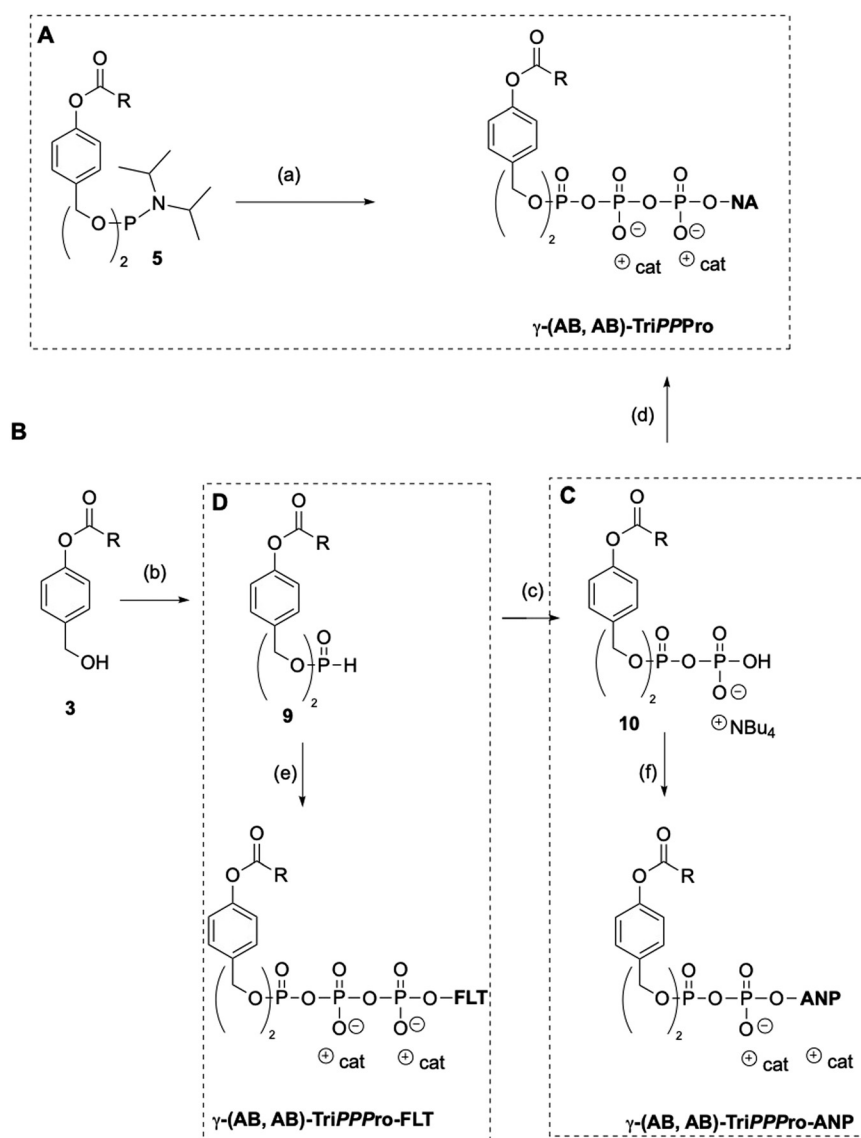
To further investigate this prodrug system, Meier *et al.* developed a sensitive hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-MS/MS) method for the simultaneous quantification of the γ -(AB,AB)-TriPPPPro lipophilic prodrug of 5FdU and the various hydrophilic resulting metabolites in a single HPLC run.⁵⁰

Interestingly, a recently reported live-cell RNA-labelling method uses γ -(AB,AB)-TriPPPPro prodrug chemistry to deliver sterically demanding NTP modified with inverse electron-demand Diels–Alder (IEDDA)-reactive groups.⁵¹ After intracellular hydrolysis, the released modified uridine and cytidine triphosphates are incorporated into nascent RNA by endogenous polymerases. A subsequent IEDDA reaction with a dual-fluorogenic tetrazine–cyanine styryl dye generates a bright, wash-free signal, enabling high-contrast imaging of RNA synthesis in living cells.

The symmetric TriPPPPro approach was also applied to the diphosphate analogue of tenofovir, where the last phosphate

of tenofovir-DP was masked with two identical AB units (Fig. 5B).⁵² The hydrolysis pathway of these prodrugs in PBS, PLE and CEM cell extract was found in agreement with previous investigation on TriPPPro of other NAs. Half-lives were longer in PBS ($t_{1/2}$ = 9 h first step, $t_{1/2}$ >200 h second step) than in PLE ($t_{1/2}$ = 0.16 min first step, $t_{1/2}$ = 0.06 min) and CEM cell extract ($t_{1/2}$ = 0.97 h). Formation of the tenofovir-DP and -MP as well as tenofovir was observed from activation of these prodrugs. These prodrugs showed slightly higher potency than the parent ANP, although their activity did not surpass that of the well-known bis-POC tenofovir prodrug. This observation suggests that the TriPPPro-ANP prodrug passes the cell membranes and is more likely converted intracellularly into the corresponding tenofovir-DP.

Synthesis. In the initial study, γ -(AB,AB)-TriPPPro of d4T⁴⁵ and T-1106 (ref. 37) were prepared using phosphoramidite chemistry previously developed for the synthesis of the DiPPPro of d4T. The key difference is that the P(III) reagent (5) bearing the two masking groups was reacted through a DCI-mediated coupling with an NDP rather than an NMP. The coupling is followed by an oxidation step with *t*-BuOOH in *n*-decane (Scheme 3A). Although this route is generally applicable, the isolated yields for d4T-TriPPPro, varied substantially (17–71%), largely due to the low efficiency of NDP synthesis and undesired cleavage of the β - and γ -phosphate anhydride bonds. This degradation pathway was supported by the detection, after chromatographic purification, of both the d4T-DP and the corresponding d4T-



Scheme 3 General synthesis of γ -(AB,AB)-TriPPPro using phosphoramidite chemistry (A), H-phosphonate chemistry (B and D) and alternative method for γ -(AB, AB)-TriPPPro-FLT (C). Reagents and conditions: (a) (i) NDP, DCI-activator solution (0.25 M in CH₃CN), CH₃CN, rt, 1 min, (ii) 5.5 M *t*-BuOOH in *n*-decane, 0 °C–rt, 15 min; (b) DPP, pyridine, 38 °C, 2 h; (c) (i) NCS, CH₃CN, rt, 1 h, (ii) (H₂PO₄) Bu₄N, CH₃CN, rt, 1 h; (d) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) NMP, rt, 1–5 h; (e) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) ANP, rt, 1–5 h; (f) (i) NCS, CH₃CN, 40 °C; (ii) FLT-DP, CH₃CN, rt.

DiPPro analogue. To achieve a more efficient conversion of NAs into the corresponding TriPPro compounds, an alternative synthetic strategy was developed in which NMP or ANP is coupled to a pyrophosphate reagent (**10**) already functionalised with the masking groups (Scheme 3B and C).^{43,46,47,52} This approach offers two major advantages: NMPs are generally easier to prepare than NDPs, and the method avoids the oxidation step, enabling the use of oxidation-sensitive nucleosides or masking groups.

When applied to d4T, this optimized route increased the overall yield of the corresponding TriPPro prodrug from 15% to 25%. This method showed improved solvent tolerance, including compatibility with methanol, although yields for some prodrugs remained limited by purification challenges. Importantly, the H-phosphonate intermediates were reported to be stable at $-20\text{ }^{\circ}\text{C}$ for more than two years, highlighting the practicality of this approach for long-term storage and batch preparation. A slight modification of the H-phosphonate chemistry was recently reported for the synthesis of γ -(AB,AB)-TriPPro of FLT (Scheme 3D).⁴⁸ In this variant, the pre-formed H-phosphonate (**9**) is first activated with *N*-chlorosuccinimide (NCS) and then reacted with the *n*-tetrabutylammonium salt of FLT-DP, affording the γ -(AB,AB)-TriPPro-FLT analogue in 37% yield.

3.1.2. Non-symmetric TriPPro. As in their earlier work on DiPPro systems, Meier and co-workers also explored TriPPro prodrugs bearing two distinct acyloxybenzyl (AB and ab) masking groups (Fig. 5C). These non-symmetric derivatives combined one short alkyl chain ($R^1 = \text{C2, C4, C6}$) with one long alkyl chain ($R^2 = \text{C14, C15, C17}$) to evaluate how non-symmetric masking groups affect stability, enzymatic activation, and antiviral activity. The γ -(AB,ab)-TriPPro approach was first applied to d4T,⁵³ and hydrolysis studies in PBS showed half-lives between 45 h and 64 h with d4T-TP and d4T-DP as main products, in addition to a small amount of d4T-MP. As seen for the non-symmetric DiPPro, no obvious selective cleavage occurred between short acyl ester and long acyl ester. Studies in CEM cell extracts and PLE proved that the hydrolysis is mainly triggered by enzymes and the half-lives are lower ($t_{1/2} = 0.8\text{--}3.8\text{ h}$) compared to those in PBS ($t_{1/2} = 22\text{--}86\text{ h}$). However, in all cases in CEM cell extract, the predominant formation of d4T-DP was observed likely due to enzymatic dephosphorylation of d4T-TP, while in the presence of PLE the mono-masked intermediates were the major species along with a small amount of d4T-TP and d4T-DP (Fig. 6B). In CEM/0 cell cultures, all γ -(AB,ab)-TriPPro-d4T showed higher activities against HIV-2 ($\text{EC}_{50} = 0.10\text{--}0.76\text{ }\mu\text{M}$) and similar or slightly better activities against HIV-1 ($\text{EC}_{50} = 0.15\text{--}0.34\text{ }\mu\text{M}$) as compared to d4T (HIV-1 $\text{EC}_{50} = 0.33\text{ }\mu\text{M}$, HIV-2 $\text{EC}_{50} = 0.97\text{ }\mu\text{M}$). More importantly, almost all prodrugs were highly potent in CEM/TK[−] cells (HIV-1 $\text{EC}_{50} = 0.39\text{--}2.0\text{ }\mu\text{M}$), whereas d4T and d4T-TP lacked any relevant anti-HIV activity (HIV-1 $\text{EC}_{50} > 50\text{ }\mu\text{M}$).

This approach was applied to T-1106, yielding prodrugs that were found to undergo hydrolysis pathways analogous to

those of the d4T analogues, resulting predominately in the formation of T-1106-NDP *via* dephosphorylation of the corresponding triphosphate form.³⁷ Similarly, to the symmetric prodrugs, the non-symmetric γ -(AB,ab)-TriPPro of T-1106 showed 3–15-fold lower anti-influenza activity as compared to DiPPro-T-1106, 2–10-fold higher than the parent nucleoside, but similar to the nucleobase. They also retained antiviral activity in MDCK-TG^{res} cells.

When the symmetric prodrugs TriPPro were studied,⁴⁵ derivatives bearing alkyl carbonate groups were investigated alongside bis-acyloxy esters, as carbonate linkages enhance stability. This informed the development of nonsymmetric TriPPro prodrugs bearing an acyloxybenzyl (AB) and alkoxycarbonyloxybenzyl (ACB) moiety (γ -(AB,ACB)-TriPPro (Fig. 5D) or two different alkoxycarbonyloxybenzyl moieties (γ -(ACB,acb)-TriPPro) (Fig. 5E). The two approaches were first applied to d4T⁵⁴ and then applied to several NAs.⁵⁵ The stability of γ -(AB,ACB)-TriPPro-d4T in PBS increased with the length of the alkyl chains in both AB and ACB masking groups ($t_{1/2} = 25\text{--}111\text{ h}$). Hydrolysis profiles revealed that both masking groups can be cleaved, but the ACB group generally persisted longer, leading to selective accumulation of mono-masked γ -(ACB)-TriPPro intermediates. When two different ACB groups were present, the chemical hydrolysis was much slower due to the increased chemical stability of the two carbonate residues. Under enzymatic conditions, all prodrugs hydrolysed much faster (PLE $t_{1/2} = 0.17\text{--}13.8\text{ h}$; CEM extract $t_{1/2} = 0.9\text{--}6.4\text{ h}$) than in PBS, confirming a strong enzymatic contribution. Importantly, the AB group was preferentially cleaved also in these biological media, generating the mono-masked γ -(ACB)-TriPPro intermediates, but in the presence of PLE, the mono- and diphosphate formation was minimized, and in CEM cell extracts d4T-TP was scarcely detected due to rapid dephosphorylation to d4T-DP (Fig. 7).

Most of the TriPPro-d4T prodrugs showed similar or even slightly better activities against HIV-1 ($\text{EC}_{50} = 0.027\text{--}0.73\text{ }\mu\text{M}$) and HIV-2 ($\text{EC}_{50} = 0.0048\text{--}0.13\text{ }\mu\text{M}$) than d4T (HIV-1 $\text{EC}_{50} = 0.43\text{ }\mu\text{M}$; HIV-2 $\text{EC}_{50} = 0.31\text{ }\mu\text{M}$) in CEM/0 cells. More importantly, all TriPPro-d4T prodrugs were also highly potent in CEM/TK[−] cell cultures (HIV-2 $\text{EC}_{50} = 0.11\text{--}3.26\text{ }\mu\text{M}$), whereas d4T lacked any relevant anti-HIV activity ($\text{EC}_{50} = 31.05\text{ }\mu\text{M}$).

To demonstrate the general applicability, Meier *et al.* extended the γ -(AB,ACB)-TriPPro approach to a variety of FDA-approved as well as nonactive NAs.^{43,55} Regarding their chemical and enzymatic stability, the same trends observed for d4T were also observed for these compounds.

Importantly, as observed previously, CEM cell extracts markedly accelerated NTP formation from the TriPPro compounds. However, the generated NTPs underwent rapid dephosphorylation to the corresponding NDPs and NMPs, making the detection of significant intracellular NTP levels nearly impossible (Fig. 7). Only in a few cases, such as for TriPPro of bromovinyl-deoxyuridine (BVDU) and 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC, emtricitabine), was the

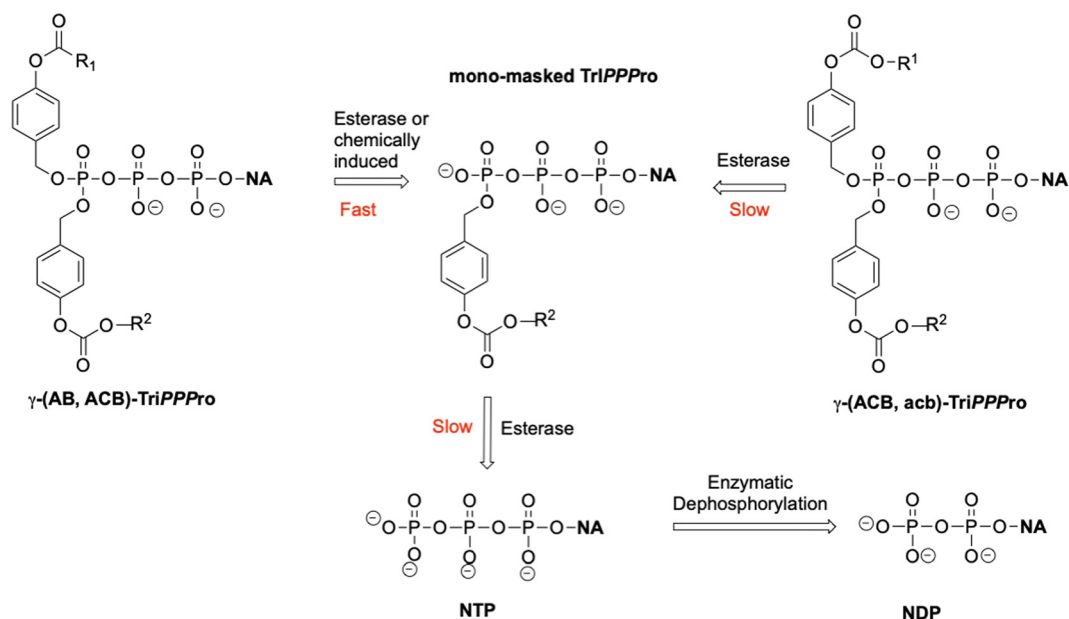
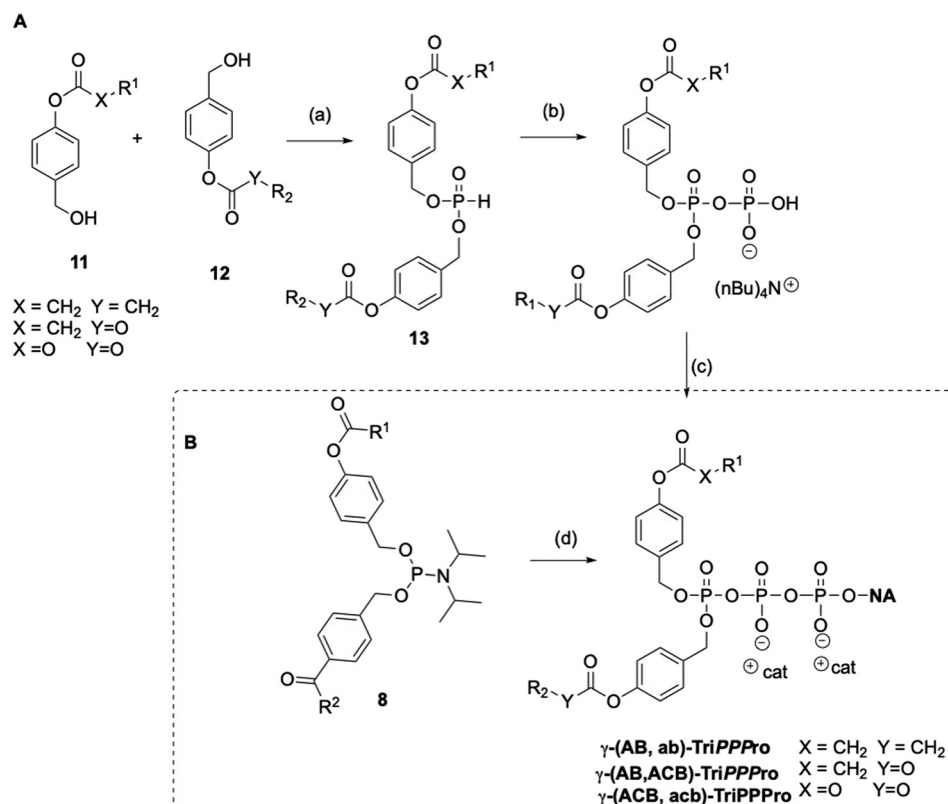


Fig. 7 Enzymatic activation pathway of γ -(AB,ACB)-TriPPPro and γ -(ACB,acb)-TriPPPro.

corresponding NTP species observed. Most TriPPPro analogues reported in these studies displayed comparable or slightly enhanced activity against HIV-1 and HIV-2 relative to

the parent nucleosides while retaining antiviral activity in CEM/TK⁻ cell cultures in a lipophilicity-dependent manner. The only exceptions were the prodrugs of 3'-azido-2',3'-



Scheme 4 General synthesis of γ -(AB,ab)-TriPPPro, γ -(AB,ACB)-TriPPPro and the γ -(ACB,ACB)-TriPPPro compounds using H-phosphonate chemistry (A) and of γ -(AB,ab)-TriPPPro T-1106 using phosphoramidite chemistry (B). Reagents and conditions: (a) DPP, pyridine, 0–38 °C, 3.5 h; (b) (i) NCS, CH₃CN, rt, 2 h, (ii) (H₂PO₄)Bu₄N, CH₃CN, rt, 1 h; (c) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) d4TMP, rt, 3–5 h; (d) (i) T-1106-DP, DCl, CH₃CN, rt, 1 min, (ii) *t*-BuOOH in *n*-decane, 0 °C–rt, 15 min.

dideoxy-5-chloro-uridine (3'-AZdd-5-ClU) and 3'-azido-2',3'-dideoxyuridine (AZU), which were found not active in CEM/0 cells as the parent nucleosides. Notably, a TriPPPPro prodrug of FddClU showed potent activity in CEM/TK⁻ cell cultures (EC₅₀ = 28 μM), whereas the parent nucleoside was inactive (EC₅₀ >250 μM). Quite interestingly, the mono-masked γ-(ACB)-TriPPPPro of d4T and FddClU were also found active in CEM/TK⁻ cells, indicating that despite the presence of an additional charge, these compounds are nevertheless taken up by the cells and were able to deliver intracellularly the corresponding NTP.

Synthesis. The non-symmetric TriPPPPro prodrugs were also prepared in moderate to good yields using the H-phosphonate route (Scheme 4A) in which the appropriate NMP is reacted with a pyrophosphate reagent (13) bearing two different masking groups (R¹ ≠ R²) (Scheme 4A). The γ-(AB,ab)-TriPPPPro of T-1106 was prepared using either the phosphoramidite protocol (Scheme 4B)^{54–56} or the H phosphonate route (Scheme 4A).⁴³

3.2. Aryloxy phosphoramidates at the γ-phosphate of NTP (γ-ProTriP)

Among the most successful monophosphate prodrug approaches is the ProTide technology pioneered by Prof. McGuigan in the late 1980s.⁵ A ProTide consists of a cyclic or acyclic nucleoside aryl phosphate (or phosphonate) masked with an amino acid ester moiety linked *via* a P–N bond. This design enables passive diffusion across cell membranes, thereby bypassing the need for membrane transporters. In addition, it circumvents kinase-dependent activation and confers resistance to catabolic enzymes. The ProTide approach has been highly successful, leading to several blockbuster antiviral drugs,^{6,7} and shows promising potential in oncology.^{9,10} In addition, its application has also expanded to non-nucleoside small molecules, demonstrating efficacy across additional therapeutic areas.^{57–59} Inspired by ProTide technology, we have developed an alternative NTP prodrug strategy termed γ-ProTriP (gamma prodrug of triphosphate), in which the γ-phosphate of an NTP is masked by an aryloxy moiety and an amino acid ester.⁴⁴ Fig. 5F illustrates the general structure of a γ-ProTriP. As in the ProTide approach, γ-ProTriP can be tuned in terms of stability and lipophilicity by varying the amino acid ester and aryloxy moieties. The amino acid component may be derived from natural or non-natural α-amino acids. The ester moiety (R¹) can include short linear or branched alkyl or benzyl chains, while the aryl substituent (Ar) can include phenyl or 1-naphthyl groups. The synthetic feasibility of this novel prodrug approach was initially demonstrated using a natural nucleoside and then applied to two FDA-approved anticancer agents, clofarabine and gemcitabine. Stability studies in PBS at pH 6.4 and 7.3 showed that the γ-ProTriP prodrugs exhibit high chemical stability (*t*_{1/2} >24 h). In contrast, significantly faster hydrolysis (*t*_{1/2} ≈ 1.5 h) was

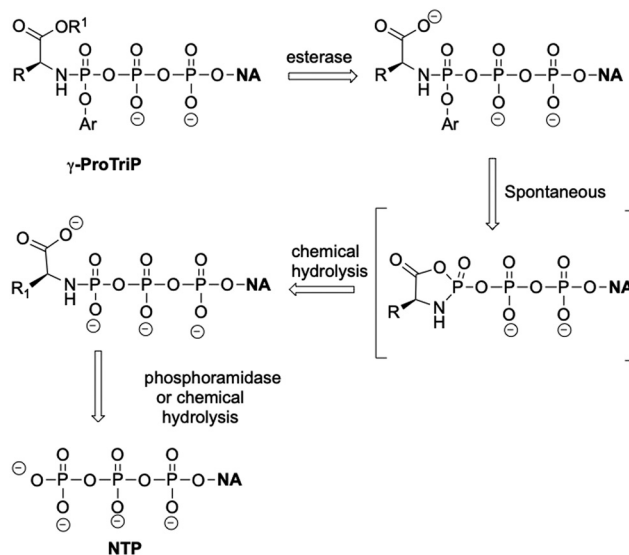
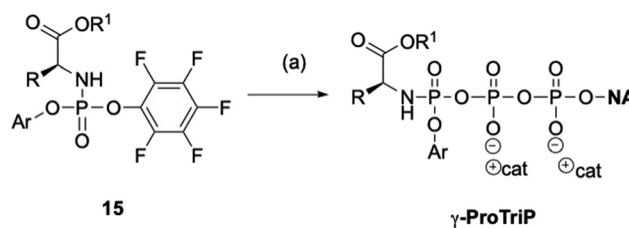


Fig. 8 Suggested enzymatic activation pathway of γ-ProTriP prodrugs.

observed in rat serum, highlighting the crucial role of enzymatic processes in prodrug activation. Although no metabolites were identified in these studies, it is proposed that activation proceeds *via* a pathway analogous to that of ProTides, ultimately releasing the corresponding NTP (Fig. 8). However, experimental evidence to support this mechanism is currently lacking.

In terms of anticancer activity, the γ-ProTriP derivative of clofarabine exhibited significant *in vitro* cytotoxicity across a panel of human cancer cell lines, including both solid tumours and haematological malignancies. The IC₅₀ values were generally comparable to those of the parent nucleoside in most cell lines.

Synthesis. γ-ProTriP prodrugs were synthesised *via* reaction of the corresponding NDP with a pentafluorophenyl phosphorylating reagent (15) bearing both the amino acid ester and the aryloxy substituents (Scheme 5).^{44,60} Optimal conditions for natural nucleosides require DMF as the solvent and the presence of DIPEA under conventional heating or microwave irradiation, with the latter significantly reducing reaction times (from 24 h to 3 h). However, when applied to clofarabine and gemcitabine, lower overall yields were obtained. This was not due to inefficiencies in the coupling step but rather to the inefficient formation of the NDP precursor.



Scheme 5 General synthesis of γ-ProTriP. Reagents and conditions: (a) NDP, DIPEA, DMF, MWI, 40 °C, 3 h.

4. Nucleoside diphosph(on)ate and triphosph(on)ate prodrugs masked with non-biocleavable masking groups

Selectivity for viral polymerases over cellular enzymes is critical in antiviral nucleotide design, as inhibition of mitochondrial DNA polymerase γ is linked to toxicity. In this context, Kravetsky *et al.*⁶¹ showed that replacing the γ -phosphate of 2'-deoxynucleoside 5'-triphosphates with a methyl or phenyl phosphonate preserved RT recognition while abolishing activity toward DNA polymerases α and β , although without antiviral efficacy. Subsequently, Meier's group found that mono-masked TriPPPro-d4T acted as RT substrates and showed anti-HIV antiviral activity in both CEM/0 and CEM/TK⁻ cells.^{62,63} Building on these findings, Meier developed a second generation of lipophilic prodrugs with the γ -phosph(on)ate of an NTP or ANP-DP and the β -phosph(on)ate of an NDP or ANP-MP masked by either one biocleavable and one non-biocleavable (Fig. 9A and B) or two non-biocleavable groups (Fig. 9C–E).

4.1. Prodrugs with one biocleavable and one non-biocleavable masking groups

Fig. 9A illustrates the general structure of a γ -modified NTP, in which a benzyloxyalkyl group (AB) serves as the biocleavable masking unit, while an alkyl (γ -(AB,alkyl)-NTP) or ketobenzyl (kb (γ -(AB,kb)-NTP) moiety functions as the non-cleavable unit. This approach was first applied to d4T^{56,64} and subsequently to other NAs.⁶⁵ Later, β -(AB,alkyl)-NDP of d4T were also reported (Fig. 9B).⁶⁶

These γ -modified d4T-TP prodrugs underwent selective cleavage of the AB group, yielding mono-masked compound (γ -alkyl-d4T-TP or γ -kb-d4T-TP *via* slow chemical hydrolysis ($t_{1/2}$ = 94–269 h) or *via* faster enzymatic activation in CEM cell extracts ($t_{1/2}$ = 0.4–5.2 h) (Fig. 10A). As seen before, cleavage rates were tuneable, with shorter chains in the cleavable moiety being less stable than longer lipophilic ones. The resulting mono-masked species showed high metabolic stability ($t_{1/2}$ $\geq$ 30 h for γ -alkyl series and $\geq$ 98 h for the γ -kb series) with negligible d4T-DP formation and no detectable d4T-TP. All these γ -modified d4T-TP prodrugs showed similar

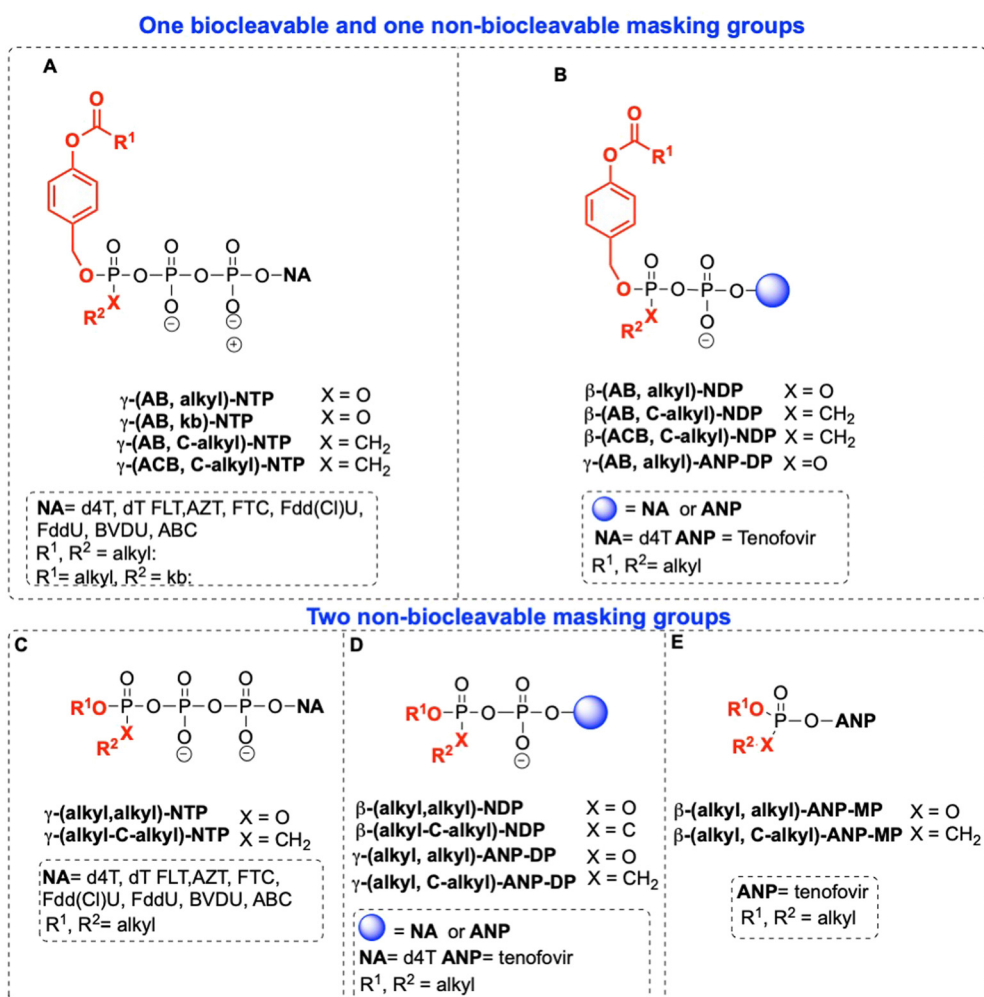


Fig. 9 NTP and NDP prodrugs with one biocleavable and one non-biocleavable masking units (A and B) and two non-biocleavable masking units (C–E).

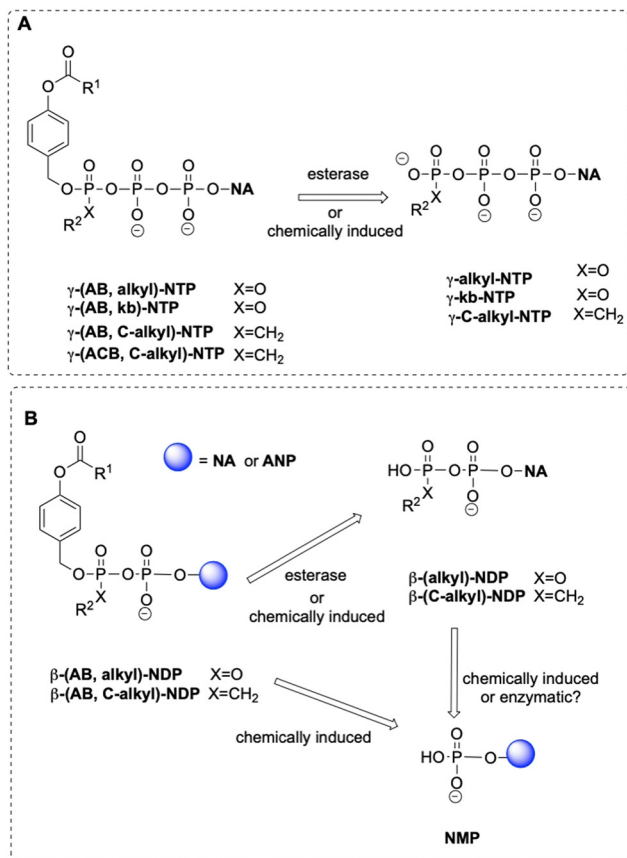


Fig. 10 Enzymatic activation of γ -(AB,alkyl)- and γ -(kb,alkyl)-NTP prodrugs, γ -(AB,C-alkyl)- and γ -(ACB,C-alkyl)-NTP prodrugs (A); β -(AB,alkyl)-NDP, β -(AB,C-alkyl)-NDP, and β -(AB,alkyl)-ANP-DP (B).

or even better antiviral activity against HIV-1 and HIV-2 (HIV-1 EC_{50} = 0.17–0.62 μ M; HIV-2 EC_{50} = 0.16–0.86 μ M) than d4T (HIV-1 EC_{50} = 0.43 μ M; HIV-2 EC_{50} = 0.22 μ M). This antiviral activity was in general retained in CEM/TK[−] cells (HIV-2 EC_{50} = 0.17–9.74 μ M), while the parent nucleoside was inactive (HIV-2 EC_{50} >50 μ M), demonstrating efficient kinase-independent delivery of the active species. Differences within the two series (R^2 = alkyl *versus* R^2 = kb) were modest, consistent with their similar overall lipophilicity. Reduced activity was observed for some prodrugs bearing short alkyl chains, confirming the critical role of lipophilicity for cellular uptake. Importantly, the antiviral activity observed in both wild type and TK[−] cells originate from the γ -alkyl mono-masked d4T compounds, since no formation of d4T-TP was detected in cellular extract. This is a striking difference to the previously reported fully masked TriPPP compounds, which were not substrates for the HIV-RT. None of the γ -(AB,kb)- and γ -(AB,alkyl)-d4T-TP analogues exhibited significantly higher toxicity than d4T, supporting their favourable antiviral selectivity profile.

Both DNA polymerase γ and α did not accept any γ -mono-masked-NTP or parent NTP prodrugs. The mono-masked compounds in the kb series as well as the parent NTP prodrugs proved to be no substrates for the human DNA

polymerase β . For the γ -alkyl mono-masked compounds, a strong chain-length dependence was observed for the β -DNA polymerase with γ -(alkyl-C4)- and γ -(alkyl-C11)-mono-masked d4T-TP being incorporated and γ -(alkyl-C18) mono-masked d4T-TP not. However, substrate efficiency to HIV-RT depended also on alkyl chain length in the γ -alkyl-NTP series. Short- and medium-chain derivatives (C4 or C11) were incorporated more efficiently than those with a long chain (C18), which required longer incubation times and showed partial stalling. Importantly, the lack of incorporation by polymerase γ is particularly relevant, as inhibition of this enzyme is commonly associated with mitochondrial toxicity. These results clearly indicate that γ -phosphate modification modulates substrate recognition by cellular polymerases while maintaining activity toward HIV-RT.

In subsequent studies, this powerful strategy was applied to FLT, an analogue of AZT which exhibited significant toxicity attributed to potent inhibition of mitochondrial DNA polymerase γ , which resulted in the termination of its phase II clinical trials.⁴⁸ Consistent with the γ -modified d4T-TP analogues, the reported γ -modified FLT-TP analogue was rapidly activated in CEM cell extract ($t_{1/2}$ = 2.4 h), releasing the mono-masked species. This compound demonstrated high resistance to both chemical and enzymatic hydrolysis, was efficiently recognised by HIV reverse transcriptase in polymerase assays and was not accepted as a substrate by human polymerases. This prodrug exhibited antiviral activity against HIV-1 (EC_{50} = 60 nM) and HIV-2 (EC_{50} = 20 nM) and was found to be 60-fold more potent (EC_{50} = 1.16 μ M) than the parent nucleoside (EC_{50} >100 μ M) in CEM/TK[−] cells while showing comparable toxicity. The mono-masked FLT compound also showed a strong activity in wild-type CEM/0 cells (EC_{50} = 1.0–2.8 nM) comparable with the parent nucleoside FLT but lost some of its activity in CEM/TK[−] cells (EC_{50} = 5.5 μ M) even if it remained 13-fold more active than FLT. These results highlight presumably partial dependence on cellular processing for γ -alkyl-modified-FLT.

The applicability of this approach was extended to several other antiviral NAs.^{43,65} The hydrolysis pathways of the resulting prodrugs followed mainly those of the previously reported for d4T and FLT analogues with formation of the mono-masked intermediates and small amount of NDPs in PBS. Only in the case of AZT was the concentration of the AZT-DP higher than that of the mono-masked compound, indicating that nucleophilic attack to the β -phosphate is the main pathway. In CEM extract, similar results to the d4T analogues were observed, with compounds undergoing quickly the first enzymatic step to produce the stable mono-masked derivative (Fig. 10A). Only a negligible amount of NDP is observed in this medium. All compounds were found highly potent in HIV CEM/0 cells and, more importantly, they exhibit even better activities against HIV-2 replication in CEM/TK[−] cells. All the mono-masked compounds proved to be HIV-RT substrates.

In more recent studies, Meier applied this approach to tenofovir preparing a series of γ -(AB,alkyl)-tenofovir-DP

prodrugs (Fig. 9B).⁵² As expected, the hydrolysis pathway in PBS and CEM cell extracts showed that the bio-reversible masking unit is cleaved (Fig. 10A). However, differently from the γ -(AB,alkyl)-NTP prodrugs, the author found that the major species is the tenofovir-MP and not the mono-masked γ -alkyl-ANP-DP, suggesting that an unexpected fast nucleophilic attack on the masked β -phosphate group may be the reason. Compounds including an $R^2 = C18$ as a non-cleavable moiety exhibited double digit nanomolar activity. The mono-masked compounds were found to be substrates of HIV-RT, while they proved to be non-substrates for human DNA-polymerases α , β , and γ . Building on these findings, Meier and co-workers subsequently described γ -monoalkyl triphosphate analogues of ANPs as inhibitors of the RNA-dependent RNA polymerase (RdRp) function of the SARS-CoV-2 nsp12 protein.⁶⁷

The same approach was applied to the synthesis of β -modified NDP of d4T (Fig. 9B).^{66,68} Consistent with previous studies, the chemical stability of these compounds in PBS increased with increasing alkyl chain length in the AB moiety ($R^1 = C4$ $t_{1/2} = 84$ h vs. $R^1 = C11$ $t_{1/2} = 279$ h). Short-chain AB compounds hydrolysed more rapidly, yielding mono-masked β -alkyl d4T-DP derivatives, while cleavage of the diphosphate unit was prevented. In contrast, long-chain compounds not only formed mono-masked β -alkyl d4T-DP compound but also released significant amounts of d4T-MP, likely due to a nucleophilic attack at the β -phosphate of the starting prodrug. In the presence of PLE and in CEM cell extracts, all compounds underwent faster hydrolysis ($t_{1/2} = 0.36$ – 4.5 h), indicating a substantial contribution of enzymatic processes. This predominantly led to the formation of mono-alkylated intermediates, with only minor amounts of d4T-MP detected. Interestingly, all β -(AB,alkyl)-modified d4T-DP compounds not only exhibited high antiviral activity against both HIV-1 ($EC_{50} = 0.059$ – 0.44 μ M) and HIV-2 ($EC_{50} = 0.036$ – 0.17 μ M) but also showed markedly improved activity in HIV-2 CEM/TK[−] cells ($EC_{50} = 0.016$ – 8.45 μ M). This suggests a mechanism of action involving their function as substrates for HIV RT and/or *via* the release of d4T-MP, which can subsequently be phosphorylated to the active d4T-TP form and incorporated into viral DNA. However, primer extension assays revealed that the mono-masked compounds were only weak substrates for HIV-RT, indicating that the presence of one negative charge at the α -phosphate and one at the β -phosphate is insufficient for efficient enzyme recognition. As these mono-masked d4T compounds were found active, the authors suggested that that the major breakdown pathway for these intermediates prodrugs is likely to be a chemical one to release the d4T-MP (Fig. 10B). Nevertheless, the authors did not rule out an enzyme-mediated breakdown involving enzymes that may be inactive or absent in CEM cell extracts but present in intact cells, such as membrane-associated enzymes. The β -modified d4T-DP and their intermediates were not found to be substrate of α , β and γ DNA polymerase and did not show any cytotoxicity.

To avoid the side reactions observed with γ - and β -alkyl-modified NTP compounds, which produced small amounts of NMP and NDP, Meier and co-workers further modified the γ -phosphate, developing a new series of γ -C-alkyl-modified NTPs. In this approach, a C-alkyl phosphonate replaces the γ -phosphate, introducing a hydrolytically stable P–C bond. A second biocleavable unit is retained, using either an AB or an ACB moiety (Fig. 9A).⁶⁹ As P–C bonds are resistant to hydrolysis, this design was expected to improve overall stability and enable rapid conversion into γ -C-(alkyl)-NTP mono-masked intermediate while preventing undesired side reactions. Again, d4T was used as a model compound⁵⁵ and then the approach was applied to different NAs.^{43,65} In the d4T series, selective cleavage of the AB or ACB group proceeds similarly as previously reported, allowing the intracellular formation of the metabolically stable γ -C-alkyl-d4T by slow chemical hydrolysis ($t_{1/2} = 1240$ – 103 h) or by fast enzymatical activation (CEM $t_{1/2} = 0.043$ – 10 h) (Fig. 10A). As expected, γ -(ACB,C-alkyl)-d4T compounds were more stable than the corresponding γ -(AB,alkyl)-d4T derivatives. Also, the resulting γ -C-alkyl-mono-masked d4T-TP compounds displayed markedly enhanced chemically and enzymatically metabolic stability, remaining intact in cell extracts for at least 30 h. Moreover, d4T-DP formation remained minor, and no d4T-TP was detected.

These γ -phosphonate d4T prodrugs exhibited robust antiviral activity against both HIV-1- and HIV-2-infected wild-type CEM/0 cells (HIV-1 $EC_{50} = 0.0018$ – 0.12 μ M; HIV-2 $EC_{50} = 0.021$ – 0.26 μ M) as well as against HIV-2-infected mutant CEM/TK[−] cells ($EC_{50} = 0.032$ – 36.8 μ M). Activity increased with lipophilicity, confirming the critical role of this parameter in cellular uptake along with the slower cleavage of the bioreversible AB or ACB moiety. When compared to the previously reported γ -alkyl-modified d4T-TP prodrugs, the inhibitory effect in CEM/TK[−] cells of these γ -C alkyl modified d4T-TP was generally enhanced. As for the mono-masked γ -alkyl d4T-TP compounds, the mono-masked γ -C-(alkyl)-d4T-TPs were found to be substrates for HIV-RT in primer extension and non-substrates for the cellular DNA-polymerases α , β , and γ .

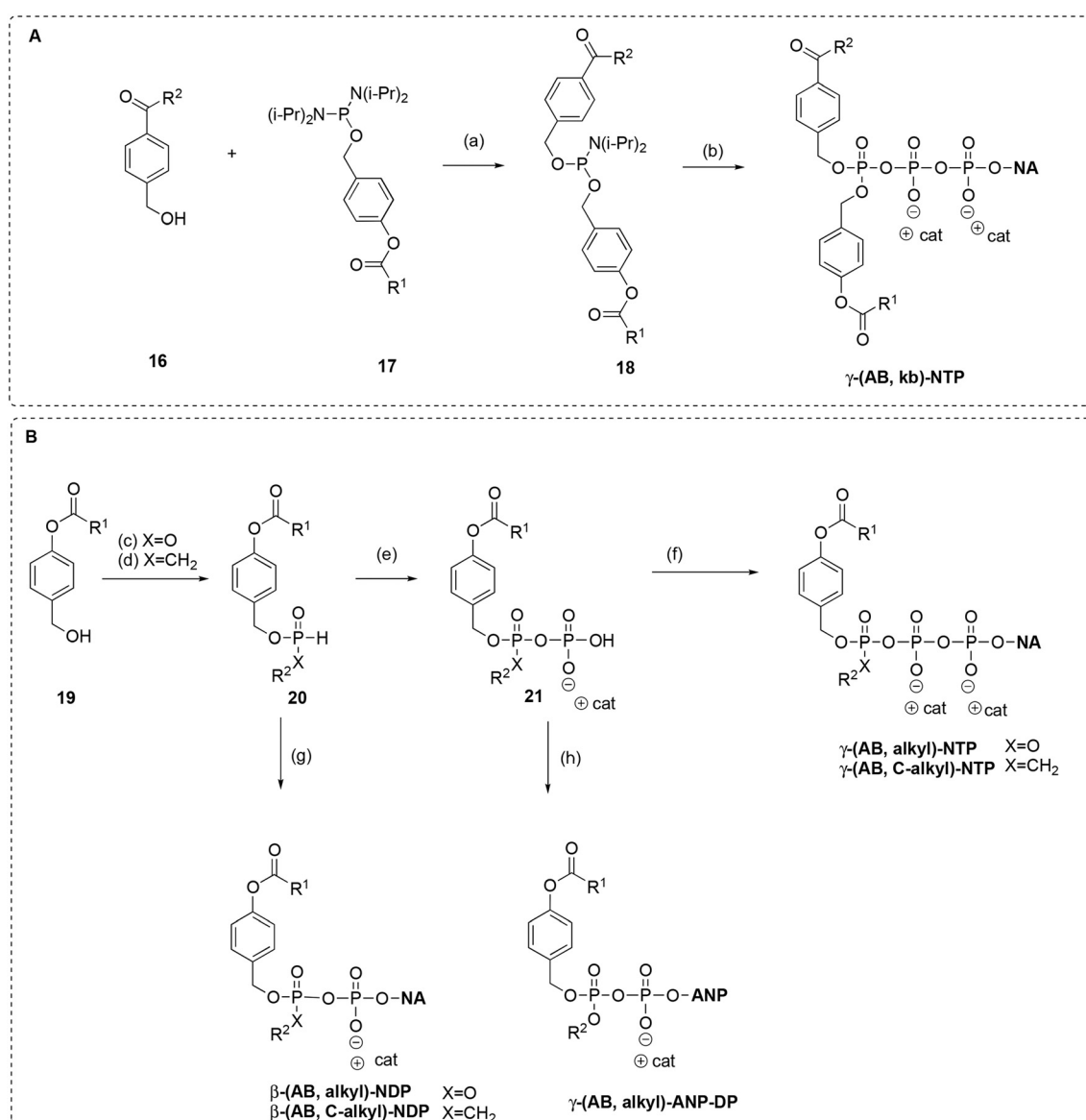
Using the optimal combination of chains ($R^1 = C4$; $R^2 = C18$) identified in this study,⁵⁵ a screening across a range of NAs demonstrated the versatility of this strategy.⁶⁵ As seen before with the d4T series,⁵⁵ these compounds were mainly hydrolysed either in PBS or in CEM cell extract to the corresponding and very stable γ -(C-alkyl)-NTPs and no formation of NDPs was observed. In agreement with the data reported previously, the enzymatic stability of γ -C-alkyl modified compounds was found to be higher compared to the corresponding γ -alkyl modified compounds.

In general, all compounds were highly active against HIV-1 and HIV-2 in CEM/0 cell lines. They also retain this antiviral activity in HIV-2 infected CEM/TK[−] cells, in contrast to their parent NAs, proving the excellent potential of this new approach. The primer extension assays confirmed for the starting prodrugs and the mono-masked compounds the

complete exclusion of human polymerases while maintaining HIV-RT substrate activity. In a similar manner, C-alkyl-modified NDP prodrugs of d4T were developed (Fig. 9B).^{66,68} Compared to the β -alkyl-modified series, this new series exhibited improved stability. The same hydrolytic pathway was observed, with preferential formation of d4T-MP when the biocleavable unit contained a long alkyl chain, resulting in slower hydrolysis that does not impede the nucleophilic attack on the β -phosphonate. These prodrugs also exhibited high potency against HIV-1 and HIV-2 in infected CEM/0 and CEM/TK⁻ cells. Notably, the β -C-alkyl mono-masked

compound acted as a substrate for HIV-RT, in contrast to the β -alkyl mono-masked analogue, which was found to be only a weak substrate for it.

Synthesis. The γ -(AB,kb)-NTP derivatives were synthesized *via* the phosphoramidite approach (Scheme 6A),⁶⁴ while the synthesis of γ -(AB,alkyl)-NTP, γ -(AB,alkyl)-ANP-DP, γ -(AB,alkyl)-ANP-DP, and γ -(AB,alkyl)-NDP was performed using H-phosphonate chemistry (X = O, Scheme 6B).^{43,48,56,65} The synthesis of γ -(alkyl,C-alkyl)-modified NTP prodrugs was achieved using the H-phosphonate route (X = CH₂, Scheme 6B).^{43,65,69}



Scheme 6 General synthesis of γ -(AB,kb)-NTP using phosphoramidite chemistry (A) and γ -(AB,alkyl)-NTP, γ -(AB,alkyl)-ANP-DP, β -(AB,alkyl)-ND, γ -(AB,C-alkyl)-NTP and β -(AB,C-alkyl)-NDP using H-phosphonate chemistry (B). Reagents and conditions: (a) R²OH, DCl, CH₃CN, rt, 1 h; (b) (i) (*n*-Bu₄N)₂⁺d4TDP 3, DCl, CH₃CN, rt, 2 h, (ii) *t*-BuOOH, CH₃CN, rt, 30 min; (c) R²OH, DPP, pyridine, 0–38 °C, 3.3 h; (d) R²P(O)OH, EDC, DMAP, CH₂-Cl₂, rt, 12 h; (e) (i) NCS, CH₃CN, rt, 2 h, (ii) (H₂PO₄)Bu₄N, CH₃CN, rt, 1 h; (f) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) ANP, rt, 3–5 h; (g) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) ANP, rt, 3–5 h; (h) (i) CH₃CN, THF, NCS, rt, 2–4 h, (ii) NMP, CH₃CN, rt, 3–4 h.

4.2. Prodrugs with two non-bioleavable masking groups

To overcome the limitations of the DiPPro system in delivering NDPs without prior degradation to the corresponding NMP, Meier *et al.* introduced a new class of γ -modified NTP prodrugs (third generation). These compounds incorporate two lipophilic, non-bioleavable masking groups attached to the γ -phosph(on)ate moiety. In this design, the γ -phosph(on)ate is masked with two alkyl chains (Fig. 9C), thereby enhancing lipophilicity and cellular uptake. The prodrugs were designed to undergo intracellular chemical hydrolysis, leading to the formation of NDP, which is subsequently phosphorylated by cellular kinases to produce the pharmacologically active triphosphate form. This strategy was also extended to the NMP delivery through the preparation of β -alkyl- and C-alkyl-modified NDPs (Fig. 9D) and to the delivery of both ANP-MP and ANP by preparation of β - and α -alkyl- and C-alkyl-modified ANP (Fig. 9D and E).

This strategy was initially applied to d4T with a series of γ -dialkyl phosphate and phosphonate derivatives being synthesized.⁶⁶ These compounds incorporated one short alkyl chain (R^1 = C4 or C8) and one long alkyl chain (R^2 = C18) to evaluate the impact of alkyl chain length and lipophilicity on prodrug stability and activity. As expected, these compounds exhibited significantly greater chemical stability than the first-generation TriPPro-d4TTP derivatives at physiological pHs. For the phosphate series, stability increased with increasing R^1 alkyl chain length. In contrast, the phosphonate analogues showed a surprising decrease in stability with longer alkyl chains. Product analysis revealed that for these γ -modified compounds, d4T-DP was the predominant species formed, accompanied by only trace amounts of d4T-MP, while no formation of d4T-TP was detected (Fig. 11A). These findings support a mechanism of NDP delivery involving cleavage of the bond between the γ -phosphate (or γ -phosphonate) and the β -phosphate. Similarly, the enzymatic hydrolysis of these prodrugs in the

presence of PLE led to d4T-DP but occurred much slower ($t_{1/2}$ >150 h) than previously reported DiPPro compounds ($t_{1/2}$ = 0.14–1.2 h). These dialkyl γ -modified prodrugs of d4T-TP showed higher antiviral activities against HIV-1 and HIV-2 than the parent nucleoside in wild-type CEM/0 cells (4–32-fold). Interestingly, all prodrugs were also highly active in CEM/TK[−] cells (EC_{50} = 0.005–0.48 μ M). Increased lipophilicity afforded more potent compounds with γ -(C8,C18)-d4T-TP being the most active (EC_{50} = 0.005 μ M in CEM/TK[−] cells). In primer extension assays, prodrugs belonging to the γ -dialkyl-modified phosphate series were found weak substrates toward HIV-RT, suggesting that binding of triphosphate derivatives within the HIV-RT remains partially feasible even when the γ -phosphate moiety is effectively neutralized. Notably, an increase in lipophilicity was associated with reduced primer elongation, which is likely attributable to decreased aqueous solubility and/or steric constraints affecting proper accommodation within the HIV-RT active site. In addition, d4T-DP was identified as a good substrate for HIV-RT, indicating that it can either be directly utilized by HIV-RT as a substrate or undergo further phosphorylation by cellular kinases to generate the active d4T-TP, which is also efficiently recognized by the enzyme. This observation correlates with the high antiviral potency of the dialkylated NTP prodrugs and confirms the intracellular release of d4T-DP from these compounds. In contrast, the γ -dialkyl phosphonate prodrugs were not substrates for HIV-RT. In this study, none of the compounds acted as substrates for human DNA polymerases α , β , or γ .

This strategy was subsequently extended to a range of different NAs,^{43,65} confirming that prodrugs bearing two non-cleavable alkyl moieties at the γ -phosphate or γ -phosphonate exhibit high stability toward hydrolysis in both chemical and biological media. In all cases, no formation of the corresponding NTPs or mono- γ -alkylated NTPs was observed. Instead, NDP was identified as the predominant product, accompanied by only trace amounts of NMP (Fig. 11A). As

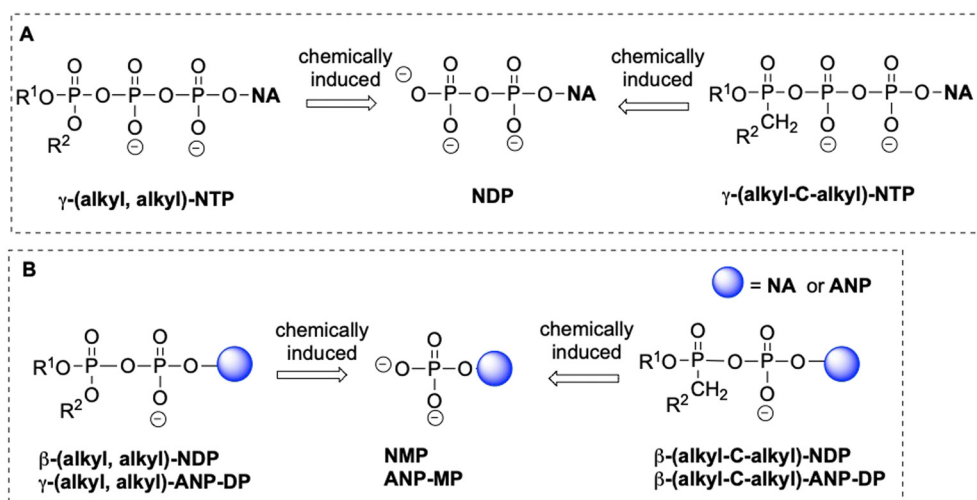


Fig. 11 Activation of γ -(alkyl,alkyl)- and γ -(alkyl-C-alkyl)-NTP prodrugs (A); β -(alkyl,alkyl)- and γ -(alkyl-C-alkyl)-NDP (or -ANP-MP) prodrugs (B).

previously observed for the d4T series, these prodrugs exhibited antiviral activity against HIV-1 and HIV-2 in infected CEM/0 cells and, importantly, retained or even enhanced this activity in CEM/TK[−] cells.

In a similar manner, β -(alkyl,alkyl)- and β -(alkyl,C-alkyl)-modified NDP prodrugs of d4T were developed (Fig. 9D).^{43,66,68} As expected the hydrolysis pathway in PBS, in PLE and CEM cell extract, showed preferential formation of the d4T-MP but not d4T-DP or mono-masked compounds (Fig. 11B).

These dialkyl diphos(on)ate prodrugs of d4T showed high antiviral activities against HIV-1 and HIV-2 in CEM/0 and CEM/TK[−] cells. None of these dialkylated d4T-DP compounds were substrates for HIV-RT. Thus, as compared to d4T-DP itself, the neutralization of both or one of the negative charges at the β -position counteracts the binding abilities of the compounds to the active site of HIV-RT.

In a more recent study, Meier applied this “two non-cleavable double-masked units” approach to the γ - and β -phosphate group of tenofovir-MP and -DP analogues, preparing a series of γ -(alkyl,alkyl)- and γ -(alkyl,C-alkyl)-tenofovir-DP as well as β -(alkyl,alkyl)- and β -(alkyl,C-alkyl)-tenofovir-MP prodrugs (Fig. 9E).⁵² Dialkylated tenofovir-DP prodrugs, like other doubly masked NTP/NDP analogues, released tenofovir-MP and tenofovir, while tenofovir-MP prodrugs produced only tenofovir in PBS. In CEM cell extracts, the same products were formed but hydrolysis was much faster, indicating significant enzymatic involvement beyond esterases (Fig. 11B). Antiviral evaluation showed that most of the dialkylated compounds exhibited superior activity against HIV-1/2 in infected CEM cells (HIV-1 EC₅₀ = 0.035–4.27 μ M; HIV-2 EC₅₀ = 0.0031–0.14 μ M) compared to tenofovir (HIV-1 EC₅₀ = 2.08 μ M; HIV-2 EC₅₀ = 1.76 μ M), although their activity remained in general lower than that of the well-known bis(POC)

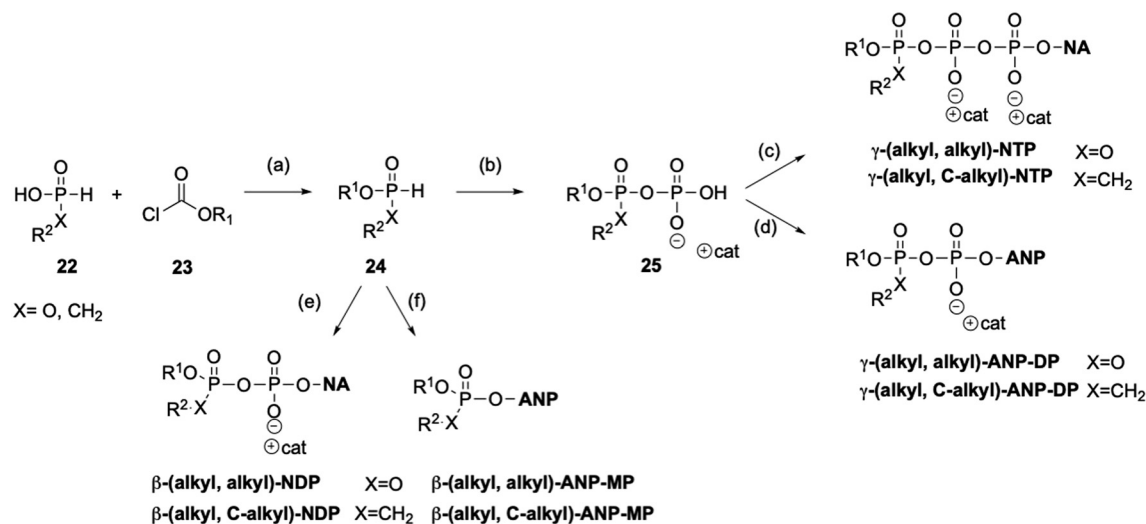
tenofovir prodrug (HIV-1 EC₅₀ = 0.013 μ M; HIV-2 EC₅₀ = 0.0039 μ M). These di-masked prodrugs were also found to be substrates for HIV-RT but not for the human DNA polymerase α , β , and γ .

Since the activation of γ -(alkyl,alkyl)- and γ -(alkyl,C-alkyl)-NTP prodrugs in CEM cell extracts results in the release of the corresponding NDP through chemical phosphoanhydride bond cleavage between the β -phosphate and the γ -phosph(on)ate moieties, in the attempts to deliver NTP, Meier and co-workers extended this concept to tetraphosph(on)ate derivatives of d4T. In these compounds, the δ -phosphate was masked with two non-bioreversible groups.⁷⁰ Evaluation of the activation pathway demonstrated the efficient generation of d4T-TP resulting from a purely chemical cleavage of the terminal δ -phosphate or δ -phosphonate moiety. These findings highlighted the potential of this strategy to achieve intracellular delivery of nucleoside triphosphates without relying on enzymatic cleavage of the masking groups.

The synthesis to obtain γ -(alkyl,alkyl)-NTP and -ANP-DP and β -(alkyl,alkyl)-NDP and -ANP-MP prodrugs was achieved using the H-phosphonate route,^{43,65,66} whereas the corresponding γ -(alkyl,C-alkyl)-NTP and -ANP-DP, and β -(alkyl,C-alkyl)-NDP and -ANP-MP prodrugs were obtained using the H-phosphinate route (Scheme 7).^{43,65,66}

5. Conclusion

The successful development and clinical approval of monophosphate prodrug technologies, most notably ProTides, have demonstrated the potential of pronucleotide approaches to overcome the limitations associated with inefficient NA phosphorylation. By bypassing the often rate-limiting first phosphorylation step, monophosphate prodrugs have



Scheme 7 General synthesis of γ -(alkyl,alkyl)- and γ -(alkyl,C-alkyl)-NTP, and β -(alkyl,alkyl)- and β -(alkyl,C-alkyl)-NDP using the H-phosphonate route and the H-phosphinate route respectively. Reagents and conditions: (a) *N*-methylmorpholine, Et₂O, toluene, 0 °C–rt, 4 h; (b) (i) NCS, CH₃CN, rt, 2 h, (ii) N(Bu)₄(H₂PO₄), CH₃CN, rt, 1 h; (c) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) NMP, rt, 2–5 h; (d) (i) TFAA, Et₃N, CH₃CN, 0 °C, 10 min, (ii) 1-methylimidazole, Et₃N, CH₃CN, 0 °C–rt, 10 min, (iii) ANP, rt, 2–5 h; (e) (i) CH₃CN, THF, NCS, rt, 2–4 h, (ii) NMP, CH₃CN, rt, 3–4 h; (f) (i) CH₃CN, THF, NCS, rt, 2–4 h, (ii) ANP, CH₃CN, rt, 3–4 h.

substantially improved the antiviral and anticancer efficacy of numerous NAs. However, despite their success, these approaches remain dependent on subsequent intracellular kinase-mediated phosphorylation steps to generate the pharmacologically active NTP. In some cases, the conversion of the NMP to the NDP, or of the NDP to the NTP, remains inefficient, limiting drug activity and contributing to resistance.^{11–16} Furthermore, accumulation of phosphorylated intermediates may lead to toxicities. Notable examples include AZT-MP, whose accumulation has been associated with severe anaemia and skeletal muscle toxicity⁷¹ as well as ribavirin mono- and diphosphate metabolites, which contribute to the haemolytic anaemia characteristic of ribavirin therapy.⁷²

These limitations provide a strong rationale for the development of diphosphate and triphosphate prodrugs. Compared with monophosphate approaches, they offer the potential to bypass additional phosphorylation steps, reduce dependence on intracellular kinases, overcome metabolic bottlenecks at the NMP and NDP levels, and minimise the accumulation of potentially toxic intermediates. Despite these advantages, the development of NDP and NTP prodrugs has

received significant attention only during the past 15 years, and no such prodrug has yet received FDA approval.

Collectively, the studies reviewed here demonstrate that although highly challenging, NDP and NTP prodrugs are synthetically accessible and can display good chemical stability when the γ -phosphate (or β -phosphate in the case of NDP prodrugs) is fully masked while the remaining phosphate residues are only partially modified.

Among the different approaches, liponucleotides were the first examples of NA diphosphate prodrugs to be explored. However, they generally release the NMP rather than the intended NDP and suffer from limitations related to solubility and toxicity. Likewise, although alkoxy ester derivatives of ANPs appear promising, there is currently no clear evidence that they release ANP-MP species in biological systems. From a synthetic perspective, liponucleotide approaches are relatively straightforward but typically require long reaction times and laborious purification.

A major breakthrough in this field was achieved by Meier and co-workers through the development of three generations of diphosphate and triphosphate pronucleotide strategies (Fig. 12),²⁰ which demonstrated the feasibility of intracellular

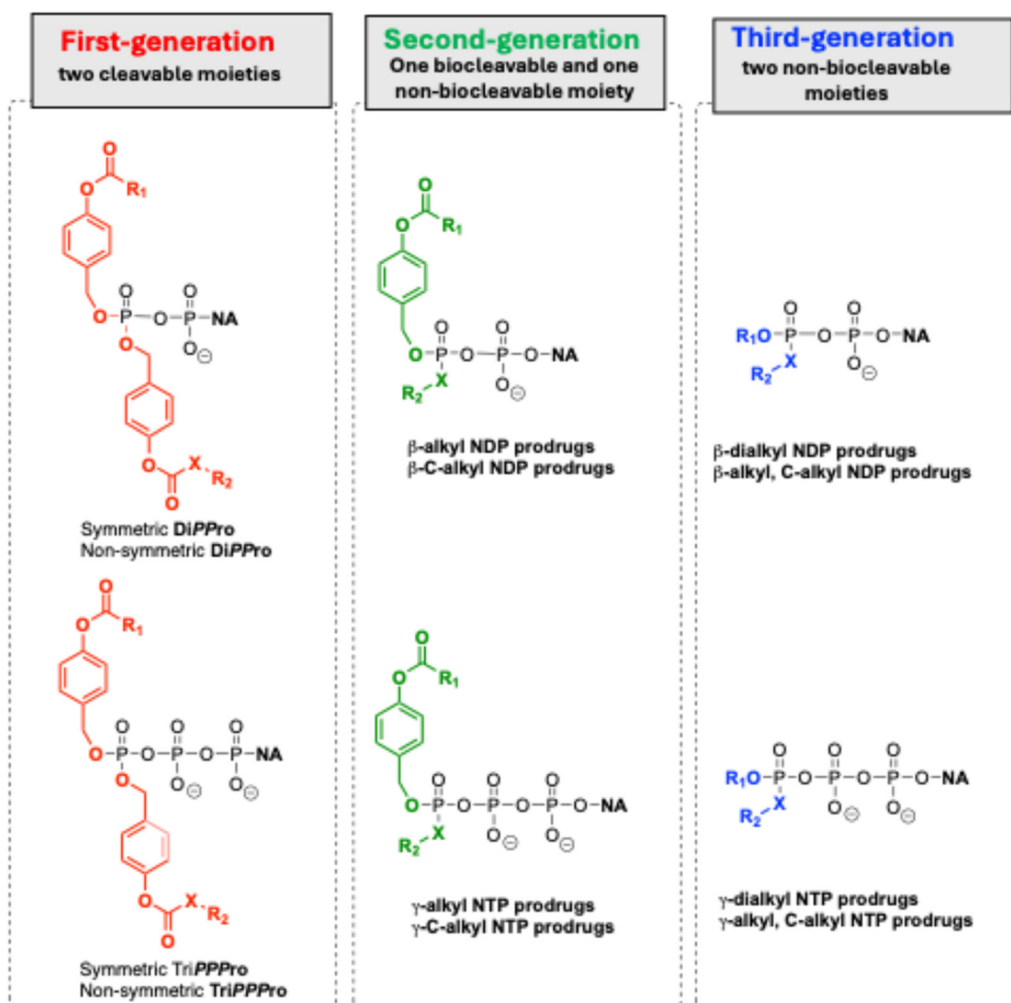


Fig. 12 The three generations of di- and triphosphate nucleoside prodrugs developed by Meier.

NDP and NTP delivery while highlighting the opportunities and challenges of these approaches.

The first-generation features either symmetric or non-symmetric biodegradable masking groups attached to the terminal phosphate (β -phosphate for DiPPro and γ -phosphate for TriPPro). For both DiPPro and TriPPro systems, biological performance depends on achieving an optimal balance between lipophilicity, stability, and activation rate. Insufficient lipophilicity can result in poor cellular uptake and premature extracellular decomposition, whereas slow intracellular activation promotes competing degradation pathways. Consequently, both NDP/NMP and NTP/NDP mixtures are often observed following activation of this series of prodrugs. Nevertheless, appropriately designed first-generation DiPPro and TriPPro derivatives can successfully release the corresponding NDP and NTP species in cell extracts and display biological activity in TK⁺ cells. The extent of competing dephosphorylation is strongly dependent on the NA scaffold, with some nucleosides displaying significantly greater susceptibility than others.

The second generation was designed to improve the drawback of the first by introducing a non-biocleavable substituent at the β -phosphorus of NDP prodrugs and the γ -phosphorus of NTP prodrugs while retaining a single biocleavable masking group. These modifications improved stability but resulted in the formation of mono-masked nucleotide intermediates that cannot undergo further intracellular processing. Consequently, their application is largely restricted to cases where the mono-masked species itself is biologically active. In the third generation, two non-biocleavable groups were incorporated at the last phosphate, allowing activation *via* phosphoanhydride bond cleavage. However, these systems release NMP and NDP species from the diphosphate and triphosphate series, respectively.

When comparing Meier's diphosphate and triphosphate prodrug approaches, it is not straightforward to identify a universally superior strategy. Conceptually, a triphosphate prodrug represents the ultimate pronucleotide approach because it seeks to deliver the active NTP directly and bypass all kinase-dependent activation steps. However, only the first generation achieves genuine NTP delivery. The second generation relies on the biological activity of mono-masked intermediates, while the third generation was specifically designed to release NDPs. Likewise, the second- and third-generation diphosphate strategies do not directly deliver NDPs, instead relying on biologically active mono-masked intermediates and the release of NMPs, respectively. Therefore, only the first-generation DiPPro and TriPPro systems can be regarded as true NDP and NTP delivery technologies, respectively. With an appropriate membrane permeability and biolability, these prodrugs can achieve efficient intracellular delivery and release of the target nucleotide. Nevertheless, dephosphorylation remains a major challenge, particularly for NTPs, which are generally more susceptible to degradation than the corresponding NDPs.

Meier's strategies provide robust and efficient synthetic routes, although the P(III)-based phosphorylation chemistry employed is generally restricted to nucleosides lacking additional hydroxyl groups unless protection-deprotection strategies are introduced. TriPPro compounds are also synthetically more demanding than DiPPro analogues because the presence of two phosphoanhydride bonds increases synthetic complexity. The presence of two phosphoanhydride bonds also gives rise to a larger number of potential intracellular metabolites, making the optimisation of masking groups and intracellular activation pathways considerably more challenging for TriPPro than DiPPro systems.

More recently, the γ -ProTriP approach has emerged as an alternative strategy. Although still at an early stage of development and lacking a fully established activation mechanism, it offers greater synthetic versatility because it can be applied to NAs containing multiple hydroxyl groups without extensive protecting-group manipulations. However, both Meier's prodrugs and γ -ProTriP synthesis rely on preformed NMP or NDP intermediates, the preparation and purification of which remain significant synthetic challenges. On the contrary, monophosphate prodrug technologies are supported by highly developed and robust synthetic methodologies that enable the efficient preparation of diverse prodrug classes, including stereochemically defined compounds and pure phosphorus diastereoisomers.

Despite significant advances, evaluation of NDP and NTP prodrugs remains largely limited to *in vitro* and cellular studies, with no reported *in vivo* preclinical validation. This highlights the persistent challenges associated with the synthesis, chemical and metabolic stability, intracellular delivery, and pharmacokinetic behaviour. Overall, the major obstacles arise from the need to balance efficient prodrug activation with competing side reactions and host-cell-mediated dephosphorylation. Future progress will therefore depend on improving phosphoanhydride bond stability while maintaining selective intracellular cleavage of the masking groups. Achieving this balance between stability and controlled activation will be critical for translating these promising prodrug strategies into clinically useful therapeutics.

Author contributions

MS, CT, DK and FP wrote the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All the data discussed and analysed in this review are taken from previously published literature and are cited appropriately within the manuscript. No new datasets were generated or

analysed during the current study. All relevant references have been carefully checked and verified by the authors.

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