

The ProTide Prodrug Technology

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Perspective

The ProTide Prodrug Technology: From the Concept to the Clinic

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The ProTide Prodrug Technology: From the Concept to the Clinic

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Abstract

The ProTide technology is a prodrug approach developed for the efficient intracellular delivery of nucleoside analogue monophosphates and monophosphonates. In this approach, the hydroxyls of the monophosphate or monophosphonate groups are masked by an aromatic group and an amino acid ester moiety, which are enzymatically cleaved-off inside cells to release the free nucleoside monophosphate and monophosphonate species. Structurally, this represents the current end-point of an extensive medicinal chemistry endeavour that spans almost three decades. It started from the masking of nucleoside monophosphate and monophosphonate groups by simple alkyl groups and evolved into the sophisticated ProTide system as known today. This technology has been extensively employed in drug discovery and it has already led to the discovery of two FDA-approved (antiviral) ProTides. In this work, we will review the development of the ProTide technology, its application in drug discovery and its role in the improvement of drug delivery and efficacy.

Keywords: ProTide, Prodrug, Nucleoside, Antiviral, Cancer.

1. Introduction

Nucleoside analogues were first introduced a number of decades ago as an effective way of treating various diseases, such as cancer and viral infections like herpes simplex virus (HSV), human cytomegalovirus (HCMV), human immunodeficiency virus (HIV), hepatitis B virus (HBV) and recently also hepatitis C virus (HCV).¹⁻⁶ Currently, there are more than twenty different nucleoside analogues available in the clinic to treat the viral infections and cancer.^{7, 8} These molecules enter the cell with the aid of transporters, such as concentrative nucleoside transporters as well as peptide transporters, or via passive diffusion.^{9, 10} Once inside the cell, the nucleoside analogues are activated by a number of nucleoside and nucleotide kinases which phosphorylate them in a stepwise manner resulting in the formation of the mono-, di- and tri-phosphorylated nucleoside analogue metabolites (Figure 1).^{6, 11}

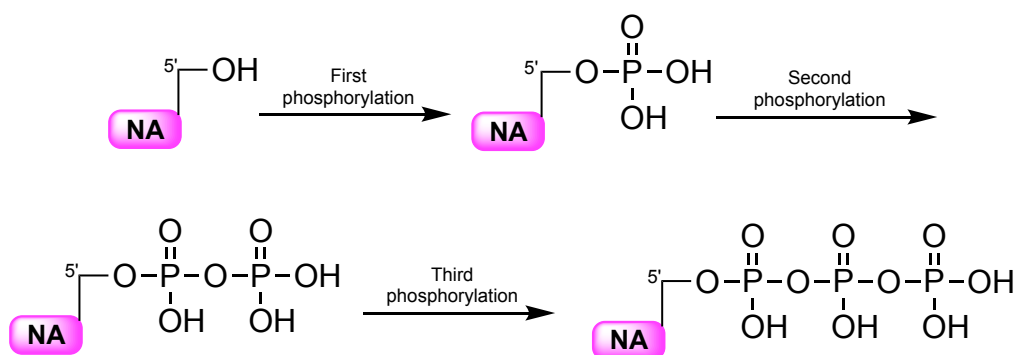


Figure 1. Intracellular activation of therapeutic and experimental nucleoside analogues. NA: nucleoside analogue.

The activated (phosphorylated) antiviral nucleoside analogues exert their therapeutic effects by targeting and inhibiting intracellular enzymes (usually the virus-encoded DNA or RNA polymerases) and/or by being incorporated into the viral nucleic acid chains leading to the termination of the elongation process.^{7, 8, 12} However, there are a number of limitations associated with the administration of nucleoside analogue drugs. Nucleoside analogues are structurally different to natural nucleosides making their phosphorylation by cellular or viral

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3 kinases often inefficient.^{13, 14} This ultimately limits the formation of the active triphosphate
4 metabolites, often the nucleoside analogue active metabolite.¹³ They also often demonstrate
5 poor oral bioavailability due to their low intestinal permeability, as they are usually polar
6 molecules that hinder their transport through the cell border *via* the paracellular route.¹⁵
7
8 Additionally, the therapeutic use of many antiviral and anticancer nucleoside analogues is
9 further limited by resistance, which occurs via a number of mechanisms⁸ that include 1)
10 down regulation of the nucleoside kinases responsible for activating nucleoside analogues,¹⁶
11 2) depletion of transporters that affect their efficient trafficking into the target cells,¹⁷⁻¹⁹ 3)
12 activation of phosphatases (i.e. 5'-nucleotidases) that dephosphorylate the active
13 metabolites of nucleoside analogues,^{20, 21} and 4) increased catabolic bioconversion of the
14 nucleoside analogues, e.g. (deoxy)cytidine deamination, which may yield inactive versions
15 of some of the nucleoside analogues^{22, 23}.

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29 To overcome these limitations, it was initially suggested that delivering phosphorylated
30 metabolites of the nucleoside analogue would address some of these shortages. This notion
31 was further supported by numerous studies that reported the poor phosphorylation of
32 nucleoside analogues into their nucleoside monophosphates as the rate-limiting step in their
33 *in situ* activation.²⁴⁻²⁷ However, the idea of using nucleoside analogue monophosphates as
34 therapeutics faced two main challenges. First, these compounds would have poor *in vivo*
35 stability due to de-phosphorylation in the blood stream, and second, their transport into cells
36 will be inefficient because of the charged nature of the phosphorylated nucleoside. To
37 address the P-O bond instability, Holý and De Clercq switched the P-O- bonds in the
38 monophosphate group of nucleoside analogues into monophosphonate P-CH₂- bonds.²⁸
39
40 This led to improved *in situ* stability, which translated into longer half-lives of the nucleoside
41 monophosphonates as compared to their monophosphates.²⁸ This advance in the design of
42 stable nucleotide therapeutics inspired the discovery of many nucleoside analogue
43 monophosphonates some of which were eventually approved for clinical use as antivirals
44 against HCMV [i.e. cidofovir], HBV [i.e. adefovir and tenofovir], and HIV [i.e. tenofovir]
45 (Figure 2).⁷

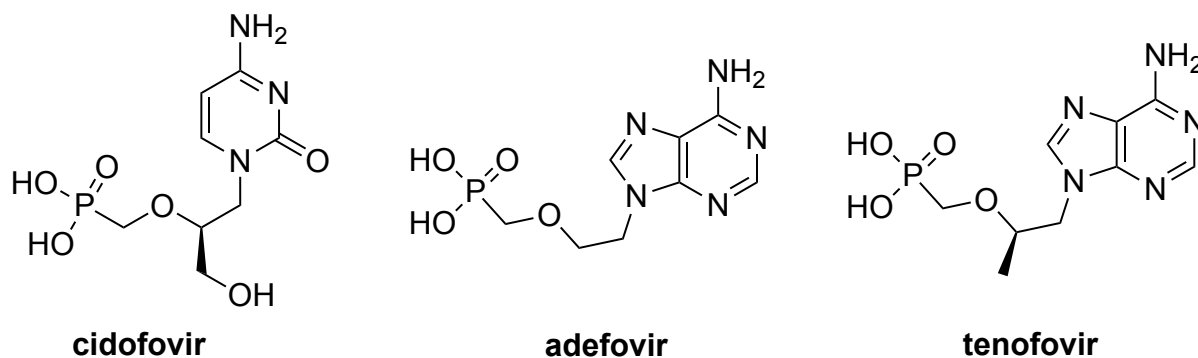


Figure 2. Chemical structures of therapeutic nucleoside analogue monophosphonates, cidofovir, adefovir and tenofovir.

Although the use of monophosphonates efficiently addressed the *in situ* and *in vivo* instability issue associated with monophosphate (P-O-) bonds, these compounds still had poor cellular uptake due to the negative charges on the monophosphonate group at physiological pH (< 7.4). This limitation acted as a challenge in the medicinal chemistry field to develop prodrugs of nucleoside analogue monophosphates and monophosphonates that can deliver them into cells more efficiently. Numerous prodrug technologies that mask either one or two of the oxygens of the monophosphate and monophosphonate groups making the molecule more lipophilic and hence improve transport into the cells have been developed.¹³ Among these prodrug approaches is the ProTide prodrug technology that was pioneered by Prof. Chris McGuigan (Cardiff, UK).³² This prodrug technology has proved to be a powerful tool in the discovery of efficacious nucleoside monophosphate and monophosphonate prodrug therapeutics.³³ To date, it has delivered at least ten clinical candidates that reached, or successfully passed, phase I clinical trials.³³ Although most of these nucleotide prodrugs were developed as antiviral agents against HIV, HBV, HCV and Ebola virus, the most recent ones are also being pursued as potential anticancer agents. Moreover, the ProTide prodrug technology has so far led to the discovery of two FDA-approved (antiviral) drugs, tenofovir alafenamide (TAF)³⁴ and sofosbuvir³⁵ (Figure 3).¹³

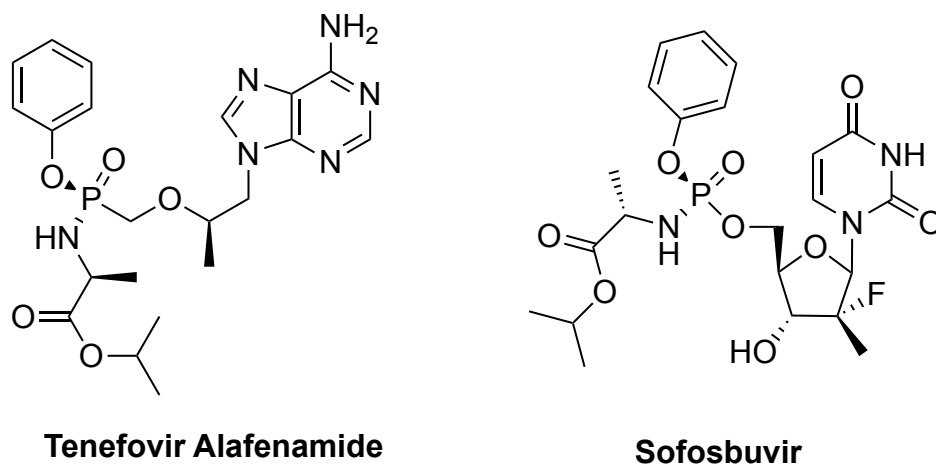
**Tenofovir Alafenamide****Sofosbuvir**

Figure 3. Chemical structures of the two FDA-approved antiviral ProTides, tenofovir alafenamide (TAF) and sofosbuvir.

Due to the effectiveness of the ProTide technology in the *in vivo* intracellular delivery of nucleoside analogue monophosphates and monophosphonates, there has been a growing interest in the application of this technology in drug discovery. The last comprehensive review on the development of the ProTide technology appeared in 2009 (Mehellou, Balzarini and McGuigan)³². However, at that time, the ProTides had just entered clinical trials and significant advances in their synthesis, metabolism and clinical studies have been achieved since then. Thus, we felt that there is a need for an updated and comprehensive report on the ProTide technology, which this review aims to address and therefore provide a much needed updated resource for the scientific community.

2. Inception and evolution of the ProTides

Initial work on the ProTides can be tracked to the late 1980s.³⁶⁻³⁸ The mission was to mask the oxygen atoms of phosphate groups in nucleoside monophosphate analogues so that they are neutral at physiological pH and hence have a better uptake into cells. The process started from the simple masking of the phosphate groups by alkyl groups but eventually evolved into the intelligent masking of the phosphate with aryl groups and amino acid esters, which can be cleaved-off inside the intact cells. Such development process, which took at least two decades, could be divided into six phases as discussed below.

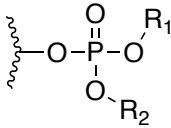
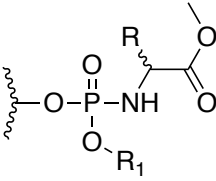
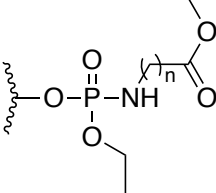
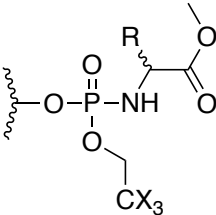
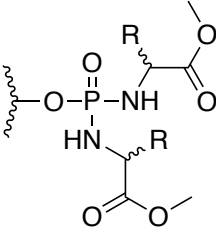
Stage One: Alkyl and haloalkyl phosphate esters

The first attempt of making nucleoside analogue monophosphate prodrugs was to mask the phosphate group of 9- β -D-arabinofuranosyladenine (AraA)³⁹ monophosphate, which exerts antiviral activity, by simple dialkyl triesters (*Stage 1*, Table 1).³⁶ Although these prodrugs led to increased stability towards adenosine/adenylate deaminases, they demonstrated a moderate biological activity.³⁶ Similar results were found when the same principle was applied to the anti-cancer agent β -D-arabinofuranosylcytosine (AraC)^{40,37}. However, when the dialkyl phosphate approach was used to mask the monophosphate group of the anti-HIV agent 3'-azidothymidine (AZT)⁴¹, no significant activity was observed *in vitro*.⁴² This was attributed to several parameters, but the most likely explanation for the lack of activity was due to the high stability of the dialkyl triesters, which hindered the eventual conversion to the biologically active 5'-O-triphosphate form of AZT.⁴³ Masking the monophosphate groups of AZT and 2',3'-dideoxycytidine (ddC)⁴⁴ with haloalkyl derivatives also yielded agents with poor biological activity.⁴⁵ In order to enhance the intracellular cleavage of the haloalkyl masking groups, more haloalkyl phosphate masking groups with halogen atoms incorporated either on one or in both masking groups were used for making AZT monophosphate prodrugs. However, these prodrugs also displayed moderate antiviral activities.⁴⁶ However, the use of haloalkyl groups in AraA and AraC monophosphates displayed significant increases in biological activity (*Stage 1*, Table 1).⁴⁷ This increase in activity was mainly attributed to increased lipophilicity, resulting in better membrane permeability of the prodrug.⁴⁷ The lack of increased AZT activity through administration of haloalkyl prodrugs of AZT could now be explained by the fact that the first phosphorylation step of AZT is not the rate-limiting step in its activation pathway while it certainly is for AraA and AraC.⁴⁸ Nevertheless, the alkyl and haloalkyl phosphate masking groups, especially for their application to AraA and AraC, indicated that these masking groups showed good activity due to better membrane-crossing ability, since the biological activity correlated with lipophilicity, rather than increased intracellular levels of the phosphate species.^{36, 37, 47} Such conclusion shifted the thinking towards phosphate prodrugs to a different strategy.

Stage Two: Alkyloxy and haloalkyloxy phosphoramidates

Subsequently, the design of nucleoside phosphate prodrugs focused on alkyloxy and haloalkyl phosphoramidate prodrugs (Stage 2, Table 1). The move towards phosphoramidate prodrugs was provoked by the findings that the HIV protease can cleave an oligopeptide from the phosphate group of blocked nucleotide phosphoramidates.⁴⁹ Initially, a selection of AZT alkyloxy phosphoramidates with methyl-esterified amino acids were synthesised.⁴² These compounds showed better anti-HIV activity than the dialkyl AZT prodrugs.⁴² Although the anti-HIV activity did not correlate with the alkyl phosphate chain, it did correlate with the amino acid side chain with L-alanine showing the better activity.^{42, 50} Retrospectively, this observation proved to be critical for the success of the ProTide technology in drug discovery since all of the ProTides that entered clinical trials and those that have been approved for clinical use bear an L-alanine moiety.³³ Further studies into different, i.e. α , β and γ , types of amino acids, indicated the superiority of α -amino acids.⁵¹ Encouraged by the influence of the amino acid moiety on the anti-HIV activity of AZT alkyloxy phosphoramidates, the masking of phosphate groups by the methyl-esterified amino acids and haloalkyl groups, which increased the membrane-crossing ability, were explored next. Haloalkyl phosphoramidates of AZT were synthesised with glycine, alanine and valine amino acids as well as ethyl, trifluoroethyl or trichloroethyl as the haloalkyl motifs.⁵² These, surprisingly, did not show good anti-HIV activity with the exception of the trichloroethyl alanine prodrug. Since no clear structure-activity relationship was apparent from the haloalkyl phosphoramidates, they were not pursued any further. However, the key finding of the enhancement of activity with nucleoside phosphoramidate prodrugs, especially with L-alanine, proved to be important for the future evolution of this nucleoside monophosphate prodrug strategy.

Stages of development	Phosphate masking group(s)
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<p>1 2 3 4 5 6 7 8 9 10</p> <p>1. Alkyl and haloalkyl phosphate triesters</p>	 <p>R_1 and R_2 = Me, Et, Pr, $-\text{CH}_2\text{CCl}_3$ and $-\text{CH}_2\text{CF}_3$</p>
<p>11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51</p> <p>2. Alkyloxy phosphoramidates</p>	 <p>R = H, Me, $-\text{CH}(\text{CH}_3)_2$, $-\text{CH}_2\text{CH}(\text{CH}_3)_2$, $-\text{CH}(\text{CH}_3)\text{Et}$ or Bn R_1 = Me, Et, Pr, Bu or Hex</p>  <p>n = 1-6</p>  <p>R = H, Me or <i>i</i>Pr X = H, F or Cl</p>
<p>52 53 54 55 56 57 58 59 60</p> <p>3. Phosphorodiamidates</p>	 <p>R = H, Me, <i>i</i>Pr, $-\text{CH}_2\text{Ph}$ or</p>

	-CH ₂ CO ₂ CH ₃ .
4. Lactyl-derived systems	<p>R = CH₃ or Et, R₁ = H or Me, R₂ = Me, nPr or nC₁₂H₂₅.</p>
5. Diaryl phosphates	<p>Ar = Ph or p-X-Ph (x = various)</p>
6. Aryloxy phosphoramidates	<p>Ar = Ph or substituted Ph R = H, Me, -CH₂iPr, -CH₂Ph...etc. R₁ = Me, Et, iPr, tBu, Bn...etc.</p>

Table 1. Stages of the development of the ProTide prodrug technology.

Stage Three: *Phosphoramidates*

Given that the introduction of amino acids to generate phosphoramidates led to enhanced anti-HIV activity, it was logical that the next step would be to mask both oxygen groups of the phosphate group by amino acid esters. Indeed, a series of amino acid methyl esters with different side-chains were used to mask both oxygens of the phosphate group of AZT monophosphate (Stage 3, Table 1).⁵³ Unlike previous studies where L-alanine appeared to give the better biological activity, in these phosphoramidate systems amino acids with aromatic side-chains, e.g. phenylalanine, showed better improvement of biological activity.⁵³ Although this was surprising in the context of the ProTides' development, such finding was consistent with other studies where a preference was found for amino acids with aromatic side-chains in the development of unrelated phosphoramidate diesters.^{29, 31} Although no

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3 further studies with these phosphorodiamidates were conducted since the early nineties and
4 the development of the ProTides moved on, phosphorodiamidates as phosphate prodrugs
5 recently (in 2011) made a comeback.⁵⁴
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8 9 **Stage Four: *Lactyl-derived systems***

10 Continuing with the probing of the phosphoramidates and particularly studies on the
11 importance of the P-N bond, isosteric O-linked nucleoside analogues derived from lactic and
12 glycolic acids of AZT were prepared and evaluated for their biological activity (Stage 4,
13 Table 1).⁵⁵ Although no significant improvement in the anti-HIV activity was noted for the
14 lactyl-derived AZT monophosphate prodrugs, the observed activity was found to correlate
15 with the lengthening of the alkyl phosphate chain (R_2) since longer chains resulted in poorer
16 anti-HIV activity. As for the glycol systems ($R_1 = H$), the prodrugs were generally more active
17 than the lactyl systems. The poor anti-HIV activity of these phosphate prodrug systems was
18 not very encouraging and they were therefore not optimised or explored any further.
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31 **Stage Five: *Diaryl phosphates***

32 The next masking groups studied in the design of a novel phosphate prodrug strategy were
33 aryl motifs. Indeed, the masking of AZT monophosphate by diaryl motifs was pursued (Stage
34 5, Table 1).⁵⁶ These prodrugs showed good anti-HIV activity and, remarkably, some of them
35 showed much better anti-HIV activity than the parent compound AZT.⁵⁶ In the journey
36 towards the ProTides, this was the first time a phosphate prodrug was more active than the
37 parent compound. Interestingly, these phosphate prodrugs retained their anti-HIV activity in
38 cell lines that were considered to be resistant to AZT.⁵⁶ However, when these cells (T
39 lymphocyte cell line, JM) were used for this study back in 1992, they were considered as
40 AZT insensitive due to poor phosphorylation of AZT.⁵⁷ But, it was later shown that such AZT
41 resistance was a result of an active AZT-efflux pump rather than inefficient intracellular
42 phosphorylation.⁵⁸ Regardless of the exact AZT-insensitivity mechanism in this cell line, the
43 AZT diaryl phosphate prodrugs were more active than their parent compound providing a
44 clear indication of an increased delivery of AZT monophosphate into the cells. This notion
45 was further supported by the fact that *para*-nitrophenyl phosphate masking groups had ca.
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3 100-fold better anti-HIV activity than their unsubstituted phenyl masking groups most
4 probably due to the electron-withdrawing effect of the *para*-nitro group that speeds-up the
5 de-masking of the prodrugs in cells.⁵⁶ A further study on the effect of the nature of the phenyl
6 substituents on the antiviral activity of diaryl phosphate prodrugs of AZT was then
7 conducted.^{59, 60} This study confirmed that stronger *para*-electron-withdrawing groups on the
8 phenyl phosphate masking groups gave better anti-HIV activity.^{59, 60} Intriguingly, these AZT
9 diaryl phosphate prodrugs as well as AZT did not exhibit any anti-HIV activity in a thymidine
10 kinase (TK)-deficient human T-lymphocyte cell line (CEM/TK⁻).⁶⁰ McGuigan and colleagues
11 concluded that this might have been due to poor intracellular release of AZT
12 monophosphate. The development of diaryl monophosphate prodrugs was not progressed
13 any further and the focus turned back to phosphoramidates, which showed good biological
14 activity.⁵⁶

29 **Stage Six: Aryloxy phosphoramidates (generally referred to as ProTides)**

31 Inspired by the improvement of the biological activities of the earlier examples of nucleoside
32 monophosphate prodrugs particularly the *para*-substituted aryls and the amino acid esters,
33 McGuigan and co-workers took the next logical step, which was to combine these two
34 masking groups in the design of new monophosphate prodrugs.⁶¹ Indeed, the masking of the
35 AZT monophosphate group with *para*-substituted aryls and an amino acid ester was
36 conducted and these efforts yielded prodrugs that showed improved anti-HIV activity as
37 compared to the parent nucleoside AZT. In particular, the combination of a phenyl and L-
38 alanine methyl ester showed the most potent anti-HIV activity in this study, $EC_{50} = 0.8 \mu\text{M}$
39 (AZT, IC_{50} (toxicity) ca. $100 \mu\text{M}$).⁶¹ Interesting structure-activity relationships on these
40 aryloxy phosphoramidate prodrugs emerged from this study. Notably, there was a
41 preference
42 for the L-alanine amino acid as compared to L-leucine (> 10-fold) and glycine (>
43 100-fold) while electron-withdrawing *para*-substituted aryls were less potent than their
44 unsubstituted moieties.⁶¹ Further studies into these substitutions concluded that *para*-fluoro
45 aryls did not largely affect the biological activity while *para*-nitro-substitutions had detrimental
46 effects on the biological activity, namely a 100-fold loss in activity.⁶² In a separate study it

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3 was shown that *para*-nitrophenyl-containing nucleoside monophosphate prodrugs were toxic
4 even to uninfected cells.⁶³ Remarkably, AZT aryloxy phosphoramidates retained their anti-
5 HIV activities in thymidine kinase deficient (TK⁻) cells.⁶² These observations provided a direct
6 proof of the ability of aryloxy phosphoramidates to deliver intact nucleoside analogue
7 monophosphates into intact cells. Hence, the masking of nucleoside analogue
8 monophosphates and monophosphonates with an aryl motif and an amino acid ester, which
9 is nowadays known as the ProTide technology, was extensively pursued in the future
10 discovery of novel antiviral and anticancer nucleotide therapeutics.
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20 **1. Synthesis of the ProTides**

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22 Although the synthesis of the aryloxy triester phosphoramidate ProTides has recently been
23 excellently reviewed by Pradere *et. al.*,²⁹ for the completion of this Review, we will briefly
24 discuss the different strategies used in the synthesis of the ProTides. We will also include
25 recent reports on the development and use of new catalysts that improve the synthesis of
26 the ProTides, which have been published after the comprehensive review by Pradere *et.*
27 *al.*²⁹. There are three different strategies for synthesising ProTides: 1) the coupling of a
28 nucleoside with a diarylphosphite followed by subsequent oxidative amination 2), the
29 coupling of the nucleoside with a phosphorochloridate reagent, and 3) coupling of an amino
30 acid to a nucleoside aryl phosphate (Figure 4).
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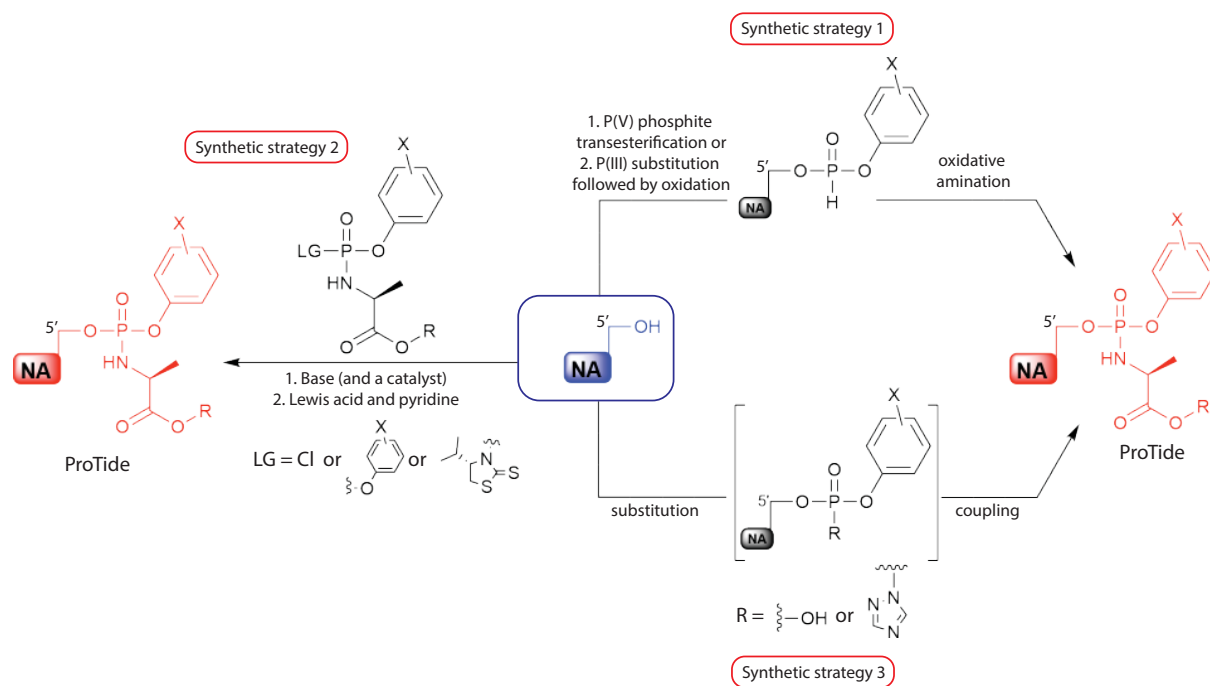


Figure 4. A representation of the three main strategies for synthesising ProTides. LG: leaving group. NA: nucleoside analogue. R: any ester. X: any aromatic substitution.

Among these three strategies, the second synthetic strategy, which involves the coupling of the nucleoside to a phosphorochloridate is the most common method and has been the method of choice of the initial synthesis of the ProTides. The coupling reaction takes place in the presence of either the Grignard reagent *tert*-butyl magnesium chloride (*t*BuMgCl) or *N*-methylimidazole (NMI), which act as a base. The choice of the base is often influenced by the nucleoside substrate, most precisely the number of hydroxyl groups that could couple to the phosphorochloridate. For instance, in ribonucleosides, the base used is often NMI and this generates mostly the 5'-*O*- phosphoramidates due to steric hindrance and the fact that it is a weaker base than *t*BuMgCl. However, in cases where the nucleoside has only one hydroxyl group, the base of choice is *t*BuMgCl, which often gives higher yields of the product as compared to NMI. Notably, the approach of coupling the nucleoside to a phosphorochloridate yields a mixture of diastereoisomers. In some cases, the two ProTide diastereoisomers exhibit similar potency and their development therefore is pursued as a mixture of diastereoisomers (see examples in Figure 10).⁶⁴ In other cases, the two

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3 diastereoisomers may have different rates of metabolism and hence varying potency. For
4
5 instance, the *Sp*-diastereoisomer of the clinically used ProTide sofosbuvir exhibits a 10-fold
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7 increase in antiviral activity than its *Rp*-diastereoisomer.³⁵ In these instances, the separation
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9 of the two diastereoisomers becomes necessary. The separation of these by column
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11 chromatography has proved to be a stern challenge.

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13 To address this, Ross et al⁶⁵ developed a system that allows for the facile generation and
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15 crystallisation of single isomer phosphoramidating reagents that could be coupled to
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17 nucleosides to yield pure single ProTide isomers. From this study, phosphorochloridates
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19 with a pentafluorophenol were found to be optimal since the desired *Sp*-isomer
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21 phosphorochloridate was readily crystallised, stable and allowed for the formation of the *Sp*-
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23 isomer ProTides, which is often the more active isomer.⁶⁵

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26 Continuing on the theme of synthetic procedures with stereocontrol of the phosphorous
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28 chirality of the ProTides, researchers from Merck recently reported the discovery of a small
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30 molecule catalyst that, upon the addition to the reaction of a nucleoside with a
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32 phosphorochloridate, leads to the generation of a single isomer with significant preference
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34 for the 5'-hydroxyl compared to the 3'-one.⁶⁶ However, the reported catalyst generates the
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36 *Rp*-isomer and not the *Sp*-one, which often shows better pharmacological activity. This
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38 represents a challenge for the wide use of this catalyst. Impressively, more work from Merck
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40 researchers led to the identification of various Lewis acids as being able to improve the
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42 yield, stereoselectivity and regioselectivity of the reaction of phosphorochlorides with
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44 nucleosides to generate ProTides.⁶⁷ Indeed, a selection of Lewis acids were studied and
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46 dimethyl aluminium chloride emerged as the optimal catalyst for this reaction.

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49 In the synthesis of nucleoside monophosphonate ProTides three different synthetic
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51 strategies are employed (Figure 5). The first strategy involves the *bis*-chlorination of a
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53 phosphate group to form a monophosphonate dichloridate. This is then followed by the
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55 addition of 1:1 molar equivalents of the phenol and amino acid ester yielding the desired
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57 ProTides. Alternatively, in the second synthetic strategy, the monophosphonate group is
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initially coupled to one phenol group using *N,N*-dicyclohexylcarbodiimide (DCC) and the product is then chlorinated. Finally the chloride group is substituted by the amino acid ester.

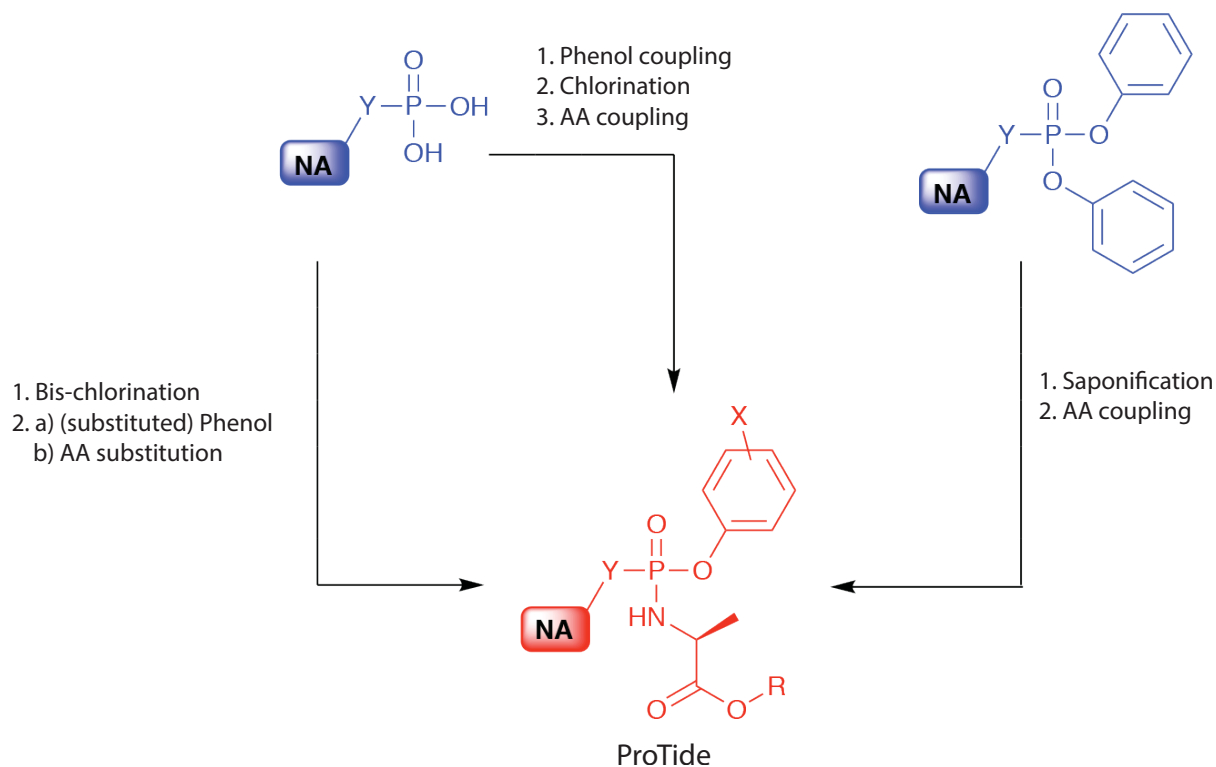


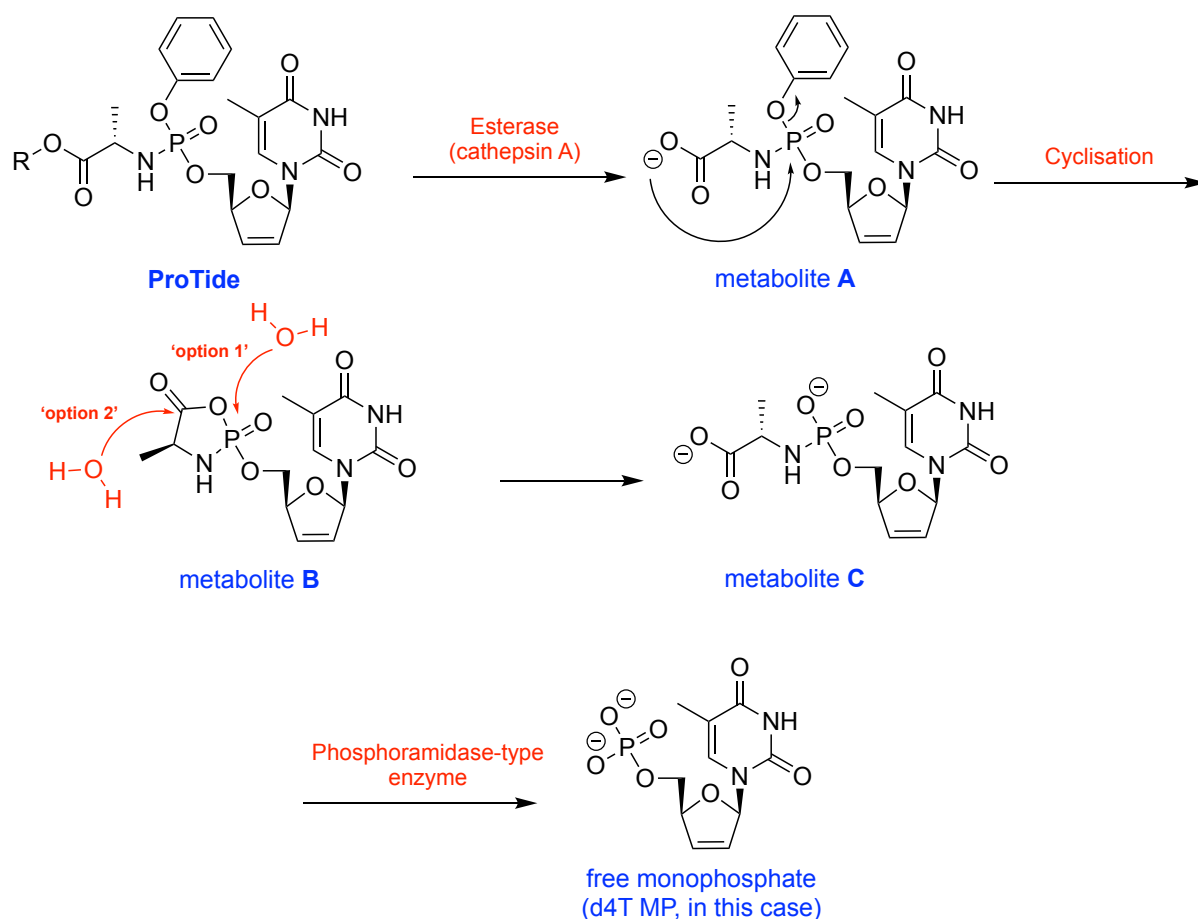
Figure 5. Synthetic strategies for accessing monophosphate ProTides. AA: amino acid. NA: nucleoside analogue. R: any ester. X: any aromatic substitution. Y: O or CH₂.

For the third synthetic strategy, the starting material employed is the diphenyl-masked nucleoside monophosphate, which first undergoes selective saponification that results in the removal of one of the phenyl masking groups. The product is subsequently coupled to the amino acid ester to generate the desired nucleoside analogue monophosphate ProTide. Further details on the synthesis of the ProTides can be found in Pradere *et al.*²⁹ and Serpi *et al.*⁶⁸.

2. Metabolism of the ProTides

Once inside the cell, the ProTides require metabolism to release the nucleoside monophosphate. The metabolic conversion of the 2',3'-didehydro-2',3'-dideoxythymidine (d4T)⁶⁹⁻⁷¹ monophosphate (d4T-MP) ProTide to the eventual active metabolite d4T-TP has been extensively studied in several cell systems, including human lymphocytes (Figure 6).

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3 Beside efficient formation of d4T-MP, d4T-diphosphate (d4T-DP) and d4T-triphosphate
4 (d4T-TP), an additional metabolite markedly accumulated upon exposure of the cell culture
5 to the d4T-MP ProTide, which was identified as being the alaninyl d4T-MP. This metabolite
6 may act as an intracellular depot form of d4T-MP, explaining not only the superior antiviral
7 activity of the ProTide *versus* the parental d4T, but also the marked retainment of antiviral
8 activity in thymidine kinase-deficient cells in which the parental d4T markedly loses antiviral
9 efficacy.^{72, 73} Also, the activation pathway of the ProTides of d4T-MP and AZT-MP containing
10 different amino acids and/or ester moieties has been investigated. It was found that overall,
11 there was a close correlation between the anti-HIV activity of these prodrugs and their
12 conversion rate to the amino acyl nucleotide metabolite (metabolite **C**, Figure 6).^{74, 75} The
13 metabolic conversion process is believed to be mediated by a two-step enzymatic process,
14 which eventually releases the active monophosphate (or monophosphonate) form of the
15 parent nucleoside as summarised in Figure 6.
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34 **Figure 6.** Postulated mechanism of ProTide metabolism illustrated using the ProTide of the
35 anti-HIV agent d4T as an example.
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40 The first step involves the cleavage of the amino acid ester by intracellular esterases.
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42 Researchers at Gilead Sciences identified cathepsin A as a major esterase that mediates
43 the hydrolysis of ester motifs of ProTides.⁷⁶ Such finding has made cathepsin A widely used
44 in the field in probing the hydrolysis of ProTides *in vitro*.^{64, 77-79} Notably, a second enzyme
45 that was able to hydrolyse the ester group of the two antiviral ProTides studied, TAF and 9-
46 (R)-4-(R)-[[[(S)-1-[(ethoxycarbonyl)ethyl]amino]phenoxy-phosphonyl]methoxy]-2-fluoro-1-
47 furanyladenine (GS-9131)⁸⁰ (structure not disclosed), was also reported in this study but its
48 identity was not determined.⁷⁶ In 2011, however, this identity of this second enzyme was
49 established and was shown to be carboxylesterase 1 (CES1).⁸¹ Although both cathepsin A
50 and CES1 were found to be expressed in primary hepatocytes, CES1 expression was
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3 undetectable in clone A replicon cells.⁸¹ This indicated that in primary human hepatocytes,
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5 both cathepsin A and CES1 are involved in the hydrolysing of the carboxyl ester motif of the
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7 ProTides, whereas in clone A cells, cathepsin A is the major enzyme that mediates such
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9 process. Beyond cathepsin A and CES1, a series of cysteine and (lysosomal) serine
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11 proteases have also been identified as being able to hydrolyse the carboxyl ester motif of
12
13 the ProTides in quiescent cells and peripheral blood mononuclear cells (PBMC).⁸²
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16 Earlier work by McGuigan and colleagues⁸³ into the efficiency of the ester group hydrolysis
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18 in various ProTides indicated that this is dependent on the structure of the ester moiety. In
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20 particular, it was noted that ProTides with a *tert*-butyl ester group were slower hydrolysed
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22 than those bearing other esters, e.g. methyl, isopropyl and benzyl, which correlated with the
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24 biological activity.⁸³ Although the lipophilicity of the ester motif seemed to give an increase in
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26 biological activity most likely due to increased cellular uptake, the rate of ester hydrolysis is
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28 another influencing factor in the ultimate biological activity of the ProTides.⁸³ For instance,
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30 ProTides with naphthyl esters yield more active ProTides than their phenyl ester
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32 counterparts due to higher lipophilicity and faster hydrolysis (Figure 7). However,
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34 propylbenzene esters, which are more lipophilic, are not as active as phenyl esters probably
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36 due to poor *in vivo* hydrolysis.
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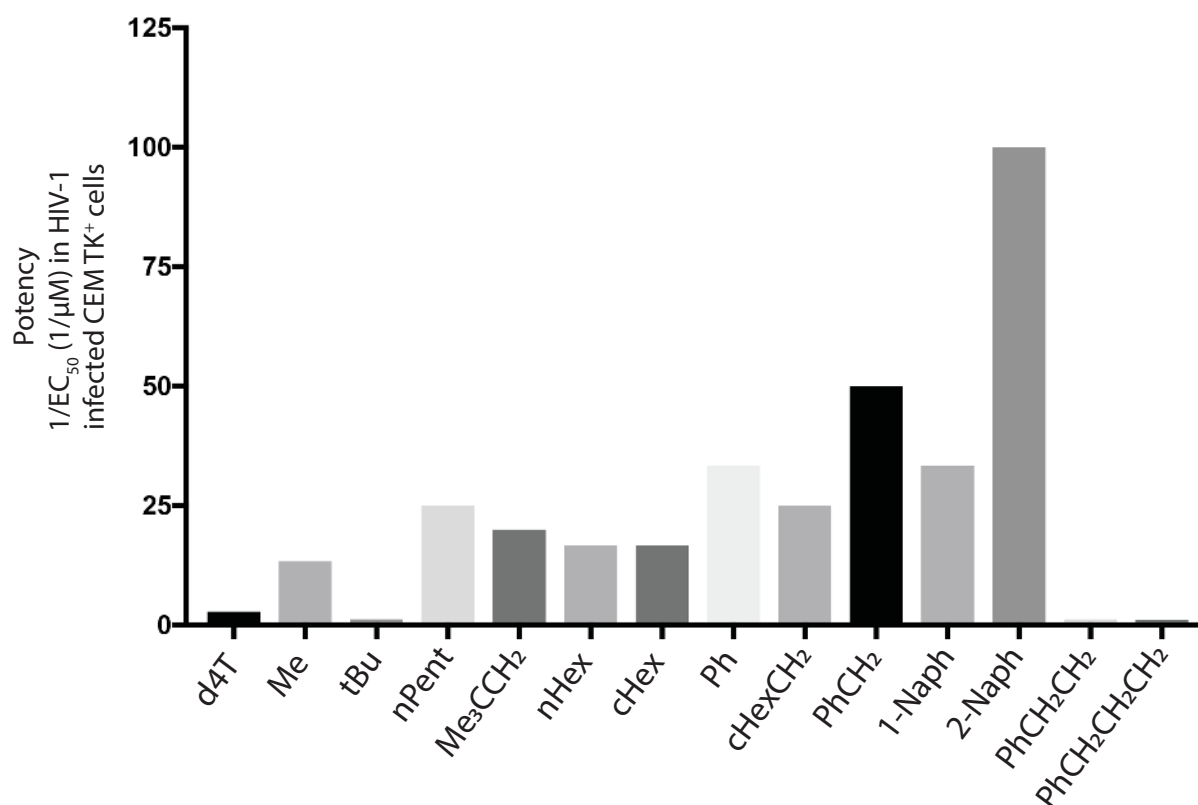


Figure 7. Effect of the ester motif on the ProTides' biological activity. The data presented regard d4T, ProTides and were obtained from McGuigan et al.⁸³

In addition to the ester group's influence on the overall lipophilicity of the ProTides and its effect on the biological activity, the aryl motif has significant impact on the lipophilicity. In McGuigan's work, the aryl group mostly used was the phenyl group though the naphthyl group has also been explored. The biological activity between these two aryl motifs often correlated with lipophilicity and hence the naphthyl-containing ProTides exhibit a better biological activity than their phenyl counterparts. Notably, substitutions on these aryl motifs exerts a significant impact on the biological activity (Figure 8). Electron-withdrawing substitutions were found to be associated with increased biological activity as compared to the electron-donating groups. The position on the aryl group that allows maximum electron-withdrawing effects, i.e. ortho and para, had more pronounced effects than the meta position.

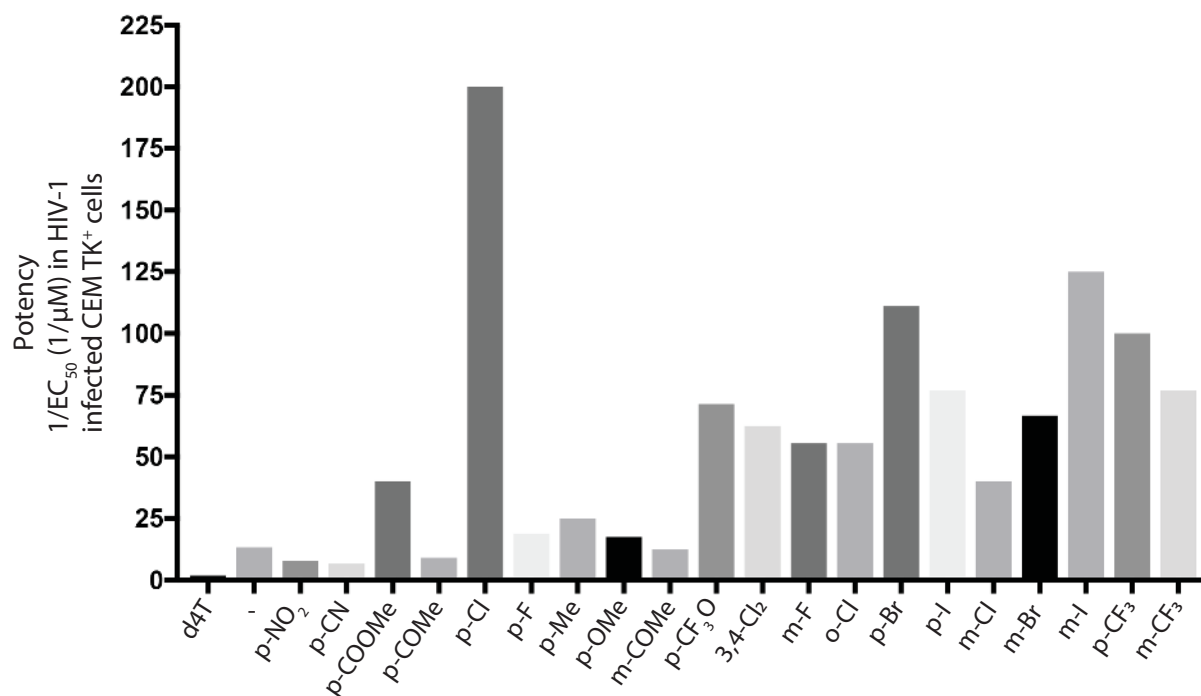


Figure 8. Effect of the aryl group substitutions on the ProTides' biological activity. The data presented regard d4T, ProTides and were obtained from Siddiqui et al.⁸⁴

Beyond the nature of the ester group, it has been shown that the nature of the amino acid of the ProTides also affects the rate of cathepsin A-mediated hydrolysis of the ester group (Figure 9).⁷⁶ Indeed, cathepsin A was unable to hydrolyse ProTides with branched amino acids such as valine and isoleucine. Subsequent studies showed that the nature of the amino acid side chain, primarily its size (bulkiness) and charge, has great influence on what enzyme hydrolyzes the ester motif.⁸² Indeed, an interesting structure-activity relationship emerged from the influence of the amino acid on the rate of the metabolism of ProTides. For instance, it was found that the ester motif of ProTides with alanine were efficiently processed by esterases (e.g. leukocyte elastase and proteinase 3) and serine proteases, but not cysteine, aspartic, or metalloproteases.⁸² Amino acids with bulkier side chains, e.g. phenylalanine and leucine, were more efficiently hydrolysed by chymotrypsin-related serine proteases such as cathepsin G and chymase.⁸²

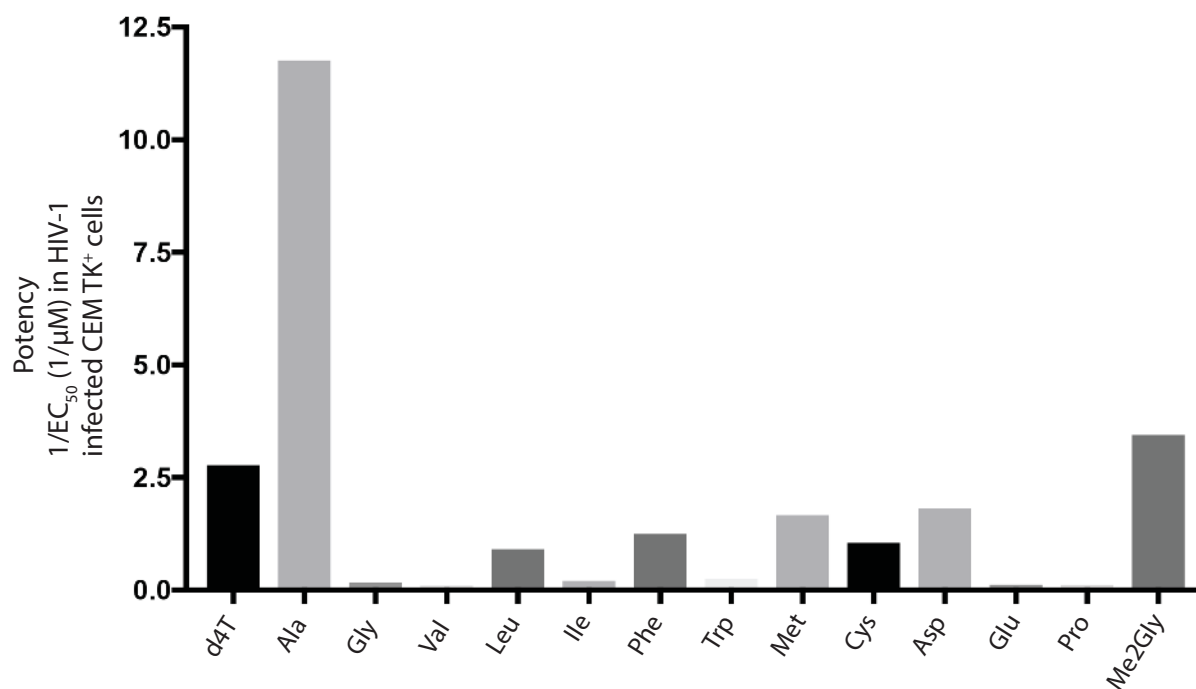


Figure 9. Effect of the amino acid on the ProTides' biological activity. The data presented regard d4T, ProTides and were obtained from McGuigan et al.⁸⁵

After the ester motif at the amino acid moiety is cleaved-off, a free carboxylate, which under physiological pH carries a negative charge, is generated. Subsequently, a nucleophilic attack by the carboxylate functionality on the phosphate or phosphonate group causes the loss of the aryl-leaving group yielding a highly unstable five-membered anhydride ring. The speed of the nucleophilic attack from the carboxylate on the phosphate or phosphonate group varies depending on the nucleoside. For instance, AZT metabolite **A** formation (Figure 6) was slower than that of d4T.⁷⁵ Interestingly, the hydrolysis of ProTides with β -alanine did not release the nucleoside monophosphate, since the hydrolysis did not proceed beyond the hydrolysis of the ester motif.⁷⁵ Metabolite **B** formation is followed by a rapid nucleophilic attack by a water molecule on the phosphate (option 1, Figure 6) or carbon of the carbonyl group of the amino acid (option 2, Figure 6) to open-up the ring and to generate the phosphoramidate metabolite (metabolite **C**, Figure 6). Although no compelling evidence exists to whether the water molecule attacks the phosphate or the carbon of the carbonyl

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3 group of the amino acid, which both yield the same product, the cleavage of the P-N bond at
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5 this stage has never been observed.⁸⁶
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8 The final step in the metabolism of the ProTides is the cleavage of the P-N bond by a
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10 second enzyme known as a phosphoramidase-type enzyme (histidine triad nucleotide
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12 binding protein 1,⁸⁷ HINT1).^{81, 88, 89} This eventually leads to the release of the nucleoside
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14 monophosphate/phosphonate. Notably, the cleavage of the P-N bond can be resistant to
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16 HINT1 as is the case with TAF. It is hypothesised that the hydrolysis of the P-N bond in this
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18 case proceeds because of the chemical instability of the P-N bond in the acidic environment
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20 of endosomes/lysosomes.⁹⁰
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22 23 **3. ProTides in Drug Discovery**

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25 Since the invention of the ProTide technology by the McGuigan laboratory in the early
26
27 1990s, this modality has been widely employed in the synthesis of many nucleoside
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29 monophosphate or monophosphonate ProTides. Although initially the structure activity
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31 relationship (SAR) studies were performed on pyrimidine nucleosides such as AZT and d4T,
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33 a wide variety of other ProTide derivatives have also been synthesized and antivirally
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35 evaluated. Notably, application of the phosphoramidate ProTide technology on purine
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37 nucleoside analogues such as 2',3'-dideoxyadenosine (ddA) and 2',3'-dideoxy-2',3'-
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39 dideoxyadenosine(D4A),⁹¹ carbocyclic adenosine derivatives,⁹² and carbovir/abacavir^{93, 94}
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41 often led to markedly enhanced antiviral potencies of such ProTides. More recently, it was
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43 even shown that ProTides of the antiherpetic acyclovir resulted in both anti-herpetic and
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45 anti-HIV activity,^{95, 96} and thus, enabled broadening of the antiviral spectrum of such drug. In
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47 this respect, it should be mentioned that the ProTide technology not only enables significant
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49 optimization of existing antiviral drugs, but it also allows conversion of apparently looking
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51 inactive molecules to active antivirals by the improved uptake and direct delivery of their
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53 activated (monophosphate) species. A series of ProTides have reached clinical trials³³ with
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55 two of them already approved for clinical use.¹³ To highlight the usefulness of this prodrug
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57 technology in drug discovery, key ProTides that are currently reported to be undergoing
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59 clinical trials and those that have received FDA-approval will be discussed.
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3 was found that its cytotoxic activity was significantly reduced, with the cytostatic activity
4 decreasing from 611 nM to > 2000 nM (CC₅₀). Contrastingly, even with blockage of hENT1,
5 the activity of **1** remained fairly constant (CC₅₀ = 162 μM), thus confirming that the cellular
6 uptake of **1** is independent of nucleoside transporters.⁶⁴ Thirdly, the structure of the ProTide
7 is such that it is able to overcome the rate-limiting first phosphorylation-mediated by
8 deoxycytidine kinase which is downregulated in certain cancers. This was confirmed by little
9 change in the activity of **1** when incubated with RT112 cancer cells in the presence of 2'-
10 deoxycytidine being the competing natural substrate for gemcitabine activation to its 5'-
11 monophosphate by 2'-deoxycytidine kinase (EC₅₀ = 0.7 μM in the presence of 2'-
12 deoxycytidine compared to 0.2 μM in the dCyd absence). Conversely, the activity of
13 gemcitabine decreased when 2'-deoxycytidine was co-administered as the two compounds
14 competed for the same active site on the deoxycytidine kinase.⁶⁴

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29 *In vivo* animal studies of compound **1** were conducted, focusing in particular on gemcitabine
30 (partially) resistant pancreatic cancers. The initial results obtained were very promising. In
31 mouse models with human tumour xenografts, **1** reduced the tumour size faster than
32 gemcitabine and significant reductions in tumour size were noticeable on day 7 post
33 administration of the ProTide. The body weight of the animals was also recorded to assess
34 their tolerance to the compound. Overall, ProTide **1** was associated with less than 4%
35 reduction in body weight and it was found that over the course of the treatment, the mean
36 body weight change was less pronounced in mice that were treated with **1** compared to
37 those treated with gemcitabine. This indicated that the gemcitabine ProTide was better
38 tolerated than the parent nucleoside analogue.⁶⁴ The pharmacokinetics of the ProTide were
39 also considerably better than those of gemcitabine: the half-life of **1** was 7.9 h compared to
40 1.5 h for gemcitabine. The ProTide also led to intracellular concentrations of the active
41 gemcitabine triphosphate metabolite that was 13 times higher than achieved by
42 gemcitabine.¹⁰⁰

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Phase I/II clinical trials were conducted involving 68 patients and showed that **1** was
effective in a variety of different cancers but most notably in patients with ovarian, pancreatic

and biliary cancers. Phase III studies will be conducted focusing on these cancers in particular. The ProTide was found to be stable *in vivo*, exhibiting a plasma half-life of 8.3 h. Maximum intracellular concentrations of the active metabolite were quickly achieved, peaked at 475 μM and were maintained for 24 h. In several patients, the tumour had shrunk by more than 30%, whereas in 33 patients, the disease had stabilised.¹⁰¹

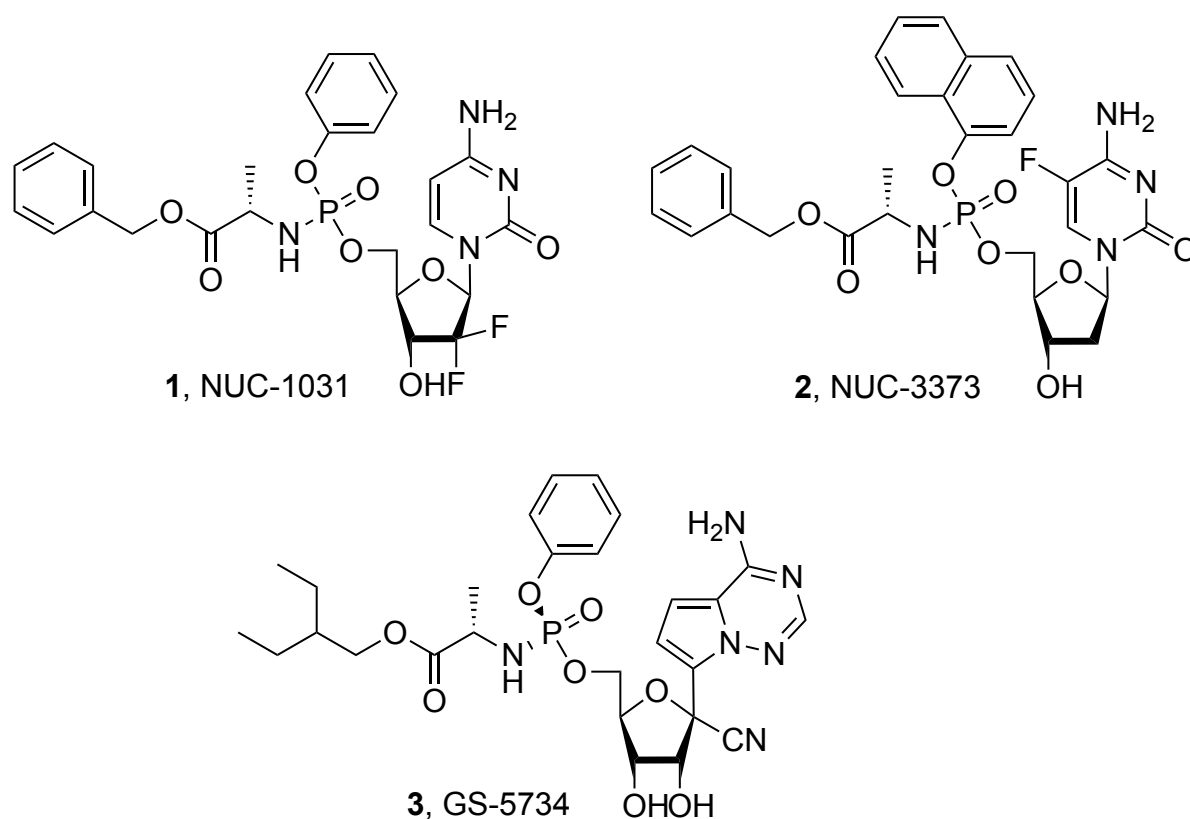


Figure 10. Chemical structures of key ProTides undergoing clinical trials.

5.1.2. Benzyl (((((2*R*,3*S*,5*R*)-5-(4-amino-5-fluoro-2-oxypyrimidin-1(2*H*)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate (NUC-3373)

Another ProTide currently being developed for clinical use by NuCana Biomed Ltd is NUC-3373 (**2**, Figure 10),^{79, 102} a phosphoramidate prodrug of 5-fluoro-2'-deoxyuridine (FdU). FdU is a metabolite which gets formed *in vivo* from the pharmacologically active anti-cancer agent 5-fluorouracil (5-FU). Initially discovered by McGuigan and his colleagues in 2011 after extensive SAR studies,⁷⁹ ProTide **2** displayed promising preclinical results and initial

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3 studies highlighted its superiority over its parent nucleoside, 5-FU. The path towards the final
4 structure of **2** began with McGuigan and his co-workers by synthesising and comparing 39
5 different ProTides, with variations in the aryl, ester and amino acid regions.⁷⁹ There was little
6 difference observed in the potency of the ProTides upon varying the amino acid ester region,
7 and also upon changing the nature of the amino acid. L-alanine has long been the amino
8 acid of choice when formulating ProTides, but there was little to discern between L-alanine
9 and the other amino acids in these experiments. Generally, the ProTides yielding the 1-
10 naphthyl group displayed more potency than phenyl-containing ProTides, thus leading to the
11 deduction that the 1-naphthyl, benzyl ester-containing ProTide was the most effective.⁷⁹ In
12 order to determine the cytotoxic activity of **2**, *in vitro* studies were conducted involving
13 human tumour cell lines (colorectal, lung, ovarian, acute lymphoblastic leukaemia and
14 cervix). Attention to thymidine kinase and the nucleoside transporter hENT1 was also given
15 to investigate the characteristics of cancer resistance. Xenograft mouse models were also
16 used to further analyse the anticancer activity of the ProTide.¹⁰³

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34 It was found that compound **2** was, on the whole, significantly more cytotoxic than 5-FU,
35 displaying IC₅₀ values 2- to 333-times lower in the majority of the tumor cell lines tested. The
36 ProTide also produced 363-times more of the active metabolite (FdU) than 5-FU, thus
37 further displaying its superiority in efficacy. Thymidine kinase inhibition only slightly affected
38 ProTide **2** (~ 4-fold) compared to FdU, that showed reductions in cytotoxicity of up to 136-
39 fold, respectively, indicating the independence of the ProTide for the FdU-activating enzyme.
40 Significant results were obtained with the colorectal tumour xenografts: ProTide **2** inhibited
41 tumour growth by 47%, compared to 25% tumour growth inhibition demonstrated by 5-FU.¹⁰³
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Compound **2** was found to overcome the main mechanisms of resistance that is associated
with 5-FU and other drugs within this family, such as decreased levels of the necessary
phosphorylating (activating) enzyme thymidine kinase, which is required to produce the
active metabolite; an overexpression of thymidylate synthase (an enzyme responsible for
DNA synthesis and a target for FdUMP (the active metabolite of 5-FU), altered levels of
thymidine phosphorylase, and reduced entry into the cells via nucleoside transporters.^{79, 102}

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3 ProTide **2** overcomes these pathways to resistance by preventing degradation by
4 phosphorolytic enzymes, bypassing the need to be activated by thymidine kinase and being
5 able to diffuse into cells, independent of nucleoside transporters such as hENT1.¹⁰⁴ Given
6 the encouraging results obtained in the pre-clinical studies, ProTide **2** entered phase I
7 clinical trials in the first quarter of 2016 in pursuance of determining the pharmacokinetic
8 profile of the ProTide, the safety of the compound in human participants and clinical activity
9 of **2** in tumour-bearing patients.¹⁰³ The trial itself consists of two parts: the first part
10 assessing the optimum dose that will maximise the risk/benefit reward to the participants
11 when given a weekly dose of the ProTide, administered as a single intravenous infusion.
12 The second part will assess the same as part one, except the dosing interval which will be
13 increased to fortnightly (www.clinicaltrials.gov, trial identifier: NCT02723240).

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16 5.1.3. 2-Ethylbutyl ((S)-(((2R,3S,4R,5R)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-5-cyano-
17 3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-L-alaninate (GS-5734)

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20 In 2014, there was a mass outbreak of the Ebola virus, most prevalent in Western Africa,
21 although there were a number of reported cases in the USA and the United Kingdom. In
22 West-Africa alone, there were a total of 28,616 confirmed cases in Guinea, Liberia and
23 Sierra Leone, leading to 11,310 deaths (WHO 2016). To this date there is no drug treatment
24 available on the market to treat this disease. However, GS-5734 (**3**, Figure 10)¹⁰⁵, developed
25 by Gilead Sciences, has shown promising results as a potential treatment for the Ebola
26 virus. Although most of the interest in this compound has been due to its activity against
27 Ebola, recently, **3** was reported to be a broad-spectrum antiviral agent since it exhibited
28 potent antiviral activity against a variety of viruses including filoviruses, coronaviruses, and
29 paramyxoviruses.¹⁰⁶

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32 Compound **3** is a ProTide of an adenosine analogue which, following intracellular delivery,
33 gets metabolised into the pharmacologically active nucleoside triphosphate form, which then
34 inhibits viral RNA dependent RNA polymerase.¹⁰⁵

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37 The activity of **3** was initially tested using cell-based assays, followed by the administration
38 of the ProTide to rhesus monkeys that were infected with the Ebola virus. Rodent studies

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3 were deemed not suitable to determine the pharmacokinetics of **3** due to the high serum
4 concentrations of esterases present in many rodent species, which would prematurely
5 degrade the ProTide and impact on the findings. Thus, rhesus monkeys were chosen as
6 they closely resemble humans and they do not express a high serum esterase
7 concentration.¹⁰⁵ 10 μM of **3** was incubated in a 2 h-pulse or 72 h-continuous incubation of
8 Ebola virus-infected human primary macrophages, endothelial cells and liver cells to deduce
9 its activity. 10 μM of the nucleoside analogue was also incubated for 72 h in human
10 monocyte-derived macrophages for comparison. ProTide **3** was found to be rapidly
11 metabolised in the primary macrophages, with maximum intracellular concentrations of
12 nucleoside triphosphate being achieved after 4 h post incubation, with a half-life of 16 h. It
13 was found that **3** displayed an EC_{50} value against Ebola virus of 0.086 μM in the human
14 primary macrophages, 0.14 μM in the endothelial cells and 0.07 μM in the Huh-7 liver cells.
15 In comparison, the nucleoside analogue displayed an EC_{50} value of greater than 20 μM in
16 both the human macrophages and endothelial cells, and 1.5 μM in the liver cells. The
17 cytotoxic concentration (CC_{50}) of the ProTide in all cell lines was above 20 μM . The different
18 cell lines were also infected with different variants of the Ebola virus (Makona and Kikwit
19 variants) and other filoviruses. The cell-based assays gave strong indication that compound
20 **3** would be effective in treating the other viruses as well.¹⁰⁵

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The pharmacokinetics of **3** were examined in rhesus monkeys. After being administered
intravenously with 10 mg/kg of the ProTide, **3** displayed a very short plasma half-life of just
0.39 h. There was rapid distribution into the peripheral compartments within 2 h post
administration, and a half-life in the peripheral blood mononuclear cells of 14 h was
observed. Levels required to achieve a 50% inhibition of the Ebola virus were maintained for
24 h.¹⁰⁵ Compiling the pharmacokinetics results and tissue distribution data led to the
conclusion that once daily dosing would be effective to achieve a clinical effect.

In order to assess the efficacy of the ProTide, a blinded placebo-controlled study was
conducted involving 12 Ebola-infected rhesus monkeys (6 per treatment group) in two
different modalities.¹⁰⁵ The first part involved intramuscular administration of **3** either on day

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3 0 (same day as being infected with Ebola), or day 2. Part two of the study administered a
4 loading dose of 10 mg/kg followed by once daily doses of 3 mg/kg, commencing at various
5 days after virus exposure or 10 mg/kg administered on day 3 post virus exposure. All of the
6 monkeys given **3** survived until the end of the trial; 50% survival was noted with the treated
7 group of animals given 3 mg/kg compared to no survival in the placebo group. It was noted
8 that monkeys given the 10 mg/kg dose after day 3 had 100% survival along with reduced
9 signs associated with the Ebola virus (thrombocytopenia, coagulopathy).¹⁰⁵

10 Given the promising results obtained in the rhesus monkey study, a phase II clinical trial is
11 due to be conducted in Liberia. Sponsored by the National Institute of Allergy & Infectious
12 Diseases, 60 to 120 men infected with Ebola virus will be recruited and will be given a five-
13 day course of **3**, administered once daily, followed by monitoring over a six-month period to
14 determine the clearance of virus from a persistent genital reservoir (www.clinicaltrials.gov,
15 trial identifier: NCT02818582).

31 **5.2. ProTides as Drugs**

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33 There have been two ProTide drugs approved by the FDA for clinical use. Sofosbuvir
34 (Sovaldi™), Figure 3, was approved by the FDA in December 2013 for the treatment of
35 HCV.¹⁰⁷ Sofosbuvir is a phosphoramidate prodrug of 2'-deoxy-2'-fluoro-2'-C-methyluridine,
36 the parent nucleoside. Having emerged as the superior ProTide out of 50 other ProTides
37 developed,³⁵ sofosbuvir was acquired by Gilead Sciences, where it adopted the identity
38 sofosbuvir and underwent further extensive clinical evaluation. Sofosbuvir was found to act
39 by inhibiting the HCV NS5B polymerase, an enzyme responsible for the replication of the
40 viral genome. Inhibition of this enzyme would result in RNA chain termination and ultimately
41 stop the replication of HCV.³⁵ Following cellular uptake by hepatocytes, sofosbuvir
42 undergoes sequential hydrolysis by cathepsin A and CES1 to form the active nucleoside
43 monophosphate. Further hydrolysis by HINT1 and subsequent phosphorylations yielded the
44 pharmacologically active triphosphate metabolite, that exerts its action on NS5B
45 polymerase.¹⁰⁸ Initial animal studies of sofosbuvir were encouraging. The ProTide was well
46 absorbed following oral administration in portal vein cannulated dogs, bioavailability was

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3 9.9%, which equated to a fraction absorbed of 36%.¹⁰⁹ In human subjects, the absolute
4 bioavailability was not determined, although over 80% of the administered dose was
5 absorbed. The absorption was rapid, with maximum concentrations (C_{max}) of sofosbuvir
6 achieved 0.5-2 h post oral administration and C_{max} of the inactive parent nucleoside
7 metabolite was achieved 2-4 h post administration and a half-life of 0.48-0.75 h. *Ex vivo*
8 studies of the distribution of the anti-HCV agent showed a plasma protein binding of around
9 82% in healthy subjects and 85% in subjects with end stage renal disease.¹⁰⁸

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Studies using the diastereomeric mixture of sofosbuvir in humans showed that it was rapidly
absorbed and metabolised in the liver according to the typical *in vivo* degradation of the
ProTides (see Figure 6). Briefly, the isopropyl ester motif was hydrolysed into the naked
carboxylate group and then the P-N bond of the phosphoramidate group was cleaved-off to
afford parent nucleoside monophosphate.¹¹⁰ Notably, the parent nucleoside, an inactive
metabolite, was detectable in the plasma and remained detectable for at least 24 h following
the 100 and 800 mg dosing of the diastereomeric mixture of sofosbuvir.¹¹⁰ Although
sofosbuvir showed little indication of being excreted renally (3.5% recovered in urine; renal
clearance = 0.238 L/min), the inactive parent nucleoside was the predominant compound
present in the urine (77.7%), indicating excretion via the kidneys. This was further confirmed
when studies were conducted observing the pharmacokinetics of sofosbuvir in patients with
impaired renal function – the renal clearance of the parent nucleoside was around 2-fold
higher than the glomerular filtration rate, signifying active renal excretion of the metabolites
of sofosbuvir.¹⁰⁹ Phase II studies of sofosbuvir in combination with pegylated interferon and
ribavirin established the doses required to provide an optimal effect, whilst minimising any
potential adverse effects.¹¹¹ Initial studies postulated that 200 mg or 400 mg doses of
sofosbuvir with pegylated interferon and ribavirin were most effective, as 100 mg dosing was
found to be sub-optimal due to low viral load decline than observed with the 200 mg and 400
mg doses. At these doses, the incidence of adverse effects was low, with the most frequent
adverse effects being fatigue and nausea.¹¹¹ Coupled with the short half-life of sofosbuvir,
once daily dosing was recommended as there was little accumulation of the drug.¹⁰⁹

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3 The safety of sofosbuvir was later confirmed in phase II clinical trials. It was postulated that
4 as sofosbuvir is not metabolised via the cytochrome P450-3A4 enzymes, drug-drug
5 interactions would be minimal. However, because it seems to induce the P-glycoprotein
6 efflux pump, caution was advised with co-administration of carbazepine and rifabutin as the
7 concentrations of sofosbuvir can decrease significantly.^{112, 113}
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10 Single-group, open label phase III study of treatment-naïve patients with HCV genotypes 1,
11 4, 5 and 6 treated with sofosbuvir plus pegylated interferon and ribavirin for 12 weeks
12 indicated high potency of sofosbuvir.¹⁰⁷ Indeed, 80% of cirrhotic patients versus 92% of
13 noncirrhotic patients achieved a viral load below the lower limit of quantifications 12 weeks
14 after completion of HCV treatment (SVR12). This indicated the efficacy of sofosbuvir in
15 treating different HCV genotypes confirming similar earlier preclinical observations.¹¹⁴ In a
16 placebo-controlled phase III study comparing sofosbuvir and ribavirin for 12 weeks with
17 matched interferon ineligible controls in genotype 2 and 3 patients, sustained viral response
18 was achieved in 78% of treated patients with 93% response rate in genotype 2 patients and
19 61% in genotype 3 patients.¹¹⁵ In a study involving patients who had previously been treated
20 with interferon who were treated with either sofosbuvir and ribavirin for 12 weeks followed
21 by placebo for 4 weeks or sofosbuvir and ribavirin for 16 weeks, response rates showed a
22 73% SVR12 in the 16-week arm compared to 50% of the 12-week arm. Impressively, 94%
23 of genotype 2 patients achieved SVR12 within 12 weeks of treatment with sofosbuvir and
24 ribavirin, while 87% achieved it within 16 weeks. As for genotype 3 patients, 30% of patients
25 achieved SVR12 within 12 weeks compared to 62% within 16 weeks. Notably, 46% of the
26 patients treated with sofosbuvir and ribavirin for 12 weeks had viral relapse compared to
27 27% of the 16-week treatment arm. In another study, the efficacy of extended treatment of
28 24 weeks of sofosbuvir plus ribavirin in genotype 3 patients was evaluated.¹¹⁶ The results
29 showed that 93% of the 73 patients with genotype 2 who took part achieved SVR12 within
30 12 weeks of sofosbuvir and ribavirin treatment, whereas 85% of the 250 patients with
31 genotype 3 treated for 24 weeks with sofosbuvir and ribavirin achieved SVR12. This was the
32 first large scale study showing the efficacy of prolonged interferon-free treatments in
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3 genotype 3 patients. Given the excellent safety profile and pan-genotypic efficacy of
4 sofosbuvir, it has been licenced for clinical use in combination with pegylated interferon,
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11 ribavirin and the NS5A inhibitors ledipasvir and velpatasvir depending on the HCV genotype
12 to be treated.¹¹⁷ Collectively, this has made sofosbuvir a cornerstone in HCV therapy.

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TAF, Figure 3, is the latest ProTide-based drug approved by the FDA for the treatment of
HIV.¹¹⁸ Developed by Gilead Sciences Inc., TAF is a ProTide of the acyclic nucleoside
phosphonate drug tenofovir. Another prodrug of tenofovir, tenofovir disoproxil fumarate
(TDF), had long been an FDA-approved treatment for HIV, however, the ProTide TAF
demonstrated increased anti-HIV activity, higher *in vivo* stability and was endowed with
lesser side-effects than both tenofovir and TDF.¹¹⁸ TAF is specifically accumulating in
lymphatic tissue, and in the liver, and thus, holds also great potential for the treatment of
HBV infections. Markedly lower plasma levels but significantly higher intracellular tenofovir
concentrations are observed with TAF as compared to TDF, explaining the lower incidence
of tenofovir-associated side-effects with respect of bone mineral density losses and kidney
nephrotoxicity.¹¹⁹ The drug itself becomes phosphorylated following cellular uptake into the
pharmacologically active diphosphate form and is a potent inhibitor of reverse transcriptase.
Inhibition of this enzyme results in DNA chain termination and viral replication ceases to
progress.¹²⁰

Early studies of TAF were conducted in dogs in order to characterise its pharmacokinetic
profile and understand its permeability and efficacy as a potential treatment for HIV. Initial *in*
vitro studies using hepatic and intestinal S9 fractions from both humans and dogs yielded
similar results, highlighting the suitability of using dogs for the pre-clinical studies. *In vitro*
experiments showed TAF to have half-lives of 31 minutes in both dog intestinal and hepatic
S9 fractions, a plasma half-life of 92 minutes and a half-life of 28.3 minutes in an MT-2 cell
extract.⁸⁰ 5 mg/kg of TAF were administered to the dogs; plasma concentrations peaked at
1.58 μM , achieved at 0.14 h post administration. The levels of TAF decreased rapidly
(undetectable after 2 h post administration), which was accompanied by an increase in
tenofovir levels. Rapid accumulation of the active metabolite tenofovir diphosphate in the

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3 peripheral blood mononuclear cells followed, with peak concentrations of 18 μM achieved at
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5 1 h post administration. The bioavailability of TAF was found to be dependent on the
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7 administered dose, with bioavailabilities of 1.7% and 24.7% at 2 mg/kg and 20 mg/kg,
8
9 respectively.¹²¹ However, other studies found that oral administration of 10 mg/kg of TAF
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11 yielded a bioavailability of 17% when compared to the intravenous dose,⁸⁰ with the majority
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13 of the drug metabolised in the kidneys and excreted in the urine.¹²²

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16 An earlier study conducted by Eisenberg et al. in 2001¹²³ compared the activity of the
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18 ProTide with tenofovir and its prodrug TDF. Following incubation with peripheral blood
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20 mononuclear cells, it was found that the levels of tenofovir and its metabolites were 10-30
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22 times higher compared to TDF and tenofovir, respectively.¹²³ The *in vitro* anti-HIV activity of
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24 TAF was determined to be 1000-fold and 10-fold greater than tenofovir and TDF.⁸⁰

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27 Following successful *in vitro* assessments of TAF, the ProTide progressed into a number of
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29 phase I and II trials, where the *in vivo* pharmacokinetics/dynamics of the ProTide was
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31 evaluated and compared to its predecessors, along with establishing the optimum dose of
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33 TAF. An initial study was conducted by Markowitz et al.,¹²⁴ where either 40 mg or 120 mg of
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35 TAF or 300 mg of TDF were administered once daily for 14 days and the activity of both
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37 prodrugs as monotherapy for the HIV-1 infection was compared. The study showed that both
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39 40 mg and 120 mg of TAF resulted in higher intracellular concentrations of tenofovir
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41 diphosphate in PBMC than tenofovir. TAF also produced a greater net change in HIV-1 RNA
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43 compared to TDF, thus illustrating its superiority over the existing prodrug in terms of
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45 potency.¹²⁴

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48 Phase II/III studies of TAF focused on its efficacy in different patient groups including, but
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50 not limited to, adults and adolescents. As tenofovir is eliminated via the kidneys, it was
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52 imperative to understand how renal function would affect both the accumulation of tenofovir
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54 in the body and also its excretion. TDF has been associated with effects on the bone and
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56 renal toxicity, leading to decreased glomerular filtration rates, increases in serum creatinine
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58 and increased risk of adverse renal events. This toxicity was attributed to its effect in the
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60 proximal tubule, with efficient uptake from plasma but less efficient efflux into urine resulting

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3 in accumulation of tenofovir in the body.¹²⁵ Therefore, a Phase 3 double-blinded study was
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5 conducted in which TAF was administered to patients with impaired renal function. Patients
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7 were given either 10 mg of TAF or 300 mg of TDF, each co-formulated with elvitegravir,
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9 cobicistat and emtricitabine. The study found that participants on the TAF treatment regime
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11 displayed a smaller increase in creatinine, less proteinuria, and significant decreases in
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13 bone mineral density.¹²⁶

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16 TAF was demonstrated to be superior not only in its antiviral activity but also in the fact that
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18 therapeutic effects could be achieved at much lower doses than TDF, thus reducing the
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20 incidence of adverse effects. This led to the FDA approval of the ProTide in the treatment of
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22 HIV-1 in a combination therapy with other anti-HIV agents.¹¹⁸ Moreover, in November 2016,
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24 the FDA granted approval for a once-daily treatment of 25 mg TAF in patients suffering of
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26 chronic HBV infection (Gilead Sciences 2016). This followed extensive studies which
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28 highlighted the ProTides superiority over current therapies used to treat not only HIV, but
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30 also chronic HBV infections.¹²⁷

31 32 33 **6. Conclusions and Outlook**

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35 The ProTide technology has facilitated the discovery and application of a variety of
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37 nucleoside monophosphate and monophosphonate analogues in the clinic. It took many
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39 years of trial and error, mainly by the McGuigan group, to optimise the masking groups of
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41 the phosphate to yield the successful ProTide technology as we know it today. Although this
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43 technology has largely been used in the discovery of nucleoside-based ProTides as antiviral
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45 and anti-cancer agents, recently it was applied in the early discovery of nucleoside-based
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47 ProTides for Parkinson's disease.⁷⁸ Additionally, the ProTide prodrug approach is receiving
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49 increasing attention as a prodrug strategy for the intracellular delivery on mono-
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51 phosphorylated non-nucleoside compounds such as glucosamine,^{128, 129} Sphingosine 1-
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53 phosphate (S1P),¹³⁰ 4-phospho-D-erythronhydroxamic acid¹³¹ and 5-phospho
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55 erythronhydroxamic acid¹³². Together, these examples show the broad usefulness of this
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57 prodrug technology and highlight its potential in the discovery of non-nucleoside
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59 monophosphate and monophosphonate prodrugs as potential therapeutics as well.
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3 Regardless of the substrates to which the ProTide technology is applied to either
4 nucleosides or non-nucleosides, it has already proven to be a powerful technology in drug
5 discovery and efficient intracellular delivery of the (activated) parental drug. Given its
6 expanding applications, there is a real possibility that this technology will deliver new
7 (nucleoside and non-nucleoside) ProTides for the treatment of a variety of other diseases
8 beyond microbial infections and cancer.
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18 **Abbreviations**

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20 5-FU, 5-fluorouracil; AraA, 9- β -D-arabinofuranosyladenine; AraC, β -D-
21 arabinofuranosylcytosine; AZT, 3'-azidothymidine; d4A, 2',3'-didehydro-2',3'-
22 dideoxyadenosine; CES1, carboxylesterase 1; d4T, 2',3'-didehydro-2',3'-dideoxythymidine;
23 ddC, 2',3'-dideoxyadenosine; ddC, 2',3'-dideoxycytidine; DCC, N,N'-
24 Dicyclohexylcarbodiimide; FDA, Food and Drug Administration; FdU, 5-fluoro-2'-
25 deoxyuridine; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; HBV, hepatitis B virus;
26 HCMV, human cytomegalovirus; HCV, hepatitis C virus; hENT1, human equilibrative
27 nucleoside transporter 1; HINT1, Histidine triad nucleotide-binding protein 1; HIV, human
28 immunodeficiency virus; HSV, herpes simplex virus; NA, nucleoside analogue; NMI, N-
29 methylimidazole; PK, Pharmacokinetics, SVR12, the lower limit of quantifications 12 weeks
30 after completion of HCV treatment; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil
31 fumarate; SAR, structure-activity relationship; tBuMgCl, *tert*-butyl magnesium chloride; TK,
32 thymidine kinase.
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51 **Dedication**

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53 This review was written in memory of Prof. Christopher McGuigan (1958-2016), who
54 provided the basis for the ProTide technology.
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Biographies

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3 **Youcef Mehellou** obtained his M.Pharm. (Pharmacy) degree from King's College London in
4
5 2005. This was followed by a Ph.D. on 'ProTide' chemistry under the supervision of Prof.
6
7 Chris McGuigan in Cardiff University, U.K. Following postdoctoral work initially with Prof.
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9 Sidney M. Hecht in Arizona State University (2009/2010) and then with Prof. Dario R. Alessi
10
11 FRS in the University of Dundee, U.K., (2010/2013) Youcef moved to the University of
12
13 Birmingham, U.K., in early 2013 as a Lecturer in Medicinal Chemistry. Since January 2017,
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15 Youcef has been a Lecturer in Medicinal Chemistry in Cardiff University, U.K., where his
16
17 group is focusing on the discovery of small molecule modulators of signal transduction and
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19 the development of novel phosphate and phosphonate prodrug therapeutics.
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23 **Hardeep S. Rattan** is currently a fourth and final year Pharmacy student in the University of
24
25 Birmingham, U.K. He has a keen interest in medicinal chemistry particularly the discovery
26
27 and development of nucleoside and nucleotide therapeutics. Hardeep's final year Masters
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29 research project focused on the ProTide prodrug technology and was carried out under the
30
31 supervision of Dr. Mehellou.
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34 **Jan Balzarini** graduated at the KU Leuven (Belgium) as Master in Biology (1975), Master in
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36 Bioengineering (1977) and PhD in Bioengineering (1984). He is affiliated to the Rega
37
38 Institute for Medical Research at the KU Leuven. In 1984, he became tenured, performed a
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40 postdoctoral stay at the National Cancer Institute of the NIH (1985/1986) with Sam Broder
41
42 and David G. Johns. In 2001, he was awarded the prestigious René Descartes Research
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44 Prize of the European Commission (in collaboration with several European Research
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46 Teams) and he received several other (inter)national awards. In 2001, he became full
47
48 professor at the KU Leuven; in 2006, he was appointed as Laboratory Head of Virology and
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50 Chemotherapy at the Rega Institute; and in 2014, he retired as Emeritus Professor.
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Table of Contents Graphic

