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Nucleoside Phosphate and Phosphonate Prodrug Clinical Candidates†

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†In memory of Prof. Chris McGuigan (1958-2016)

Abstract
Nucleoside monophosphates and monophosphonates have been known for a long time to exert favorable pharmacological effects upon intracellular delivery. However, their development as drug molecules has been hindered by the inherent poor drug-like properties of the monophosphate and monophosphonate groups. These include inefficient cellular uptake and poor in vivo stability, with this latter drawback being most relevant to monophosphates than monophosphonates. To address these limitations, numerous monophosphate and monophosphonate prodrug strategies have been developed and applied in the discovery of nucleoside monophosphate and monophosphonate prodrugs that can treat viral infections and cancer. The approval of sofosbuvir, a nucleoside monophosphate prodrug, highlighted the success to be had by employing these prodrug technologies in the discovery of nucleotide therapeutics. In this Miniperspective, we discuss the different key monophosphate and monophosphonate nucleoside prodrugs that entered clinical development, some of which may in the future be approved to treat various human diseases.
1. Introduction

The effectiveness of nucleoside analogues in treating cancer and various infections that are caused by the human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV, respectively) was established a few decades ago. A significant number of nucleoside analogues are now used daily in the clinics to treat these diseases. These molecules exert their therapeutic effects after being converted in vivo into their mono-, di- and triphosphates (Figure 1A). Since nucleoside analogues are structurally different from natural nucleosides, their phosphorylation by nucleoside/nucleotide kinases to generate the active metabolites is often of limited efficiency. This, as a result, has limited the therapeutic efficacy of many of these therapeutic agents. To overcome this shortcoming, it was initially thought that delivering the phosphorylated metabolites of these nucleoside analogues would overcome the kinase-dependent phosphorylation steps and hence achieve better potency. However, the introduction of phosphate groups (mono-, di- or tri-) into these nucleoside analogues made them more polar resulting in decreased transport into cells (Figure 1B).

**Figure 1.** (A) A general representation of the intracellular activation of nucleoside analogues by phosphorylation to yield their pharmacologically active metabolites. (B) Nucleoside monophosphates and monophosphonates are not efficiently transported into cells. (C) Masked nucleoside monophosphates and monophosphonates cross the cell membrane, undergo a de-masking step to release the monophosphate or monophosphonate derivatives,
which are subsequently further phosphorylated to the active triphosphate/phosphonodiphosphate species.

Additionally, the poor in vivo stability of the P-O bonds of phosphate groups has hindered their use. To address this latter point, the conversion of the α-phosphate groups of monophosphorylated nucleoside analogues into the more stable phosphonate groups, which have relatively longer half-lives, was adopted. This approach led to the successful development of numerous nucleoside analogue phosphonates such as cidofovir (1, Figure 2) and tenofovir (2, Figure 2). Still, the polar nature of the phosphonate group at physiological pH (< 7.4), similar to phosphate groups, limits their transport into cells.

Figure 2. Chemical structures of key phosphonates and mono(phosphate/phosphonate) prodrugs approved for clinical use.

As the first phosphorylation step, which converts nucleoside analogues into their monophosphate counterparts, is often regarded as the rate-limiting step in their bioactivation, a number of prodrug technologies that mask monophosphate and monophosphonate groups to increase their lipophilicity and thus improve their cellular uptake have been developed. These technologies have been used with success in the discovery of numerous nucleoside monophosphate and monophosphonate prodrugs that are currently used to treat viral infections in humans, e.g. HIV, HBV and HCV.

The latest nucleoside monophosphate and monophosphonate prodrugs approved by the US Food and Drug Administration (FDA) are sofosbuvir (3, Figure 2) and tenofovir alafenamide (4, Figure 2). Sofosbuvir is now used to treat patients with HCV while
tenofovir alafenamide is used in combination with other antiretrovirals for the treatment of HIV-1 infections and is also being pursued as a possible treatment for HBV.\textsuperscript{17, 18} The success of these two prodrugs highlighted again the application of monophosphate and monophosphonate prodrug technologies as a powerful strategy in the discovery of nucleotide therapeutics. Herein, we will discuss the different monophosphate and monophosphonate prodrug strategies that have delivered clinical nucleotide candidates.

2. Pronucleotide Clinical Candidates
The key ten monophosphate and monophosphonate prodrugs that will be discussed in this Miniperspective employ a range of phosphate and phosphonate masking groups that are enzymatically cleaved off inside cells to release the monophosphate or monophosphonate species. Subsequent phosphorylation of these compounds yields the active di- or triphosphate derivatives, which in turn produce the desired therapeutic effects as illustrated in Figure 1C. Notably, these monophosphate and monophosphonate prodrug approaches differ in the type of the masking group(s) as well as their \textit{in vivo} demasking mechanisms.

2.1. Phosphoramidate (ProTide) prodrugs
Considering the pipeline of nucleotide prodrugs undergoing clinical trials, it is clear that the majority of them employ the phosphoramidate (ProTide)\textsuperscript{19} technology that was invented by Prof. Chris McGuigan (Cardiff University, UK). In the ProTide approach, the negative charges of the phosphate or phosphonate group are masked by an aryl motif and an amino acid ester group (Figure 3). Upon cell entry, the masking groups are cleaved off in two enzymatic steps to release the nucleoside analogue monophosphate or monophosphonate. The first metabolic step is initiated by esterases that cleave the ester motif of the ProTide. Under physiological pH (< 7.4), the unmasked, negatively charged carboxyl group carries out a nucleophilic attack on the phosphate or phosphonate group. This results in the leaving of the aryl motif and the formation of a highly unstable five-membered ring. A nucleophilic attack from a water molecule opens up this heterocyclic ring leading to the generation of a phosphoramidate metabolite. A second enzyme, termed phosphoramidase-type enzyme
(histidine triad nucleotide-binding protein 1, HINT-1),\textsuperscript{20} mediates the cleavage of the P-N bond of this metabolite leading to the release of the nucleoside analogue monophosphate or monophosphonate.\textsuperscript{21-23}

Figure 3. Postulated mechanism of ProTides \textit{in vivo} breakdown to release the nucleoside analogue monophosphate or monophosphonate.

This particular prodrug approach was adopted in the discovery of ProTides sofosbuvir and tenofovir alafenamide (Figure 2) with numerous other nucleoside ProTides entering clinical trials (Figure 4).

Figure 4. Chemical structures of key nucleoside ProTides that entered clinical trials.
2.1.1. NUC-1031 and NUC-3373

NUC-1031 (5, Figure 4)\textsuperscript{24} is an aryloxy triester phosphoramidate (ProTide) of the anticancer drug gemcitabine (Gemzar\textsuperscript{\textregistered}). The 5'-phosphate group is masked by a phenyl group and an L-alanine benzyl ester motif. This experimental medicine is currently being developed by NuCana Biomed Ltd. for the treatment of lung, ovary, breast, colon and pancreatic cancers. Strikingly, this ProTide was effective in treating cancers that are resistant to the parent therapeutic nucleoside gemcitabine.\textsuperscript{24} The ability of ProTide 5 to overcome gemcitabine resistance\textsuperscript{25} was found to be due to three main reasons. First, compound 5 was able to overcome active cellular uptake mechanisms that limit the efficacy of gemcitabine as the prodrug is more lipophilic and enters cells through passive diffusion.\textsuperscript{24, 26} Second, the delivery of gemcitabine monophosphate using the ProTide approach overcame the dependency on the essential first activation step by deoxycytidine kinase, which is downregulated in some cancers.\textsuperscript{24, 26} Third, the delivery of gemcitabine monophosphate limited the deamination of the cytosine nucleobase, which generates the inactive uracil nucleos(t)ide metabolite, a process that reduces the efficacy of gemcitabine.\textsuperscript{24, 26}

Initial animal in vivo characterisation of 5 focused on pancreatic cancers that are either partially responsive or resistant to gemcitabine. These studies showed that 5 was superior to gemcitabine in two mouse xenograft models as it reduced the tumour size faster and the therapeutic effects lasted longer.\textsuperscript{26} Animals that lost < 2% of their weight, regained it after the treatment regimen was completed.\textsuperscript{26} In other studies, ProTide 5 dosed intravenously was better tolerated than gemcitabine as its maximum tolerated dose in Beagle dogs was 4-times equimolar higher than that of gemcitabine.\textsuperscript{27} Notably, the pharmacokinetics profile of 5 was also better than the parent nucleoside gemcitabine.\textsuperscript{27} For instance, the half-life of 5 was 7.9 h compared to gemcitabine's 1.5 h while the dosing of 5 generated 13-times higher intracellular concentrations of the active gemcitabine triphosphate metabolite than dosing with gemcitabine.\textsuperscript{27}

Results from phase I/II clinical trials showed that 5 was effective against a wide range of cancers that included pancreatic, biliary and ovarian.\textsuperscript{28} ProTide 5 achieved ca. 12-fold higher
levels of the active metabolite gemcitabine triphosphate than gemcitabine in patients similar to the levels seen in previous animal studies. This significant increase in the active metabolite was translated into better therapeutic efficacy. From 68 patients, five achieved tumour shrinkage of ≥ 30% while an additional thirty three patients had achieved stable disease. Generally, the prodrug was well-tolerated by patients with the most common adverse effects being anaemia (67%), fatigue (67%), transaminitis (64%) and thrombocytopenia (53%). Compound 5 has reportedly entered phase III clinical trials in 2015 as a potential treatment of pancreatic cancer. Additionally, a phase Ib trial examining the safety of 5 in combination with cisplatin in patients with locally advanced or metastatic biliary tract cancers is ongoing.

Continuing with the theme of ‘ProTiding’ nucleosides that are known to exert anticancer activity, NuCana are currently developing a naphthyl L-alanine benzyl ester ProTide of 5-fluoro-2’-deoxyuridine (FdU), the penultimate metabolite en route to the active metabolite of the chemotherapeutic agent 5-fluorouracil (5-FU). This prodrug, known as NUC-3373 (6, Figure 4), showed excellent preclinical properties. McGuigan and co-workers reported the discovery of 6 in 2011 and highlighted its advantageous properties as compared to the parent nucleoside FdU. Indeed, it was shown that compound 6 still exhibited significant cytotoxic properties in cells lacking thymidine kinase unlike the parent nucleoside FdU. This was thought to be due to the requirement of this nucleoside kinase to bioconvert FdU to its monophosphate analogue. Interestingly, 6 was found to be resistant to inactivation by catabolic enzymes such as thymidine uridine phosphorylases, which inactivate FdU. Further studies showed the favourable chemical and serum stability of 6 as compared to the parent nucleoside FdU. Preclinical investigation showed that compound 6 generated an impressive 363-times higher level of the active metabolite, FdU monophosphate, than the chemotherapeutic agent 5-FU. The significantly higher levels of the active metabolite were responsible for making ProTide 6 superior in efficacy compared to 5-FU in terms of tumour reduction in HT-29 colorectal xenografts. Coupling this favourable efficacy results with the
improved tolerability of 6 as compared to 5-FU,\textsuperscript{31} compound 6 has entered phase I clinical trials in Q4 2015.

2.1.2. GS-5734

GS-5734 (7, Figure 4)\textsuperscript{32} is a very interesting ProTide as it is currently the only C-nucleoside-based ProTide undergoing clinical trials following the termination of the clinical development of the initial anti-HCV C-nucleotide ProTide, GS-6620\textsuperscript{33} [structure not shown]. Although ProTide 7 is currently being developed by Gilead Sciences, Inc. for the treatment of Ebola, it exhibited broad spectrum antiviral activity against a selection of RNA viruses such as arenaviruses, coronaviruses and filoviruses. Structurally, compound 7 is a phenyl 2-ethylbutyl L-alanine ester phosphoramidate of 1'-cyano-4-aza-7,9-dideaza adenosine. Unlike other ProTides in clinical development it is being pursued as a single isomer (Sp) akin to the two FDA-approved ProTides, sofosbuvir and tenofovir alafenamide (Figure 2). ProTide 7 exhibited potent inhibition of the Ebola virus (EBOV) replication (EC\textsubscript{50} = 0.06 to 0.14 μM) while the parent C-nucleoside was not as effective (EC\textsubscript{50} = 0.77 to > 20 μM).\textsuperscript{32} This was attributed to the generation of higher levels of the C-nucleoside triphosphate active metabolite when 7 was used as compared to the parent C-nucleoside. 10mg/kg intravenous dosing of 7 in rhesus monkeys showed that the prodrug had a short half-life (t\textsubscript{1/2} = 0.39 h), due to rapid clearance and fast metabolism to generate the alanine intermediate (compound 7 with the unmasked alanine amino acid, but lacking the phenyl motif that masked the oxygen of the phosphate).\textsuperscript{32} Interestingly, the generation of the parent C-nucleoside was also observed suggesting a substantial dephosphorylation of the parent C-nucleoside monophosphate. In peripheral blood mononuclear cells (PBMCs), 7 was rapidly bioconverted to its active triphosphate derivative and achieved persistent high levels (t\textsubscript{1/2} = 14 h) that led to over 50% inhibition of EBOV replication in 24 h.\textsuperscript{32} Coupling these pharmacokinetic properties with tissue distribution studies, 7 was viewed as a suitable candidate for once-daily dosing. Compound 7 was found to be effective in inhibiting EBOV replication in rhesus monkeys infected with Ebola virus as once-daily intravenous administration of 7 led to significant suppression of the Ebola virus replication and 100%
protection of infected animals against lethal disease. Indeed, different treatment regimens that ranged from 3 mg/kg to 10 mg/kg initiated at different time points following EBOV exposure (maximum 3 days) showed pronounced inhibition of EBOV replication and improved survival.\textsuperscript{32} Impressively, 10 mg/kg dosing starting from day 3 following virus exposure led to significant improvement in EBOV-related clinical signs and greater survival rates. Compound 7 dosed intravenously is currently undergoing phase I clinical trials for the treatment of Ebola virus disease. Additionally, given the broad spectrum of antiviral activity of this ProTide, as mentioned previously, it has the potential to be used in the treatment of other RNA viral infections that are currently difficult to treat.

\subsection*{2.1.3. Stampidine and Thymectacin}

In 2009, we commented\textsuperscript{19} on the development of the two anti-HIV ProTide clinical candidates stampidine (8, Figure 4)\textsuperscript{34} and thymectacin (9, Figure 4)\textsuperscript{35}, but not much information has been published on their progress since then.

Stampidine was one of the first ProTides to enter clinical trials. It is a para-bromophenyl L-alanine methyl ester ProTide of the FDA-approved anti-HIV reverse transcriptase inhibitor stavudine.\textsuperscript{34} As the first phosphorylation of the clinically used stavudine is rate-limiting in the formation of its active triphosphate species,\textsuperscript{36} the application of the ProTide technology to overcome this rate-limiting step was a logical one. Stampidine exhibited potent anti-HIV activity against wild-type HIV-1 strains as well as virus strains carrying mutations that give rise to resistance to nucleoside and non-nucleoside reverse transcriptase inhibitors.\textsuperscript{34, 37} As expected, this ProTide’s anti-HIV activity was retained in thymidine-kinase deficient cells confirming the ability of the ProTide technology to operate independently of this kinase. Unlike other ProTides in clinical development, which do not carry any substituents in the aryl motif of the ProTides, stampidine has a unique para-bromophenol motif. This introduction of the electron-withdrawing atom makes the metabolism of the ProTide take place quicker and hence the active triphosphate metabolite is generated faster. \textit{In vivo} studies showed that stampidine was well tolerated. Indeed, no acute toxicity was detected in mice and rats dosed with up to 500 mg/kg of stampidine intraperitoneally or orally.\textsuperscript{38} Coupling this with favourable
tolerability, stampidine progressed to phase I clinical trials. Results from these studies involving oral dosing of stampidine did not show any dose-limiting toxicity at single dose levels of up to 25 mg/kg.\textsuperscript{39} Beyond its potential in treating HIV-resistant strains, stampidine was also suggested as a promising candidate in the pre-exposure prophylaxis against HIV in heterosexual women as well as serodiscordant couples.\textsuperscript{40}

Thymectacin\textsuperscript{35} is a phenyl L-alanine methyl ester ProTide of the therapeutic nucleoside brivudine (BVDU), which is used to treat herpes zoster (shingles).\textsuperscript{41, 42} Intriguingly, the ProTide of brivudine showed superior anticancer activity to its parent nucleoside (brivudine).\textsuperscript{43} This ProTide selectively targets tumour cells with a high expression of thymidylate synthase (TS) and shows at least 10-fold more cytotoxicity to 5-FU-resistant, TS over-expressing colorectal tumour cells than to healthy cells.\textsuperscript{35} As expected, thymectacin was metabolised in vivo to release the monophosphate derivative, which was expected to be further phosphorylated into the triphosphate and get incorporated into DNA causing cytotoxicity akin to other nucleoside therapeutics. However, this did not appear to be the case as cellular studies using radiolabelled thymectacin indicated that the released metabolite BVDU monophosphate was processed in a TS-dependent manner to generate reactive metabolites that resulted in the labelling of proteins.\textsuperscript{44} Although the identity of these labelled proteins remains elusive, the labelling of these proteins by BVDU monophosphate metabolites correlated well with the potency of thymectacin across the cells used in these studies. This pointed to a unique nucleotide-mediated labelling of proteins that causes cytotoxicity. Further studies into the mechanism of action of thymectacin suggested the involvement of the tumour suppressor p53 and the activation of the G2/M checkpoint eventually contributing to cell cycle arrest.\textsuperscript{45}

Phase I clinical trials of thymectacin dosed intravenously in patients with advanced colorectal cancer that failed 5-FU treatment was initiated. Initial results using doses ranging from 200 mg/kg to 1250 mg/kg showed that thymectacin was well tolerated and a number of patients achieved a stable disease state.\textsuperscript{46} Among the serious side effects that were reported were respiratory distress, vomiting, hyperglycaemia and ascites. Despite the seemingly
encouraging results from this ProTide’s initial clinical results no further details on its development have been released.\textsuperscript{46}

Admittedly, despite the promising clinical data from stampidine and thymectacin, there has not recently been an update on their further development and thus their current state of clinical development is unclear.

Beyond these ProTides that are reported to be in clinical development, the ProTide technology has delivered several other nucleoside monophosphate and monophosphonate prodrugs that entered clinical trials but eventually their development was suspended.\textsuperscript{47} This showcases the usefulness of this phosphate and phosphonate prodrug technology in the discovery of nucleotide therapeutics.

2.2. HepDirect prodrugs

Among the various phosphate and phosphonate prodrugs used in the discovery of nucleotide therapeutics, very few achieve tissue/organ targeting. Examples of these include the HepDirect\textsuperscript{48} approach, which is predominantly metabolised in the liver, via cytochrome P450 (CYP450) enzymes and thus achieves liver-targeted drug delivery (\textbf{Figure 6}).\textsuperscript{49} In this approach, the two negative charges of the phosphate are masked via the formation of a phenyldioxaphosphinane oxide.

\begin{center}
\textbf{Fig. 6.} Mechanism of CYP450-mediated metabolism of HepDirect prodrugs in the liver.
\end{center}

These prodrugs undergo oxidation in the liver by CYP3A enzymes.\textsuperscript{49, 50} Intriguingly, this step was found to be dependent on the stereochemistry at the benzylic position.\textsuperscript{49} Indeed, only
prodrugs with cis-isomerism between the phosphate group and the nucleoside undergo cytochrome P450 3A (CYP3A) oxidation. Once oxidised, the prodrug moiety is quickly opened up to generate a mono-charged phosphate group. This intermediate subsequently undergoes β-elimination to generate a free unmasked phosphate group. The resulting aryl vinyl ketone released is rapidly detoxified by conjugation to glutathione, a process mediated by glutathione S-transferase.

Due to the ability of this prodrug approach to be activated predominantly in the liver, it has been used extensively in the discovery of nucleotide therapeutics for the treatment of hepatitis infections. To date, it has been reported that two HepDirect nucleotide prodrugs have entered clinical development; pradefovir (10, Figure 7)\textsuperscript{51} and MB07133 (11, Figure 7)\textsuperscript{52}.

\begin{figure}[h]
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\includegraphics[width=0.8\textwidth]{fig7.png}
\caption{Chemical structures of two nucleotide HepDirect clinical candidates.}
\end{figure}

\subsection{2.2.1. Pradefovir}

Pradefovir is a HepDirect prodrug of the phosphonate adefovir, which is already used in the bis(pivaloyloxymethyl) (bisPOM) prodrug form (see section 2.4) in the treatment of HBV.\textsuperscript{51} The use of the HepDirect prodrug approach in this case led to the targeting of adefovir to the liver, which limits the nephrotoxicity side effects associated with adefovir. Indeed, in rat liver, pradefovir showed 12-fold improvement in liver accumulation than adefovir and was accompanied by less accumulation in the kidney.\textsuperscript{51} Since the early pharmacokinetic profiles of pradefovir supported oral dosing, this drug candidate was subsequently pursued in the mesylate salt form in order to increase water solubility. The mesylate salt formulation achieved better oral bioavailability (42\%) as compared to the parent drug (31\%).\textsuperscript{51} Pradefovir
progressed into phase I and subsequently phase II clinical trials.\textsuperscript{53} \textsuperscript{54} Indeed, in a randomised, open-label, parallel-group, multicentre study comparing pradefovir dosed at 5, 10, 20 and 30 mg/day and adefovir dipivoxil dosed at 10 mg/day for 48-weeks, pradefovir was reported to be safe, well-tolerated and had significantly greater anti-HBV activity than adefovir dipivoxil.\textsuperscript{53} Notably, the only adverse effects reported were minor or mild with most of them being associated with the patient cohort placed on 30 mg/day dosing. Intriguingly, another open-label clinical study that aimed at comparing the long-term safety profiles of pradefovir and adefovir dipivoxil was terminated due to adverse findings from nonclinical carcinogenicity studies.\textsuperscript{55} Although this observation has complicated the further clinical development of pradefovir, it is reported to be still undergoing phase 2 clinical development in China for the treatment of HBV.\textsuperscript{56}

\textbf{2.2.2. MB07133}

This HepDirect prodrug, which was granted Orphan Drug Designation by the FDA in 2007, was being developed as a possible treatment for hepatocellular carcinoma. It is a HepDirect prodrug of cytarabine, a nucleoside analogue used clinically to treat acute myelocytic leukaemia.\textsuperscript{57} Like many other therapeutic nucleosides, one of the limitations for using cytarabine is its poor in vivo phosphorylation (activation) to generate the active metabolite, cytarabine triphosphate. Attempts aimed at overcoming this bioconversion process to reach the therapeutic levels of the active triphosphate metabolite were often associated with myelosuppression due to generation of cytarabine triphosphate in health bone marrow cells.\textsuperscript{52}

As compared to cytarabine, compound 11 generated > 12-fold and > 19-fold higher levels of cytarabine triphosphate, the active metabolite, in the liver than in the bone marrow and the plasma, respectively.\textsuperscript{52} In animal studies, no toxic by-products from the monophosphate masking motifs were observed in hepatocytes or animals treated with 11. Data from a phase 1/2 open label study to assess the safety, tolerability and pharmacokinetics of seven days continuous intravenous infusion of 11, at doses up to 2400 mg/m\textsuperscript{2}/day, in subjects with unresectable hepatocellular carcinoma showed that it was well-tolerated, though a few
hepatic toxicities were noted.\textsuperscript{58} In another report it was noted that \textbf{11} was well-tolerated at doses up to 1800 mg/m\textsuperscript{2}/day in patients suffering with unresectable hepatocellular carcinoma.\textsuperscript{59} Critically, in this study a few patients were reported to have experienced severe adverse effects, but these were suggested to be unrelated or unlikely related to compound \textbf{11}.\textsuperscript{59}

The clinical development of both nucleotide HepDirect clinical candidates, pradefovir and \textbf{11}, was passed to Chiva Pharmaceuticals, Inc in 2011. Clinical data on the progress of these agents since has been scarce.

\subsection*{2.3. Phosphate and phosphonate monoester prodrugs}

All of the phosphate and phosphonate prodrugs discussed already in this Miniperspective share a common feature of fully masking the phosphate or phosphonate group. In the alkoxyalkyl monoester prodrug approach,\textsuperscript{60} however, only one of the phosphate or phosphonate oxygens is masked while the second one is left free. The design of these ether lipid phospho-conjugates was inspired by the natural lysophosphatidylcholine. In these prodrugs, the choline moiety is replaced by the drug, and this conjugate benefits from active uptake mechanisms in the intestine leading to higher oral bioavailability. In order to reduce undesired \textit{in vivo} conversions, further optimisation of the structures of these masking groups was undertaken and this led to the hexadecyloxypropyl (HDP) monoester prodrugs. These prodrugs are thought to be metabolised \textit{in vivo} by phospholipases such as phospholipase C yielding the nucleoside monophosphate (\textbf{Figure 8A}).\textsuperscript{60, 61} This approach has proven to be successful in nucleotide therapeutics drug discovery and two nucleotide prodrugs are in clinical development; brincidofovir (\textbf{12, Figure 8B})\textsuperscript{62} and CMX-157 (\textbf{13, Figure 8B})\textsuperscript{63}. 
Fig. 8. Hexadecyloxypropyl (HDP) monoester prodrugs. A. Mechanism of nucleoside phosphate or phosphonate release in vivo from HDP prodrugs. B. Chemical structures of two nucleotide HDP clinical candidates.

2.3.1. Brincidofovir

Brincidofovir, also referred to as CMX-001, is an HDP monoester phosphate prodrug of the FDA-approved antiviral agent cidofovir. This prodrug has the advantage of being dosed orally unlike its parent drug cidofovir. Early in vitro studies showed that brincidofovir exhibited a broad spectrum of antiviral activity as it exerted potent activity against adenoviruses, smallpox, cytomegalovirus (CMV), papillomavirus, polyomavirus and orthopoxviruses. This antiviral activity was found to be due to cidofovir diphosphate, which inhibits viral DNA polymerases in double-stranded DNA viruses. Brincidofovir is more lipophilic than the parent drug cidofovir and also benefits from active transport in the small intestine. This resulted in 100-fold higher intracellular levels of the active cidofovir diphosphate from brincidofovir as compared to cidofovir. These differences in delivering the active species could explain the significant differences in (in vitro) antiviral activities between this prodrug and its parent drug, which could reach 100-fold difference.

Brincidofovir tissue distribution studies using radiolabelled brincidofovir showed it to be accumulated most in the small intestine followed by the liver, lung, kidney and spleen. Interestingly, brincidofovir had lower accumulation in the kidney as compared to the parent drug cidofovir. This suggested that brincidofovir would not cause as much nephrotoxicity as
cidofovir, a common adverse effect associated with its use. The high accumulation of brincidofovir in the small intestine was linked to the gastrointestinal toxicities observed in animals when brincidofovir was dosed at high concentration. However, in healthy volunteers, there were no adverse effects observed when brincidofovir was dosed as a single dose at 2 mg/kg or multiple doses at 1 mg/kg while at higher doses increased rates of diarrhoea were noted. In a separate study, notable gastrointestinal adverse effects, particularly diarrhoea, were observed in a cohort of patients on doses of 200 mg of brincidofovir weekly or more with 200 mg twice weekly being dose-limiting. Phase I pharmacokinetic studies in which brincidofovir was dosed at a range of concentrations between 0.025 to 2 mg/kg as single and multiple doses indicated that cidofovir maximal plasma concentration was reached between 7 and 15 h. The half-life varied according to the doses used and was found to increase from 6.15 h with a 0.025 mg/kg dose to 32.7 h with a 1.5 mg/kg dose. Regarding the possible development of resistance against this experimental therapeutic agent, a recent study employing in vitro selection identified novel CMV UL54 DNA polymerase gene mutations that confer resistance to brincidofovir. However, phase II clinical trials brincidofovir to placebo for prophylaxis against cytomegalovirus infection in hematopoietic cell transplant recipients indicated that there were no resistance-associated mutations detected in patients treated with brincidofovir. The study concluded that brincidofovir could be useful as a first-line agent in the prophylaxis of cytomegalovirus infection. Recent results from a double blind, placebo-controlled phase III trial that involved 452 subjects at high risk for CMV who received either brincidofovir or placebo twice-weekly for 24 weeks following hematopoietic cell transplantation (HCT) showed brincidofovir did not demonstrate statistically significant efficacy. Although the antiviral effect of brincidofovir was demonstrated at the end of the on-treatment period at week 14, by the primary end point of assessment (week 24) there were no significant differences in the clinically significant CMV infection in the cohorts treated with brincidofovir and those on the placebo. This unimpressive phase III data puts questions on the development of brincidofovir for the prophylaxis of CMV in HCT patients. Beyond CMV,
brincidofovir is also being pursued as a possible treatment of adenovirus and smallpox infections.\textsuperscript{74}

Interestingly, brincidofovir emerged as a promising agent in the fight against Ebola. This is quite intriguing as Ebola is not a DNA virus. Still, brincidofovir was found to inhibit Ebola virus in multiple human cell lines including HeLa and A549 cells.\textsuperscript{75} Unlike its mechanism of action of inhibiting DNA viruses, phosphorylation of brincidofovir did not seem to be required for activity. Instead, recent studies showed that the anti-Ebola activity required the lipid motif, \textit{i.e.} the masking group.\textsuperscript{75} Indeed, the parent drug cidofovir alone did not show activity against Ebola while the lipid masking group alone was 29-fold less active than brincidofovir.\textsuperscript{75} Encouraged by the promising activity of brincidofovir against Ebola, there were some efforts put into initiating human clinical trials investigating its efficacy against Ebola.\textsuperscript{76} However, the company developing brincidofovir, Chimerix, Inc., decided against this and focused its development on treating CMV and adenovirus infections.\textsuperscript{76}

2.3.2. CMX-157

Compound 13 is an alkoxyalkyl monoester of the clinically used nucleoside phosphonate tenofovir.\textsuperscript{63} It was developed on the premise of being a lipophilic lysophosphatidylcholine (LPC) mimic to allow more efficient tenofovir delivery via the alternative, LPC uptake pathway. Additionally, the application of this alkoxyalkyl monoester prodrug approach was aimed at delivering a new therapeutic agent with better oral availability, efficacy and less nephrotoxic adverse effects than the parent drug tenofovir.\textsuperscript{63, 77} Compound 13 was > 300 fold more active than tenofovir and is active against the major subtypes of the HIV-1 virus.\textsuperscript{77} Initial animal studies indicated that this agent was orally bioavailable and showed no significant toxicity following dosing of up to 100 mg/kg/day for 7 days.\textsuperscript{63} Encouraged by the preclinical data, 13 underwent phase I clinical trials where it demonstrated 267-fold higher activity than tenofovir in a dose-escalation, randomized, blinded trial where doses varied from a singular 25-400 mg dose of 13 or tenofovir.\textsuperscript{78} The results showed that single doses of 13 were well tolerated by the patients. The antiviral activity was thought to be a result of the formation of tenofovir diphosphate, which was detectable in peripheral blood mononuclear
cells from patients following a single dose of 13. Interestingly, the tenofovir diphosphate levels were detectable for up to six days following a single dose of 400 mg of 13 suggesting the possibility of once a week dosing. Compound 13 is being developed by ContraVir Pharmaceuticals, Inc. and is currently undergoing phase II clinical trials as a potential treatment of HBV.

2.4. BisPOM and bisPOC prodrugs

BisPOM and bis(isopropylxoxymethyl carbonate) (bisPOC) are two phosphate and phosphonate prodrugs that were used successfully in the discovery of nucleotide phosphonate prodrugs. Indeed, two bisPOM and bisPOC drugs, tenofovir disoproxil and adefovir dipivoxil, are currently used to treat HIV and HBV. The release of the nucleoside phosphate or phosphonate from these prodrugs is triggered by esterases (Figure 9). In the POC prodrug approach, the carbonate group is cleaved by esterases leading to the generation of the unstable carboxylate, which in turn undergoes two sequential degradation steps resulting in the formation of the nucleoside phosphate or phosphonate in addition to carbon dioxide and formaldehyde. In the POM approach, however, the process is rather simpler though still mediated by esterases and produces similar types of byproducts. The ester motif in POM is first cleaved by esterases and this leads to the formation of a highly unstable hydroxymethyl alcoholate, which undergoes spontaneous rearrangement to generate formaldehyde and the nucleoside phosphate or phosphonate.
2.4.1. Besifovir

Besifovir (14, Figure 10)\textsuperscript{82} is a bisPOM nucleotide phosphonate prodrug in clinical development as a potential treatment of HBV.\textsuperscript{83} It showed potent antiviral activity against wildtype HBV as well as lamivudine-, adefovir-, entecavir-, and telbivudine-HBV resistant strains.\textsuperscript{84} Besifovir is well absorbed and is rapidly degraded in the liver into the patent nucleotide, which is subsequently oxidized at the C6 position by oxidases such as xanthine oxidase.\textsuperscript{82,85} The triphosphate derivative of the oxidized metabolite is the active species as it inhibits viral DNA replication.\textsuperscript{85}

Fig. 10. Chemical structure of Besifovir, an anti-HBV bisPOM prodrug clinical candidate.
Pharmacokinetics data showed besifovir has a long half-life supporting once-daily dosing.\textsuperscript{85} Interestingly, the absorption of the prodrug was delayed by high fat diet.\textsuperscript{85} Initial data in mice showed that ca. 40\% of the active metabolite was generated in the liver.\textsuperscript{85} In a placebo-controlled randomized clinical trial, besifovir was dosed in patients with high viral load in escalating doses up to 240 mg/day for four weeks.\textsuperscript{85} The results showed significant viral load reduction in the cohort treated with besifovir as compared to the placebo group. In a phase II clinical trial looking into the safety and efficacy of 12-week treatment with besifovir in lamivudine-resistant patients, it was shown that viral HBV DNA was reduced in a dose-dependent manner.\textsuperscript{86} Notably, for the first week of treatment, besifovir was combined with lamivudine.\textsuperscript{86} Although besifovir was generally well-tolerated, a number of patients had fluctuations of creatinine clearance.\textsuperscript{86} Importantly, 48-weeks treatment with besifovir led to significant HBV DNA reduction in HBeAg-positive and negative HBV patients. The results from a two year, head-to-head, multi-centre trial comparing besifovir at 90 mg and 150 mg with entecavir 0.5 mg in treatment-naïve chronic HBV patients, indicated that there were no significant differences in the treatment outcome parameters between patients receiving besifovir and entecavir.\textsuperscript{87} Although, during this two year trial, there were no identifiable resistance mutations and no significant nephrotoxicity, the patients taking besifovir suffered from depletion of carnitine, which is essential for the transportation of fatty acids from the cytosol to the mitochondria.\textsuperscript{88} This indicated that long-term use of besifovir needs to be accompanied with a carnitine supplement to overcome carnitine depletion. Additionally, significant reductions in phosphate blood levels were also observed in two patients taking besifovir.

3. Conclusion

The success of recently approved nucleotide prodrugs sofosbuvir and tenofovir alafenamide highlighted the usefulness of employing nucleoside monophosphate and monophosphonate prodrug technologies in the discovery of new therapeutics. Currently there is a selection of
nucleoside monophosphate and monophosphonate prodrugs in clinical development and an increasing number of these types of prodrugs in preclinical development. Although the vast majority of these prodrug (pre)clinical candidates are being developed to combat viral infections, there is an increasing interest in applying these prodrug technologies in the discovery of anticancer nucleotide therapeutics. Together, these point to the possibility of more nucleotide prodrugs being approved in the future to treat viral infections and cancers that are currently difficult to treat. Critically, the success of nucleoside monophosphate and monophosphonate prodrug technologies in delivering clinical candidates has reignited interest in the development of nucleos(t)ide therapeutics, which not a long time ago, were being viewed as an 'outdated' class of drugs.

Conflicts of interest
The authors declare no competing financial interest.

Biographies

Pete Thornton completed his MSc degree in Chemistry at the University of Birmingham in 2010. He then went on to undertake a PhD with the group of Prof. James H. R. Tucker working on the synthesis of photo-catalysed interlocked structures. After graduating in 2015, he has since been working as a Research Fellow in the Mehellou lab carrying out the synthesis and development of new phosphate prodrugs.

Hachemi Kadri received his MPharm degree in Pharmacy from the University of Nottingham in 2006. He then pursued graduate studies at the Welsh School of Pharmacy at Cardiff University where he obtained his PhD in Medicinal Chemistry (2010) under the mentorship of Dr. Andrew Westwell. Hachemi is currently a Research Fellow in the laboratory of Dr. Youcef Mehellou at the University of Birmingham. His research interests focus on the design and development of small-molecule modulators of protein-protein interactions.
Ageo Miccoli graduated from the University of Birmingham with a first class MSc in Chemistry in 2015. He is currently a PhD student in the laboratory of Dr. Youcef Mehellou. His research interests are in the development of novel phosphate prodrugs technologies and their application in anticancer drug discovery.

Yousef Mehellou obtained his MPharm (Pharmacy) degree from King’s College London in 2005. This was followed by a PhD in medicinal chemistry under the supervision of Prof. Chris McGuigan in Cardiff University. The PhD project was focused on the application of the ‘ProTide’ phosphate prodrug technology in the discovery of nucleotide therapeutics. Following postdoctoral work with Prof. Sidney M. Hecht in Arizona State University and holding an MRC Career Development Fellowship with Prof. Dario R. Alessi FRS in the University of Dundee, Youcef moved to the University of Birmingham in 2013 as a Lecturer in medicinal chemistry. His group’s research interests are in discovery of small molecule modulators of signal transduction and the development of novel phosphate and phosphonate prodrug therapeutics.

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Abbreviations
BisPOC: bis(isopropyloxymethyl carbonate); bisPOM: bis(pivaloyloxymethyl); BVdU: Brivudine; CMV: cytomegalovirus; CYP450: cytochrome P450; CYP3A: cytochrome P450 3A; EBOV: Ebola virus; FDA: US Food and Drug Administration; FdU: 5-fluoro-2'-deoxyuridine; 5-FU: 5-fluorouracil; HBV: hepatitis B virus; HCV: hepatitis C virus; HCT: hematopoietic cell transplantation, HDP: hexadecyloxypropyl; HIV: human immunodeficiency virus, LPC: lysophosphatidylcholine; TS: thymidylate synthase.
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