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Phosphoramidates and phosphonamidates (ProTides) with antiviral activity

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Abstract

Following the first report on the nucleoside phosphoramidate (ProTide) prodrug approach in 1990 by Chris McGuigan, the extensive investigation of ProTide technology has begun in many laboratories. Designed with aim to overcome limitations and the key resistance mechanisms associated with nucleoside analogues used in the clinic (poor cellular uptake, poor conversion to the 5′-monophosphate form), the ProTide approach has been successfully applied to a vast number of nucleoside analogues with antiviral and anticancer activity. ProTides consist of a 5′-nucleoside monophosphate in which the two hydroxyl groups are masked with an amino acid ester and an aryloxy component which once in the cell is enzymatically metabolized to deliver free 5′-monophosphate, which is further transformed to the active 5′-triphosphate form of the nucleoside analogue. In this review, the seminal contribution of Chris McGuigan's research to this field is presented. His technology proved to be extremely successful in drug discovery and has led to two Food and Drug Administration-approved antiviral agents.

Keywords

Nucleoside analogues, prodrugs, phosphoramidate (ProTide), antiviral

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A tribute to Chris McGuigan, "the Drug Hunter"

In this issue, it is our immense privilege to pay tribute to Professor Chris McGuigan, an extremely dedicated and enthusiastic scientist who achieved remarkable success in the antiviral field.

Professor Chris McGuigan's achievements in the development of ProTide technology are undoubtedly extremely significant and remarkable. ProTide technology is currently the most successful prodrug strategy applied in the antiviral field, also demonstrating promising results in other therapeutic areas.

As the remaining three members of his group, it will be our mission to continue his legacy for many years to come. We firmly believe that the best way to honor him will be to put the same efforts and passion into research for new medicines as he did.

Introduction

Nucleoside analogues

Viral infections represent a major problem to human society. Viruses are often difficult to eradicate due to the fact that they are easily spread, and are able to use the host biochemical pathways to replicate. Therefore, targeting viral machineries often presents the challenging task of reducing the viral load in the human cell without damaging it.

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One of the most successful approaches to fight viral infections is using nucleoside analogues (NAs).[1] NAs are synthetic compounds that exhibit structural similarities to natural nucleosides. In the cell, they can undergo the same physiological processes as the endogenous nucleosides from the uptake to the metabolism, so that in their phosphate forms they can act on cellular functions. Mono-, di-, and triphosphorylated nucleosides are therefore the active forms of these drugs. These compounds act by interfering with viral enzymes as competitive inhibitors of their natural substrates as well as by being incorporated into newly synthesized viral DNA and RNA strands. Their incorporation into nucleic acids may induce either the termination of chain elongation or the accumulation of mutations in the viral genome.[2] Through these mechanisms NAs interfere with the viral genome replication and thereby work as antiviral drugs. However, human enzymes can also recognize NAs, which act as antimetabolites. Antimetabolites can have toxic effects on cells, such as halting cell growth and division and/or inducing apoptotic processes. Consequently, these NAs are specifically used as chemotherapy for cancer. NAs have been in clinical use for almost 50 years and have become cornerstones of the treatment for patients with viral infections or cancer conditions.[3]

In the antiviral arena, NAs are commonly used in the therapy of human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV), herpes simplex virus (HSV), cytomegalovirus (CMV), and varicella zoster virus (VZV) infections. These agents are generally safe and well tolerated as they are recognized by the viral, but not human polymerases in DNA replication. The NAs used to treat HIV infections are often referred to as nucleoside reverse transcriptase inhibitors (NRTIs), a viral DNA polymerase essential for HIV replication. However, they have activity against both DNA-dependent and RNA-dependent DNA polymerases. They inhibit viral replication by several mechanisms, either by competitive inhibition of the viral polymerase or by DNA chain termination. Many of the antiviral NAs either are missing or are blocked at the 3′-hydroxyl group, which results in failure of elongation of the nascent DNA molecule. Other antiviral NAs are negative enantiomers (L-forms) of the natural (D-forms) nucleosides and interfere with replication, partially because of steric hindrance when they are taken up by the viral polymerase or added to the DNA molecule.

There are currently 14 approved cyclic NAs **[1–14]** (Figure 1) that are used as antiviral agents for several indications. The antiviral research field started with the discovery of the NA idoxuridine (IdU, **[1]**),[4] approved in 1962 against herpes simplex keratitis,[5] followed by trifluridine (TFT, **[2]**)[6] licensed in 1980 for the topical treatment of herpetic keratitis. Currently, these two NAs are used in the topical treatment of herpetic eye infections.

Vidarabine (AraA, **[3]**)[7] was approved later on in 1986 against HSV and VZV and is now used for the treatment of acute keratoconjunctivitis, recurrent superficial keratitis caused by HSV-1 and HSV-2 and herpes zoster infections in AIDS patients. In many conditions vidarabine, due to its lower selectivity, higher inhibitory concentration and lower potency together with a low aqueous solubility, which implies intravenous dosing, has been replaced by the more powerful and effective drug acyclovir (ACV) (see infra).

The combination of pegylated interferon-α with ribavirin (RBV, **[4]**), approved in 1986, has for the last 10 years been the standard of care for the treatment of HCV infections.[8] In addition since RBV has a broad activity spectrum including various DNA and RNA viruses, it has been used (topically, as aerosol) in the treatment of respiratory syncytial virus (RSV) infections.

Several other NAs were described as antiretroviral agents and were later approved and marketed for antiviral therapy. Among them there are brivudine (BVDU, **[5]**) approved in 1980 against VZV,[9] zidovudine (AZT, **[6]**) used since 1986 to prevent and treat HIV infections,[10] didanosine (ddI, **7**) approved in 1991 to treat HIV infections in combination with other medications as part of highly active antiretroviral therapy (HAART)[11] and stavudine (d4T, **[8]**) approved in 1994 against HIV.[12] Zalcitabine (ddC, **[9]**) was the third antiretroviral drug to be approved in 1992 for the treatment of HIV infections.[11] Lamivudine (3TC, **[10]**) is an antiretroviral medication used since 1995 to prevent and treat HIV infections and it is also used to treat chronic HBV infection when other options are not possible.[13] Other NAs approved include abacavir (ABC, **[11]**) approved in 1998 against HIV,[13] emtricitabine (FTC, **[12]**),[14] approved in 2004 in combination with tenofovir disoproxyl fumarate (TDF) (see infra) against HIV, entecavir (ETV, **[13]**),[15] and telbivudine (LDT, **[14]**),[16] both approved against HBV in 2005 and in 2006, respectively (Figure 1).

The clinical development of a second generation of antiviral NAs, including at present six approved compounds (Figure 2), started 35 years ago with the discovery of the acyclic NA ACV **[15]**, a selective anti-herpetic agent used in the treatment of VZV and HSV-1 and HSV-2 infections.[17,18] ACV possesses elevated selectivity of action, low cytotoxicity, and limited side effects. Since it was originally described, until now, ACV can still be considered as the "gold standard" for the treatment of HSV and VZV infections. In the same family is penciclovir (PCV, **[16]**),[19,20] the guanine analogue of ACV with similar activity spectrum and mechanism of action. Currently, PCV is used as topical

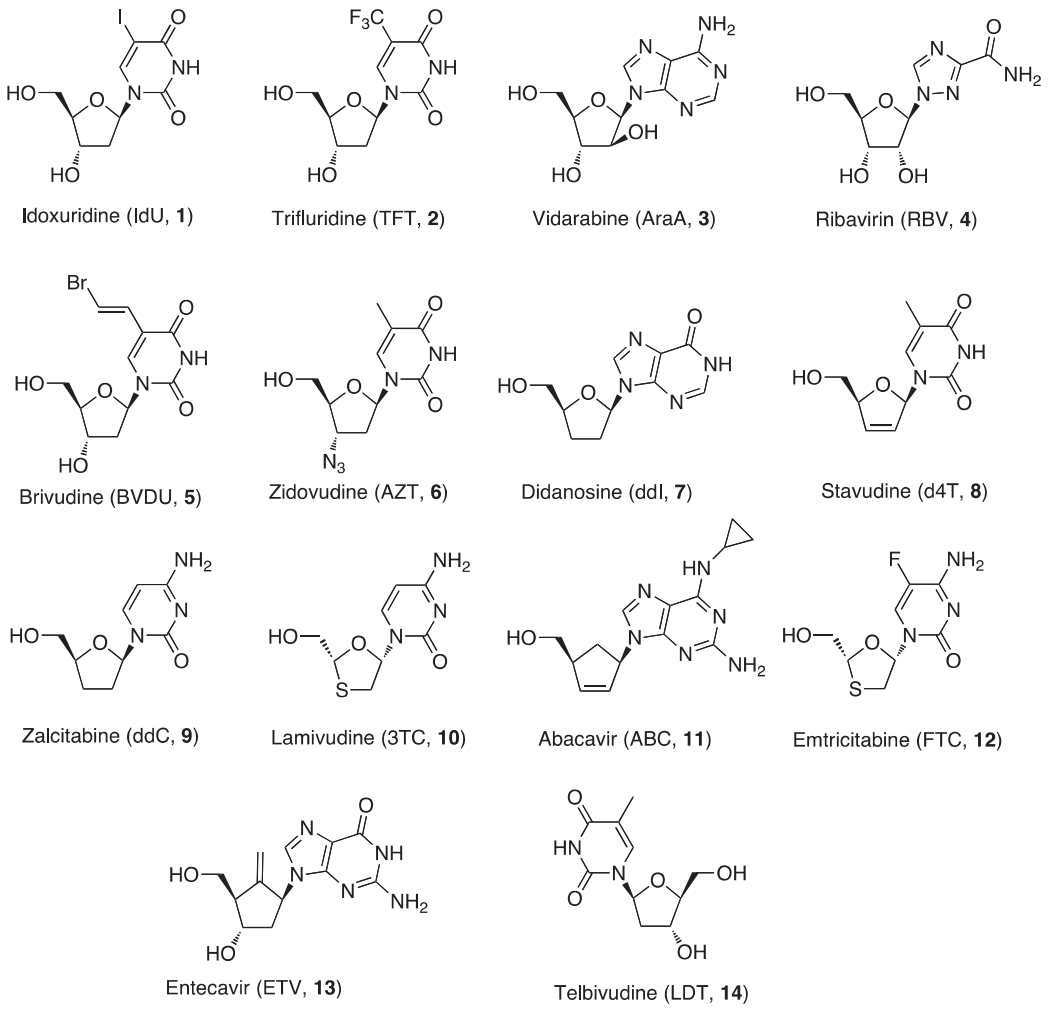


Figure 1. Cyclic NAs in clinical use as antiviral agents.

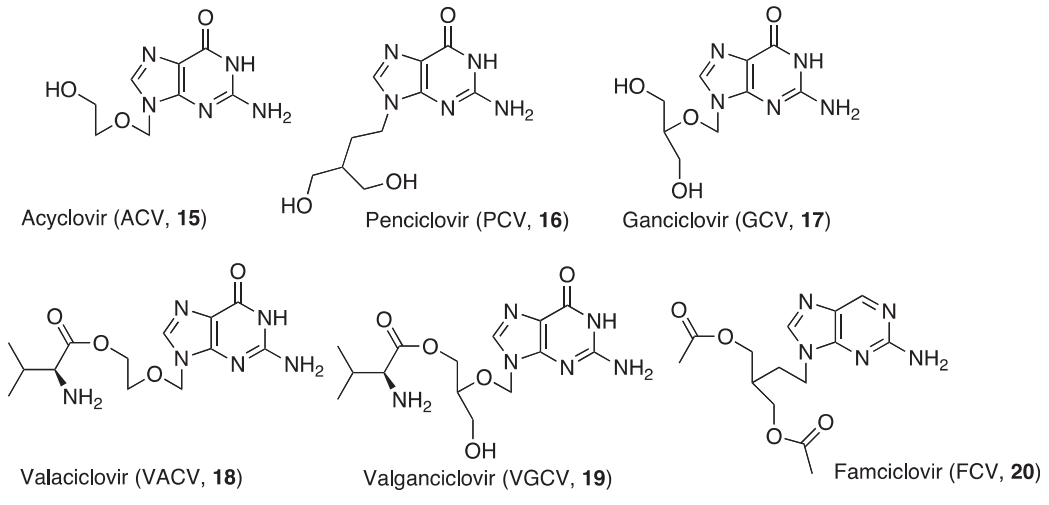


Figure 2. Acyclic NAs in clinical use as antiviral agents.

cream against cold sores caused by the HSV infections. Ganciclovir (GCV, **[17]**),[21] is another guanine analogue with an extended spectrum of activity. It has been approved to treat CMV retinitis with a slow release formulation and topical ophthalmic use for acute herpes simplex keratitis. The pharmacokinetics and the oral bioavailability of this second generation of antiviral NAs were enhanced with valaciclovir (VACV, **[18]**) and valganciclovir (VGCV, **[19]**), amino acid ester derivatives of ACV and GCV, respectively and with famciclovir (FCV, **[20]**),[22] which is the diacetyl prodrug of PCV.[20] VACV, the valine ester of ACV was synthesized to increase the aqueous solubility and to increase the oral bioavailability in order to use it in eye drops or in intramuscular injections. VACV is used for preemptive prophylaxis of CMV infections after renal transplantation. FCV is used to treat shingles (caused by reactivation of VZV), genital herpes (HSV-2), and herpes labialis (HSV-1). After rapid in vivo adsorption, FCV is converted into PCV through selective deacetylation followed by final oxidation of the nucleobase at the 6-position. VGCV, used in the treatment of CMV and HSV, is the valine ester prodrug of GCV, synthesized with the aim to increase oral bioavailability and solubility in water. Following oral administration, the ester is cleaved by esterases in the intestines and in the liver to release the parent nucleoside **[17]**.

In order to exert their antiviral activity, NAs have to be phosphorylated (in vivo) via three consecutive phosphorylations with the first one being usually the rate-limiting step.[23] However, if the first phosphorylation of the nucleoside to its 5′-monophosphate cannot take place all these drugs are inactive. This happens when the virus either does not induce a specific kinase or has developed resistance to the compound through mutations in this enzyme while the human cell fails to secure phosphorylation. Thus, to overcome this issue and improve therapeutic properties, nucleosides with a phosphate group already present in the structure have been targeted. The idea of replacing the phosphate group by an isosteric and isoelectronic phosphonate moiety was also investigated leading to the discovery of nucleoside phosphonate analogues (NPs).[24] Since the CH_2–P bond, unlike CO–P bond, is not susceptible to esterase and phosphatase hydrolysis, the resulting phosphonate compounds proved to be chemically and enzymatically more stable than the phosphate analogues. NPs are classified into major groups: cyclic nucleoside phosphonates (CNPs) and acyclic nucleoside phosphonates (ANPs).[25] CNPs are natural-like NAs as they contain a nucleobase and a sugar moiety. Compared to the large number of the ANPs described in the literature, only a few examples of CNPs with some antiviral activity have been reported.[24,26] This scarcity of examples is due the fact that in general CNPs are characterized by weak (if none) antiviral activity, which is generally explained by their poor substrate properties for cellular and viral kinases. For this reason until now, none of these compounds have reached the clinic.

On the contrary, ANPs have acquired a prominent therapeutic position.[25] They exhibit a broad spectrum of antiviral activities, particularly against DNA viruses and retroviruses, which are ascribed to their ability to undergo intracellular phosphorylation to the diphosphate forms and to be incorporated in the growing nucleic acid strand. The common structural attribute of ANPs is a nucleobase attached to an aliphatic side chain containing a phosphonomethyl residue. A methylene bridge between the phosphonate moiety and the rest of the molecule excludes the possibility of enzymatic dephosphorylation; absence of the glycosidic bond in the structure of ANPs further increases their resistance to chemical and biological degradation. Flexibility in the acyclic chain is assumed to enable these compounds to adopt a conformation suitable for interaction with active sites of different enzymes involved in DNA replication.

This new family of marketed antiviral drugs, includes cidofovir (CDV, **[21]**)[27] formally approved only for the treatment of human cytomegalovirus HCMV retinitis in AIDS patients, but also used successfully off-label in the treatment of various other DNA virus infections, particularly human papilloma virus (HPV)-associated lesions (Figure 3).

Nucleoside phosphate and phosphonate prodrugs

NAs as hydrophilic molecules do not rapidly penetrate cell membranes by non-facilitated diffusion. Instead, they permeate the cell by carrier-mediated endocytosis,[28] which is an active or facilitated transport mechanism that requires energy and a specific receptor or protein on the cell surface. Unfortunately, carrier-mediated transport often requires very close structural resemblance to natural products.

As previously mentioned, nucleoside 5′-monophosphates or 5′-phosphonates bypass the slow first phosphorylation step performed by viral kinases. However, these two classes of compounds are subject to poor cell penetration as a consequence of the negative charges in the phosphate and phosphonate groups, at physiological pH. Similarly to NAs, they require active transportation to enter the cells, and might present a risk of being deactivated in vivo by several cellular enzymes. In addition, they are not ideal for oral administration, an extremely desirable requisite for the treatment of

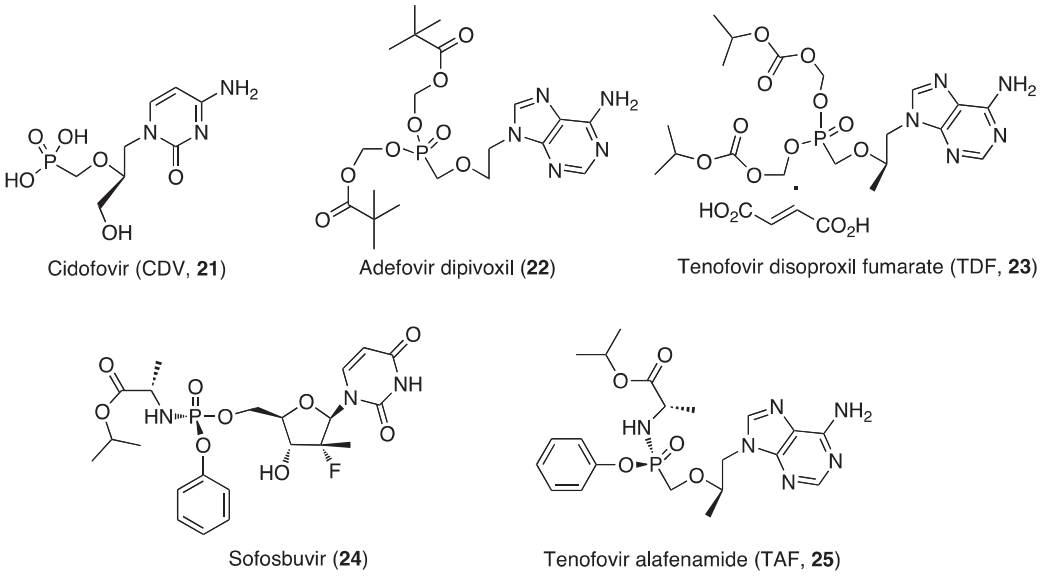


Figure 3. Nucleotide analogues in clinical use as antiviral agents.

chronic diseases. To overcome these limitations, several prodrug structures of biologically active phosphate and phosphonate analogues have been developed.[29–37] The rationale behind the design of such agents is to achieve temporary blockade of the free phosphonic functional group until their systemic absorption and delivery, allowing the in vivo release of the active drug only once at the target site. Such compounds have increased lipophilicity and as such are capable of altering cell and tissue distribution/elimination patterns of the parent drug.[38] Passive transcellular absorption is the most general route for absorption of lipophilic molecules. Many prodrug approaches have been utilized to overcome the limitations of phosphate- and phosphonate-containing drugs. Some of these approaches are still under development and until now no clinical investigations as antiviral agents of compounds, belonging to these classes of prodrugs, have been reported. Among them, there are aryl and phenyl esters,[39] cyclosaligenyl esters (CycloSal),[40] bis-S-acylthioethyl esters (Bis-SATE),[41] and peptidomimetic prodrugs.[42–44] Nevertheless, other approaches have been more successful and include prodrugs that have reached the clinic for antiviral therapy. An example of such a well-investigated class of prodrugs is represented by the alkoxyalkyl ester prodrugs of ANPs, designed by Hostetler group.[45] These include brincidofovir (CMX001, hexadecyloxypropyl-CDV), an experimental antiviral drug in clinical development by Chimerix for the treatment of CMV, adenovirus, smallpox, and Ebola virus infections[46] and CMX157 (hexadecyloxypropyl-tenofovir) another novel lipid ANP that has completed a Phase I clinical trial in healthy volunteers, demonstrating a favourable safety, tolerability, and drug distribution profile.[47] ContraVir Pharmaceuticals is planning further clinical development of this compound against HBV and HIV.

Cyclic 1-aryl-1,3-propanyl ester prodrugs (HepDirect), are another example of phosphate prodrugs effective as antiviral agents. This class of prodrugs features pradefovir, the 3-chlorophenyl HepDirect prodrug of adefovir,[48] which has been advanced to human clinical trials for hepatitis B infection therapy. The clinical development of pradefovir, as an oral prodrug for chronic HBV infection, although discontinued in USA and Europe, is still progressing in China by Chiva Pharmaceutical.[49] Currently, IDX184, a (SATE)-phosphoramidate diester prodrug of 2′-*C*-methylguanosine, is the only example of the successful application of this prodrug technology to reach human study. Unfortunately, the development of this antiviral agent for HCV treatment was stopped in Phase IIb in 2012.[50] Another example of a successful phosphate prodrug approach is represented by the phosphonodiamidate GS-9191,[51] a double-prodrug of the ANP 9–(2-phosphonylmethoxyethyl)guanine (PMEG).[52] In 2011, GS-9191 has completed Phase II clinical trials by Gilead Sciences as a topical prodrug to treat external genital warts due to HPV infections.[53] After that, Gilead Sciences has granted Graceway pharmaceuticals an exclusive worldwide license to GS-9191 for topical use,[54] but since then no further development has been reported to date. The Pharmasset agent PSI-352938, a novel cyclic phosphate prodrug of β-D-2′-deoxy-2′-α-fluoro-2′-β-*C*-methylguanosine,[55] was first-in-class prodrug to be clinically evaluated for the treatment of HCV infection. It progressed up to Phase II clinical trial[56] but after that its clinical development was discontinued due to observed hepatotoxicity.[57]

Two examples of prodrug approaches (Figure 3) applied to ANPs represented by the acyloxy and alkoxycarbonyl esters, were very effective and led to compounds that entered clinical trial studies and further obtained Food and Drug Administration (FDA) approval. Such compounds currently marketed for antiviral therapy are: adefovir dipivoxil **[22]**,[58] the *bis*-pivaloyloxymethyl ester of adevofir, approved in 2002 for the treatment of HBV infections; and tenofovir disoproxil fumarate **[23]**[59] the *bis*-diisopropyloxycarbonyloxymethyl ester of tenofovir fumarate licensed in 2001 for the treatment of HIV infections, and in 2008 also approved to treat chronic HBV infections.

Finally, the ProTide approach, invented almost 25 years ago by Chris McGuigan has recently been proven very successful in the intracellular delivery of nucleoside monophosphate into the cell, improving the activity of the parent drug.[36,60,61] During these 25 years, the ProTide technology was applied to a vast array of nucleosides and these studies have paved the way to the discovery of sofosbuvir (phosphoramidate of 2′-α-C-fluoro-2′-β-C-methyl uridine, **[24]**)[62] and tenofovir alafenamide (TAF, phosphoramidate of tenofovir, **[25]**)[63] (Figure 3), both launched in the market by Gilead Sciences for the treatment of HCV (2013) and HIV infections (2015), respectively.[63] A year later, TAF was approved for the treatment of HBV infections. To further confirm the success of this phosphate prodrug approach in the antiviral arena, it is important to mention that several other phosphoramidates of NAs are in clinical or preclinical development to treat viral infections (see infra).

Given the tremendous importance of phosphor(n)-amidate prodrugs in the antiviral arena and beyond, after the approval of sofosbuvir **[24]** and TAF **[25]**, the application of the ProTide technology has increased considerably. We herein attempt to report the ProTide development from its discovery to the recent application in the antiviral arena. After the brief introduction on the topic in the following sections, we will focus our attention on the ProTide approach, first discussing the synthetic methodology toward phosphor(n)amidates and summarizing the studies performed to prove their metabolic activation pathway. We will then report in details on the application of this approach to antiviral nucleosides starting from McGuigan's pioneering studies until the most recent use of his technology. We will give a full account on sofosbuvir **[24]** and TAF **[25]** and on those ProTides that are currently in clinical development.

Aryloxyphosphor(n)amidate prodrugs (ProTides)

McGuigan and his team at Cardiff University researched design of novel chemically protected phosphate prodrug groups or motifs, which later became known as "ProTide" technology. A ProTide (pronucleotide) is a nucleoside aryl phosphate or phosphonate masked with an amino acid ester promoiety linked via P–N bond (Figure 4). Such a prodrug is able to enter the cell via facilitated passive diffusion through the cell membrane and when cleaved, it delivers the nucleoside monophosphate or monophosphonate releasing the two masking groups. The amino acid motif is normally selected from a range of natural and unnatural amino acids, although usually L-alanine is found to be preferred one and is featured by all ProTide drugs that have reached the clinic. In fact, in vitro study of d4T ProTides with β-amino acids as phosphoramidate moiety revealed an almost complete lack of anti-HIV activity in comparison with their α-amino acid derivatives.[64] Short linear (methyl, ethyl, pentyl) or branched alkyls (isopropyl, neopentyl) and benzyl esters are usually employed. The *tert*-butyl is usually excluded due to its poor bioactivation.[65] Phenyl and 1-naphthyl are commonly used as aryl components and are indeed those incorporated in the drugs in clinical use or development. However, the 5,6,7,8-tetrahydronaphthyl group has more recently appeared as a valid and

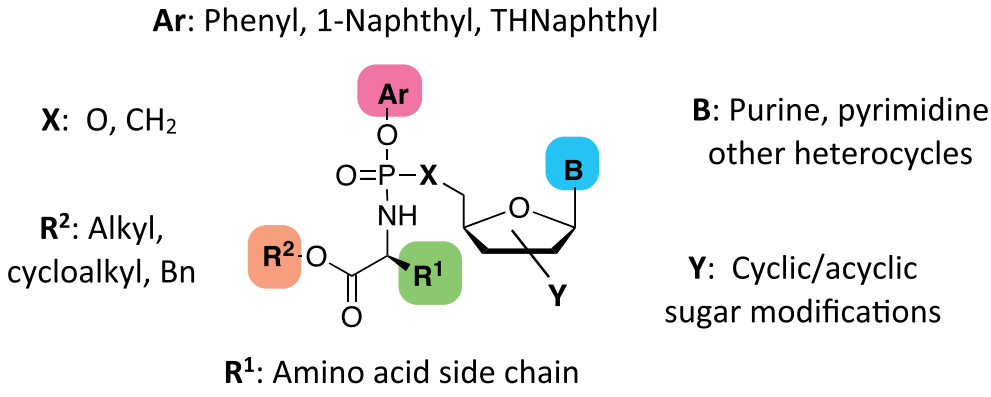


Figure 4. General structure of ProTide scaffold.

effective moiety.[66,67] Although the potency of ProTides varies with all the individual components of the phosphoramidate core, amino acid ester has been proven to drive predominantly the antiviral activity of the prodrugs, as it is closely linked with their stability and metabolic activation. Therefore, an extensive structure-activity relationship (SAR) study of amino acid ester and aryl moieties is generally performed to find an optimal combination of the promoieties for biological activity.

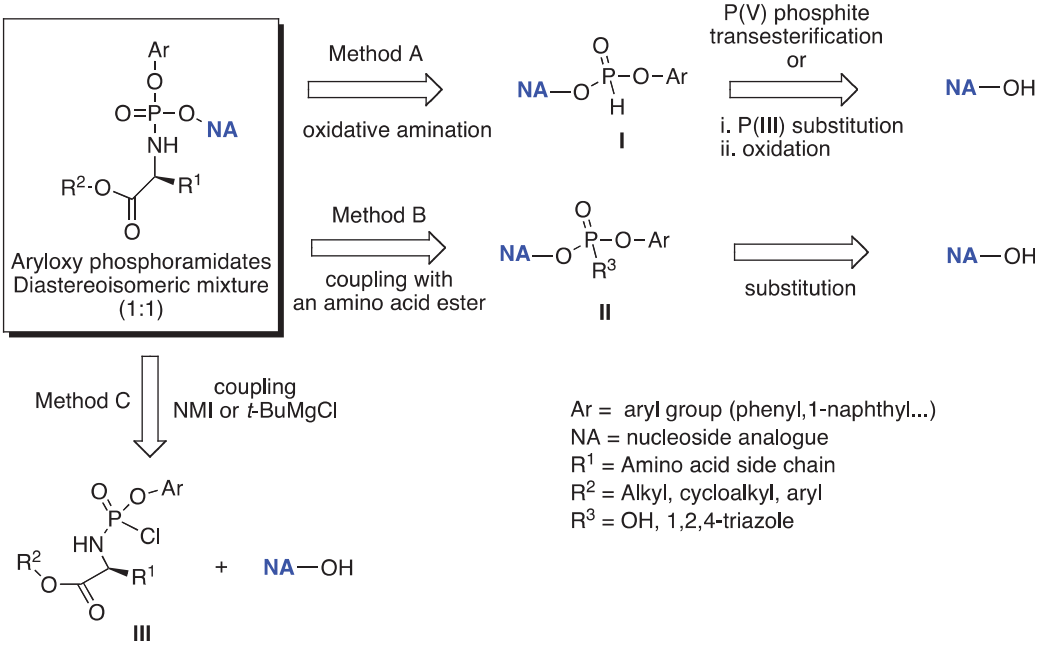
The ProTide approach as a strategy to circumvent an impeded 5′-monophosphate formation was extensively applied also to anticancer NAs.[66,68–80] An Edinburgh-based clinical-stage pharmaceutical company NuCana, is currently pioneering this technology in the oncology setting in collaboration with our laboratories.

The established position of the ProTide approach in the antiviral and anticancer nucleotide prodrug field provided a foundation for its further expansion into additional research area and/or non-nucleoside type compounds. These include phosphoramidates of carbohydrates such as *N*-acetyl-D-glucosamine for the treatment of osteoarthritis,[81,82] 2-fluoro-2-deoxyribose-1-phosphate,[83] 2,2-difluoro-2-deoxyribose-1-phosphate,[83] 2-deoxy-D-ribose-1-phosphate,[84] and phosphonamidates of 2-deoxy-D-ribose-1-phosphonate[85] as antivirals. More recently, application of the ProTide technology to *N*-(3–(5-(2′-deoxyuridine)) prop-2-ynyl)octanamide, the *Mycobacterium tuberculosis* thymidylate synthase X inhibitor[86] and to fingolimod, an immunomodulating drug used for treating multiple sclerosis disease[87] was reported. Other research groups have also explored this prodrug approach in different areas of medicinal chemistry.[88–91]

Synthetic methods

Aryloxyphosphoramidates

There are three methods for the preparation of aryloxyphosphoramidates as highlighted in the retrosynthetic approaches presented in Scheme 1. These procedures differ in the way the phosphoramidate moiety is introduced at the 5′-hydroxyl group of a NA. Method A is based on a coupling reaction of a NA with a diarylphosphite to form a NA-5′-monoaryl-*H*-phosphonate intermediate **[I]** suitable for subsequent oxidative amination.[92] In method B, a nucleoside aryl phosphate **[II]** is coupled to an amino acid. Method C employs a phosphorylating agent [**III**] which is coupled to a NA in the presence of either *N*-methylimidazole (NMI) or *tert*-butyl magnesium chloride (Grignard reagent, *t*-BuMgCl).[93] Over the past two decades, method C has been recognized as the most common strategy for the synthesis of aryloxyphosphoramidates and it has been applied to a wide number of antiviral and anticancer NAs. The choice of base for the coupling reaction largely depends on the presence and susceptibility of other free hydroxyl groups in the substrate. These additional groups might compete with the 5′-OH toward phosphorylation. The key phosphorylating



Scheme 1. General retrosynthetic approaches for the conventional synthesis of aryloxyphosphoramidates.

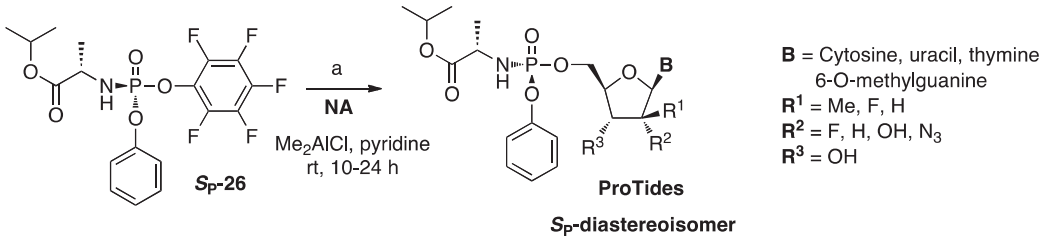
agents **[III]**, obtained as a pair of diastereoisomers at the phosphate centre (1:1 *Rp: Sp* ratio) are formed from an amino acid ester (HCl or tosyl salt) and dichlorophosphate upon the reaction with triethylamine at low temperature.[94,95]

The Grignard reagent is not selective thus when used for the coupling reaction, the formation of undesired 3′-phosphoramidate and 3′,5′-bis-phosphoramidate is usually observed. This methodology suffers some limitations such as the need of extensive purification from a complex mixture of 3′,5′-bis-phosphorylated by-products, which is not suitable in case of large-scale synthesis.

Coupling mediated by NMI, which forms a labile imidazolium intermediate with phosphorylating agent, favors the selective phosphorylation of the primary hydroxyl group at the 5′-position of the nucleoside. However, the final outcome in terms of regioselectivity and yield with NMI or *tert*-BuMgCl is difficult to predict. The comprehensive review by Pradere et al.[96] summarizes these results for a wide range of NAs.

To avoid the formation of undesired 3′,5′- and 3′-phoshoramidates, usually the selective protection of the free 3′-hydroxyl group in the sugar part of a NA prior to the coupling reaction with a phosphorylating reagent **[III]** is often necessary. This methodology requires further deprotection of the 3′-position at the phosphoramidate stage. In the case of ribonucleoside analogues, commonly used protecting strategies include 2′,3′-diol protection with isopropylidene or cyclopentylidene moieties as reported by McGuigan and colleagues for *β*-2′-*C*-methylguanosine (2′-MeG),[97] and *β*-2′-*C*-methyladenosine (2′-MeA)[98] and 5-substituted uridine-based NAs such as 5-iodouridine (5-IU) and 5-bromouridine (5-BrU).[99] The *tert*-butoxycarbonyl (Boc) and silyl-containing groups such as *tert*-butyldimethylsilyl (TBDMS) are often used to protect either 3′-OH[100] or 2′- and 3′-OH functional groups in the 2′-deoxy-, 2′-modified-2′-deoxyribonucleoside or ribonucleoside analogues,[101,102] respectively. The selective removal of these protecting groups at the phosphoramidate stage is usually performed under acidic conditions. Isopropylidene or cyclopentylidene moieties are normally removed using 60% acetic acid at 90–95°C, or 80% formic acid at room temperature.[97–99] Whereas, *tert*-butoxycarbonyl (Boc) and silyl-containing groups are detached using a mixture of formic acid/water (1:1 v/v) or trifluoroacetic acid/water/THF (1:1:4) as described for gemcitabine and pseudoisocytidine-based ProTides.[76,80] In general, the yields of deprotection under acidic conditions are rather low, despite the somewhat acid-stable nature of ProTides. Although, yields of the coupling reaction are generally significantly improved using 2′,3′-protected nucleoside, the overall yields of the protection–deprotection sequence remain moderate. The benzyloxycarbonyl (Cbz) group can be efficiently employed to protect hydroxy- and amino-groups in both sugar and nucleobase moieties as reported by Cho et al., for cytidine, uridine, adenosine, and guanosine analogues.[103] The ease with which the Cbz is introduced on both the sugar and the nucleobase, coupled with its facile and clean removal via hydrogenation under neutral conditions, make this protecting group particularly attractive. To mask a competitive site such as NH_2 in a nucleobase unit of NAs and to significantly improve general solubility of NAs, temporary protection of this functional group with either benzoyl or dimethylformamidine groups can be introduced prior to coupling reaction as reported for pyrimidine methylenecyclopropanes[104,105] and the anti-HSV agent ACV, respectively.[106] A more straightforward method toward the 5′-regioselective synthesis of phosphoramidates was recently published by Simmons et al.,[107] consisting of a direct and highly selective 5′-phosphorylation reaction, mediated by dimethylaluminum chloride without employing 3′-protection manipulations. Moreover, when using the single isomer of 2,3,4,5,6-pentafluorophenyl-bearing phosphorochloridate (S_P-**[26]**),[100] both stereoselectivity (see infra) and regioselectivity of resulting prodrugs can be achieved. This method, reported in Scheme 2, was successfully applied to a wide number of modified NAs leading to pharmaceutically relevant compounds such as anti-HCV clinical agents sofosbuvir and INX-08189 with good yields.

When the key phosphorylating agents are used as a pair of diastereoisomers at the phosphorus centre (1:1 ratio R_P and S_P), formation of two diastereoisomeric aryloxyphosphoramidates in the same ratio is achieved. Such diastereomeric mixtures are often very difficult to



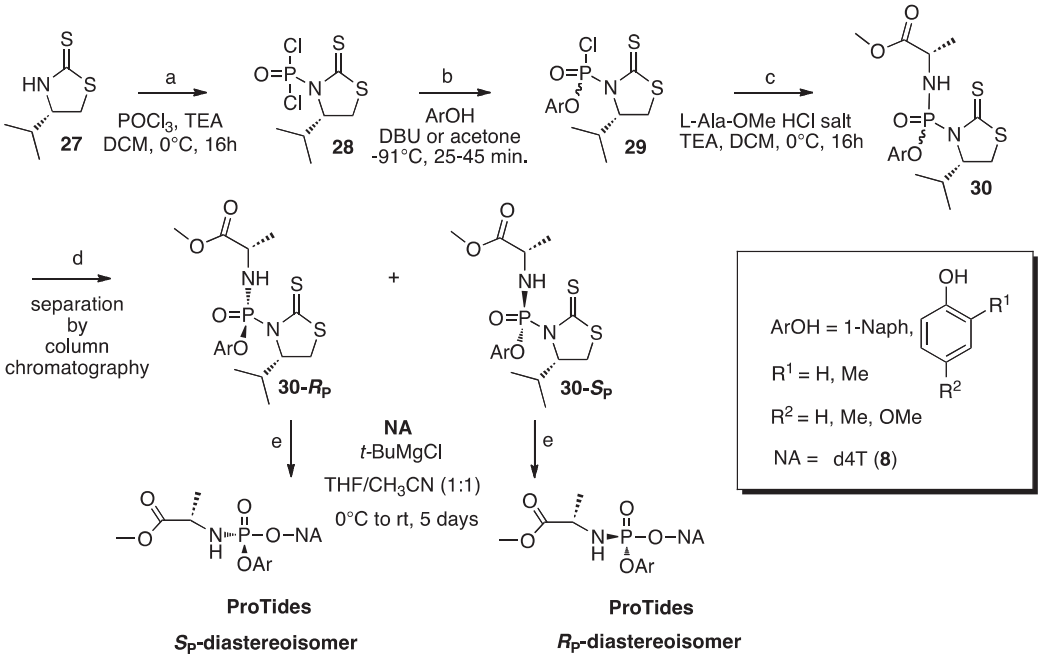
Scheme 2. Regioselective synthesis of 5′-O-phosphoramidates.

separate by standard chromatographic methods, including reverse-phase chromatography or crystallization.[108,109] Therefore, a lack of stereoselectivity represents one of the major limitations of these approaches.

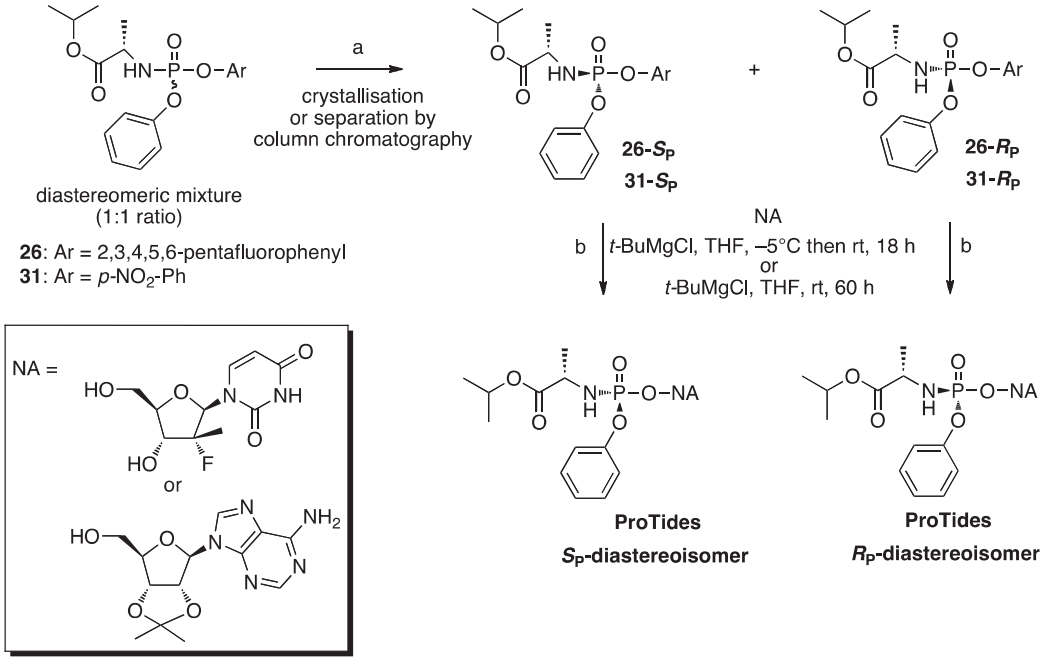
Over the past 10 years, the main focus of researchers was concentrated on the development of diastereoselective strategies toward phosphoramidates obtained as a single isomer. Demand for efficient diastereoselective methods appeared to increase particularly after the discovery of a significant difference in the antiviral activity between S_P and R_P isomers as reported for S_P-isomer of TAF **[25]**, and *S*p-isomer of sofosbuvir **[24]** (Figure 3), which showed a 10-fold increase in potency against HIV[110,111] and an 18-fold difference in HCV activity versus the corresponding *R*p isomer,[62] respectively.

A diastereoselective method to obtain phosphoramidates using a chiral auxiliary-bearing phosphoramidating reagent **[30]** was developed by Meier and colleagues (Scheme 3).[112] In this approach, (*S*)-4-isopropylthiazolidine-2-thione **[27]**[113] acts as a chiral auxiliary and introduces the stereochemistry at the phosphorus atom in the intermediate **[29]**, which is formed following the reaction of phosphorodichloridate **[28]** with aryl derivatives (1-naphthol or substituted phenols) at −91°C in the presence of 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) for 25–75 min. The ratio (up to 14:1 S_p:R_p) of the two diastereoisomers S_p-**[29]** and R_p-**[29]**, obtained in this step, where the chirality transfer is taking place, is dependent on the phenol derivative used in the reaction. The (S_P)-configured diastereomer is assumed to be preferentially formed as the result of an addition/elimination mechanism.[114] When diastereomerically enriched mixtures of phosphorochloridates **[29]** are reacted with L-alanine methyl ester hydrochloride, the phosphorodiamidates **[30]** are obtained as a mixture of chromatographically separable R_P and S_P diastereoisomers (de > 95%). The diastereoselectivity ratio is achieved irrespectively of the variations in the amino acids.[115] In the final S_N2-type reaction each of the diastereoisomer **[30]** (configuration of **[30]**-R_P isomer confirmed by X-ray crystallography) was separately reacted with NAs to give phosphoramidate prodrugs as single R_P and S_P-isomers. In particular, this procedure was reported for d4T **[8]** ProTides obtained in 11–50% yield with 85–95% diastereomeric excess (Scheme 3).

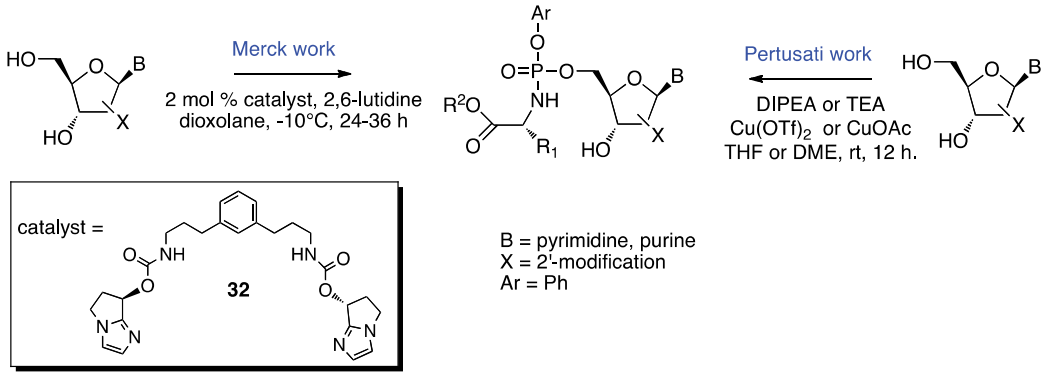
A novel approach to develop a diastereoselective synthesis of aryloxyphosphoramidates was reported more recently by Ross and colleagues.[100] In this approach, a diastereomerically pure phosphoramidating agent with substituted phenolic leaving groups such as *p*-nitrophenyl or 2,3,4,5,6-pentafluorophenyl (Scheme 4), was isolated by crystallization with additional supercritical fluid chromatography and subsequently used in the coupling reaction with two NAs, yielding ProTides as a single isomer. Among a series of different phosphoramidating reagents investigated in these studies, the reagent with 2,3,4,5,6-pentafluorophenyl as the aryl moiety **[26]** was identified as the optimal reagent and its S_P-isomer was used to prepare the HCV clinical agent PSI-7977 (sofosbuvir) in multi-gram scale.[100]



Scheme 3. Asymmetric synthesis of phosphoramidates via chiral auxiliary.



Scheme 4. Diastereoselective synthesis of aryloxyphosphoramidates using a single isomer of 2,3,4,5,6-pentafluorophenyloxy or para-nitrophenyloxy phosphorylating agents.



Scheme 5. Catalyst-mediated diastereoselective synthetic approaches to phosphoramidates.

A diastereoselective method for the synthesis of *P*-chirogenic phosphoramidate prodrugs *via* copper-catalyzed reaction in the presence of a base was recently developed by Pertusati and McGuigan in the McGuigan group.[116] Among several catalysts screened in this study, $Cu(OTf)_2$ and CuOAc proved to be the most effective when used in the synthesis of purine-based and pyrimidine-based ProTides, respectively. An assessment of the effect of the base and solvent on the stereoselectivity and yield of the coupling reaction showed that diisopropylethylamine (DIPEA) and dimethoxyethane (DME) are able to provide the phosphoramidates with good diastereoselectivity and moderate yields (diastereomeric ratio 1:8.3 R_P/S_P; 40–60% yield) (Scheme 5).

Another catalytic stereoselective method that attains high selectivity for nucleoside phosphoramidation was reported in 2017 by researchers at Merck. In this methodology a metal free small-molecule catalyst **[32]** enabled the phosphoramidation of different 2′-modified nucleosides with high stereoselectivity at the phosphorus center towards R_P configuration (Scheme 5).[117] When using this methodology to optimize the synthetic route to the therapeutic anti-HCV agent, the desired R_P-isomer MK-3682 was obtained in high diastereomeric ratio of 99:1 (R_P:S_P) in 92% yield.

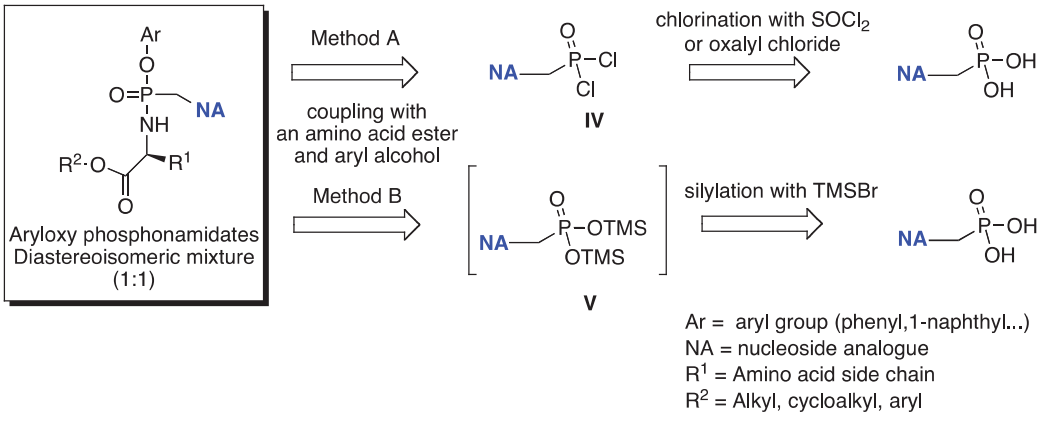
All aryloxyphosphoramidates synthetic strategies described above are based on the phosphoramidation process performed at the level of either protected or unprotected NAs. A different synthetic approach, involving the preparation of an aryloxyphosphoramidate ribose derivative as the key building block was recently testified by Gao et al.[118] This aryloxyphosphoramidate ribose bearing L-aspartic acid diisoamyl ester can be further coupled with a number of nucleobases under Vorbrüggen conditions to afford the desired products. The presence of a 2-acyloxy-group in the sugar moiety is a prerequisite for the neighbouring group participation, which allows N-glycosylation. However, the exclusive formation of the nucleotide analogue with β-configuration is obtained only in certain cases and it depends on the sugar and nucleobase (pyrimidine vs. purine) used for N-glycosylation reaction. Moreover, basic conditions required for the deacetylation (at the ProTide level) might not always be compatible with promoieties other than L-aspartic diisoamyl ester.

Aryloxyphosphonamidates

The preparation of phosphonamidate prodrugs of ANPs is generally accomplished from the corresponding phosphonic acid via two general procedures A and B (Scheme 6).

Method A, developed in McGuigan's laboratory[119] consists of the formation of the nucleoside phosphorodichloridate **[IV]** by treatment of the phosphonic acid with thionyl chloride, followed by reaction with the desired aryloxy-compound and amino acid ester to obtain the corresponding prodrug. During the first step, partial hydrolysis of the second P–Cl bond has been reported. For this reason, before the reaction with the appropriate amino acid, the intermediate must be treated again with thionyl chloride. Under these conditions, tenofovir and adefovir aryloxyphosphonamidate prodrugs were obtained in very low yields (5–10%). Modification of this method was reported by Gilead Sciences.[120] In the first step, a mixture of DMF/sulfolane is replaced by dichloromethane, whereas trimethylsilyl phenolate is instead used as the nucleophile. The temperature is also increased up to 100°C. In this case, the intermediate monochloride is intentionally hydrolyzed with sodium hydroxide and isolated from the reaction mixture. Next, the amino acid ester is introduced via prior chlorination of the mono-acid. This method was further modified for the industrial synthesis of TAF **[25]**.[120–122] The introduction of the aryl alcohol is accomplished by its coupling with the free phosphonic acid using *N,N'*-dicyclohexylcarbodiimide (DCC) as a coupling agent in the presence of an organic base, usually triethylamine. The reaction is generally performed in *N*-methyl-2-pyrrolidinone (NMP) at 100°C. The aryloxy phosphonic acid is then isolated and transformed to the corresponding chloride with thionyl chloride in acetonitrile. The chloride is then reacted at low temperature (−30°C) with the appropriate amino acid ester. In comparison with method A, yields for the formation of phosphonate prodrugs improved up to 25% over two steps.

Method B is based on a modified methodology for the synthesis of symmetrical bis-amidate prodrugs of ANPs, which was reported first by Janeba and colleagues.[123] This latter procedure consists of the synthesis of the silyl ester of the phosphonic acid via the reaction of a selected ANP with an excess of trimethylsilylbromide (TMSBr) in acetonitrile. The silyl ester was not isolated but immediately reacted with the desired amino acid ester in pyridine and triethylamine used as a coupling reagent with a mixture of triphenyl phosphine and 2,2′-dipyridyldisulfide (Aldrithiol-2). Phosphonodiamidates of several ANPs were obtained in high yields. This method is operatively simpler when



Scheme 6. Retrosynthetic analysis for the conventional synthesis of phosphonamidates.

compared to the procedures described above and offers the advantage that either the free phosphonic acids or the corresponding alkyl esters can be used as a starting material. This is of a great advantage, considering the difficulties generally encountered in the purification of free phoshonic acids. McGuigan's group adapted this methodology to the synthesis of phosphonamidate ANPs prodrugs.[66] To accomplish that, the silyl ester **[V]** must be treated with a 1:1 mixture of aryl alcohol and amino acid ester hydrochloride. Under these conditions, both adefovir and tenofovir phosphonamidates were isolated in moderate yields.[66] Later, the same authors discovered that under this protocol for C5-pyrimidine ANPs, functionalized with a but-2-enyl-chain, only traces of the desired phosphonamidate were detected with the phosphonodiamidate being the major product. To circumvent this problem, the addition of an excess (6 equivalents) of aryl alcohol with respect to the amino acid ester (1 equivalent) was essential.[67]

Metabolic activation pathway

The biological activity of aryloxyphosphor(n)amidate prodrugs is expressed after their metabolic activation to the intracellularly released corresponding monophosph(on)ate nucleoside, further phosphorylated to the corresponding active di– and –triphosphate forms by nucleotide kinases.[124]

The early reports on the phosphoramidate activation pathway date back to the late 1990s.[64,125] In these preliminary investigations, the metabolism of d4T phosphoramidates with pig liver carboxylesterase (CES) was studied[125] using in situ ^{31}P NMR analysis, a technique following that is now routinely employed in McGuigan's laboratories as a predictive tool for the likely in vitro biological activity as well as for SAR establishment. The original protocol of this enzymatic experiment was later adapted to study the ProTide first activation step with carboxypeptidase Y enzyme, to prove the nucleoside monophosphate release in biological matrix such as cell lysate[126] or to test the prodrug stability in human serum.[76]

Over the same period, these investigations were then extended to AZT phosphoramidates, where stability was tested with pig liver CES and different biological media such as human lymphocyte CEM cell extract, human serum, and mouse serum.[64,125]

The results of these studies suggested that the carboxylester group linked to the amino acid moiety has pronounced influence on the pharmacokinetics of the prodrugs and their associated stability. Introducing a *tert*-butyl ester was shown to lead to a significant reduction in antiviral potency due to a poor esterase-mediated activation. The metabolism of the prodrug was found to be markedly dependent on the amino acid moiety, with α-amino acids necessary for biological activity. Although compounds bearing longer amino acids (*β*–amino acids) showed efficient ester cleavage, they were found biologically inert and displayed no phenyl loss. L-Alanine was identified as the preferred amino acid, thus fully agreeing with the superior antiviral activity of the L-alaninyl-containing phosphoramidates of d4T seen at that time and consistently observed for other nucleosides analogous in following programmes. These results clearly suggested three key points for the metabolic activation of this class of prodrug: (1) the amino acyl liberation is necessary for biological action, (2) an α-amino acid is necessary for the phenyl cleavage (by intramolecular cyclization), and (3) phenyl loss proceeds after ester cleavage. Based on these findings, for the first time McGuigan proposed the metabolic pathway of the phosphoramidate prodrug,[127] which is commonly accepted and considered valid also for phoshonamidate prodrugs. A general scheme representing metabolic activation of phosphor(n)amidate ProTides is depicted in Figure 5. The mechanism involves an initial carboxylic esterase or carboxypeptidase-mediated hydrolysis of the carboxylic ester of the amino acid leading to intermediate **[A]**. As evidence of this first step Gilead Sciences was able to show that cathepsin A is the primary enzyme that activates TAF **[25]** and GS-9131 (see infra) in human lymphatic tissues.[127] The ester cleavage is followed by an internal nucleophilic attack of the acid residue on the phosphorus centre, displacing the aryloxy group and giving the transient formation of the putative five-membered cyclic intermediate **[B]**. This cyclic mixed anhydride is rapidly hydrolyzed to the corresponding aminoacyl phosphor(n)amidate **[C]**. The intermediate **[C]** is then believed to undergo P–N cleavage, mediated by an enzyme with phosphoramidase activity[128] or may result from simple hydrolysis in a more acidic subcellular compartment, to eventually release the parent drug **[D]**. Phosphoramidase-type enzyme belongs to the human histidine triad nucleotide-binding protein (Hint)[129] and its enzymatic efficiency and substrate specificity is believed to determine the eventual activity of ProTides.[130–132] The postulated mechanism of activation of ProTides was supported by the cellular metabolism study of PSI-7851, a phosphoramidate prodrug of 2′-deoxy-2′-α-fluoro-*β*-*C*-methyluridine-5′-monophosphate, in clone A and primary human hepatocytes. Murakami and colleagues isolated and characterized the metabolites of PSI-7851 and its diastereoisomer PSI-7977 (sofosbuvir), including the intermediate metabolite **[C]** formed upon a stereospecific hydrolysis of the carboxyl ester (catalyzed by cathepsin A and CES1), and subsequent rapid chemical reaction (steps a–c). The succeeding cleavage of the

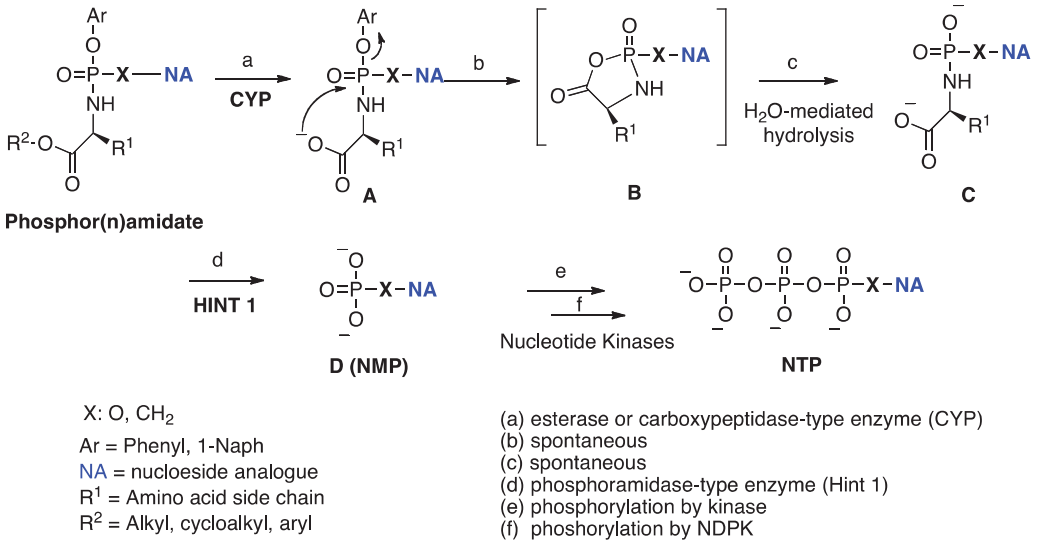


Figure 5. General metabolic pathway for phosphor(n)amidate ProTides.

P–N bond (catalysed by Hint1) lead to a formation of a 5′-monophosphate intermediate further phosphorylated to di- and triphosphate forms (steps d–f).[133]

A detailed mechanism of the hHint1-catalyzed hydrolysis of nucleoside phosphoramidates (in particular sofosbuvir) was recently proposed on the basis of crystallographic studies using a combination of more slowly hydrolyzed substrates and a catalytically inactive mutant enzyme.[134] Molecular modeling of phosphoramidates and their corresponding amino acyl intermediate **[C]** in the catalytic site of a model of either carboxypeptidase[135] or hHint[128] are often employed to analyze the data.

ProTide approach application to antiviral nucleosides

Chris McGuigan's work: From early reports to his latest investigations

The first prototype of phosphoramidates of a nucleoside, reported by McGuigan et al., in 1990, displayed two alkyl amines masking the monophosphate group on AZT.[136] AZT suffers from an absolute dependence on host cell kinase-mediated activation, which can lead to poor activity, emergence of drug resistance, and clinical toxicity. In order to address AZT limitations, McGuigan designed the above-mentioned compounds as membrane-soluble prodrugs of the bioactive nucleotide, capable to bypass the first phosphorylation step. Among different compounds, terminal substituted alkyl amines showed pronounced anti-HIV effect in vitro, which was observed to decline when increasing the length of the methylene spacer. These results were considered consistent with a mechanism of action involving intracellular cleavage of the phosphoramidate P–N bond, and the release of the nucleotide, or a derivative thereof. Thereafter, phosphate triester derivatives of AZT were designed and evaluated against HIV-1 in vitro.[137] During these studies, it was found that simple dialkyl phosphate derivatives of AZT as well as other NAs such as d4T, were inactive as anti-HIV agents, whereas substituted dialkyl phosphates were active. In particular, compounds bearing at the phosphorus centre a trichloro- or trifluoroethyl group and a carboxyl-protected, amino-linked amino acid displayed potent anti-HIV activity and low host toxicity.

Continuing to explore different structures, the phosphorus center was then masked with an ester-containing group in combination with either a simple alkyl moiety or a trichloroethyl group or another ester-containing group.[138] The results of these investigations revealed the presence of two ester-substituted groups enhances activity relative to having only one substituted group. Furthermore, suggesting that a trihaloethyl group may substitute for an ester-containing group but with reduced potency. In several cases, these phosphate derivatives were found to be more selective in their antiviral activity than AZT due to their low toxicity in comparison to the parent nucleoside. Overall, the data supported the conclusion that these phosphate derivatives exert their biological effects via intracellular release of the nucleotide form.

In this report McGuigan stated: "If these in vitro findings could be translated into a demonstrable in vivo advantage, such phosphate pro-drugs could have merit as candidates for clinical development."[138] This was clearly an anticipation of what would have happened 25 years later. His investigations underlaid

the importance of the masked phosphate approach, and had significant implications for what became the future design of chemotherapeutic NAs.

The ProTide series of AZT is the earliest example of aryloxyphosphoramidate technology reported by McGuigan's group in the early 1990s.[139,140]

In vitro evaluation revealed these compounds had a pronounced, selective anti-HIV activity in CEM cells; the magnitude of the biological effect varied considerably depending on the nature of the phosphate-blocking groups. Moreover, several of the compounds retained marked antiviral activity in TK- (thymidine kinase-deficient) mutant CEM cells in which AZT was virtually inactive. Diaryl phosphate derivatives of the anti-HIV NA AZT were also investigated as potential prodrugs of the bioactive free nucleotide. The compounds were shown to be inhibitors of HIV replication in several cell lines, and show reduced cytotoxicity in vitro, by comparison to the parent nucleoside. However, in contrast to the previously reported aryloxyphosphoramidate derivatives, the diaryl phosphates of AZT showed to be poorly active in HIV-infected TK-deficient CEM cells. The results clearly pointed to the aryloxyphosphoramidate as the most promising structure for the delivery of the nucleotide and paved the way for the development of this class of prodrugs.

Thereafter, the ProTide technology was extensively and successfully applied to a high number of nucleoside phosphates with antiviral activity. In particular, following AZT studies, extensive SAR studies investigating the aryl, amino acid, and ester moieties were carried out on d4T phosphoramidates.[141–145] In these reports, the preliminary results on the activation mechanism of such prodrugs were also described.[125,146] The nature of the amino acid appeared to be extremely important for the eventual antiviral action. Among the amino acids studied, L-alanine was the most efficacious, whilst L-proline and glycine were particularly poor. However, an unnatural amino acid moiety, dimethylglycine, was shown to be able to substitute for L-alanine with little or no loss of activity.

As part of research project (sponsored by GlaxoSmithKline in Research Triangle Park North Carolina) devoted to discover anti-HIV and anti-HBV agents, McGuigan's team applied the ProTide approach to 2′,3′-didehydro-2′,3′-dideoxyadenosine and other 2′,3′-dideoxy nucleosides including 2′,3′-dideoxyuridine, -adenosine, -3′-fluoroadenosine, -uridine[147–153] and also to the carbocyclic nucleoside ABC **[11]** with significant enhancement of its antiviral activity.[154] Phosphoramidates of other carbocyclic nucleosides were also explored such as carbocyclic adenosine derivatives and 2′,3′-dideoxy-2′3′-didehydro-7-deazaadenosine and they were shown to possess potent antiviral activity against HIV and HBV.[155,156]

The phosphoramidate technology was also applied to uridine-based NAs. Although their triphosphate forms were found to posses inhibitory activity on uridine triphosphate (UTP) incorporation into RNA of influenza virus, in general they are characterized by poor antiviral activity which may be related to their inefficient phosphorylation.[99] However, in this case the ProTide approach was not very successful leading to compounds with weak antiviral activity. The slow release of the active monophosphate species of these compounds observed in cell lysate, as well as inefficient di- or triphosphorylation of 5′-monophosphate forms were considered as possible explanations for their weak antiviral activity.

A different outcome was instead obtained with ProTides of 6-modified 2′-fluoro-2′-deoxyguanosines, which showed marked antiviral activity in vitro assays proving that this class of prodrugs can be pursued for influenza virus therapy.[157] Rapid metabolic activation in enzymatic assays with yeast carboxypeptidase Y or crude cell lysate supported the antiviral results. Evidence for efficient removal of the 6-substituent on the guanine part was provided by enzymatic studies with adenosine deaminase, and by molecular modeling of the nucleoside 5′-monophosphates in the catalytic site of a model of this enzyme (ADAL1), thus indicating the utility of the double prodrug concept.

No improvement or broadening of the antiviral activity of the parent nucleoside RBV was obtained with a family of ProTides.[158] Again, a likely explanation for this lack of activity was attributed to their poor activation to the free 5′-monophosphate, as evidenced by cell lysate incubation studies. While enzymatic studies with carboxypeptidase Y indicated that the first step in the activation of RBV ProTides was efficient, molecular modeling data with the Hint enzyme suggested that subsequent amino acid cleavage to liberate the necessary free 5′-monophosphate was most probably impeded in this case.[158] Other examples in which the ProTide technology showed a lack of significant improvement of the antiviral activity versus the parent compounds are the phosphoramidates of 2-fluoro derivatives of the bicyclic NA Cf1743, the most potent anti-VZV agent reported to date,[159] and of 2′-deoxy-2′,2′-difluoro-5-halouridine.[160]

A collaboration between McGuigan's group (Cardiff University, UK) and Van Calenbergh's laboratories (Ghent University, Belgium) led to the investigation of α-L-2′-deoxythreofuranosyl nucleosides with A, T, C, and U as nucleobases.[161] Unfortunately, the ProTide of the T NA, included in these studies was devoid of antiviral activity.

More recently, the phosphoramidate approach was applied by the same authors to the family of apionucleosides[162] such as 2′,3′-dideoxy-β-D-apio-D-furanonucleosides (ddANs), which, synthesized in the early 1990s as potential antiviral agents, were found inactive.[163,164] In 2014, Van Calenbergh and colleagues discovered that the 3′-*O*-phosphonomethylated adenine and thymine phosphonate exhibited promising anti-HIV properties. Since these phosphonates act as bioisosteres of the corresponding phosphorylated species, the authors reinvestigated the biological activity of such ddANs and in collaboration with McGuigan's group designed and synthesized nucleosides and their corresponding ProTides, respectively.

While all target nucleosides failed to show significant antiviral activity, the authors demonstrated that the triphosphate of 2′,3′-deoxy-D-apio-D-furanoadenosine was readily incorporated into a DNA template by HIV reverse transcriptase to act as a DNA chain terminator. The ProTides of this nucleoside were found active against HIV-1 and HIV-2, indicating that the lack of activity of the parent nucleoside, and possibly also of other members of the D-apio-D-furanose nucleoside family needed to be sought in the inefficient cellular conversion to the 5′-monophosphate form.

Application of the ProTide approach to ACV was also extensively investigated in the McGuigan laboratories. Although ACV makes an important contribution to the therapy of herpes infections, it has some limitations such as low oral bioavailability and drug resistance caused by mutation in either the TK or DNA polymerase.[17,18] Though the oral bioavailability can be increased by the amino acid prodrugs of ACV, these compounds can be cleaved in the gut and the liver by hydrolase enzymes. Interestingly, ACV was reported to inhibit HIV in human herpes virus (HHV) coinfection in tissue cultures.[165] This activity was found to be correlated with the phosphorylation of the parent drug to the monophosphate form mediated by HHV-encoded kinase(s), whereas further phosphorylation steps provided the active triphosphate form of ACV able to inhibit HIV-RT. Because, HIV does not encode an enzyme that recognizes ACV as a substrate for its activation (phosphorylation) step, the HHV coinfection is needed for ACV to exhibit activity. ACV was therefore a perfect substrate for the application of ProTide technology. Based on these observations, McGuigan and colleagues presented the synthesis and initial biological evaluation (against HSV-1 and HSV-2 and against HIV-1 and -2) of a series of ACV ProTides.[135,166,167] The application of this strategy was efficient to overcome two main issues associated with ACV: to bypass its poor efficiency of diffusion through intact cell membranes and the first limiting phosphorylation step. SAR studies showed that in general ester and aryl variations were well tolerated, whereas the variation of the amino acid moiety seemed to be tolerated only in the case of HSV. Regarding in vitro HIV screening, good results were obtained only for the L-alanine and L-phenylalanine derivatives. Differences in the activity demonstrated by these prodrugs may be due to different substrate specificities and/or different intracellular levels of enzyme necessary for the activation of these compounds. Although the compounds lacked any improvement in activity against HSV-1 and -2 compared to the parent, they retained activity against the TK-deficient HSV-1 strain while ACV showed loss in activity.

In the absence of HHV infection, the prodrug compounds showed antiviral activity, demonstrating their nucleoside kinase independence. These findings were also supported by a different study, where ACV phosphate prodrugs showed a full retention of antiviral activity against HSV-1 and VZV TK-deficient strains. Enzymatic and molecular modeling studies were performed to better understand the antiviral behavior of these compounds. These indicated that ProTides with diminished biolability toward carboxypeptidase translate to poor anti-HIV agents and vice versa. Given that, this enzymatic assay became a predictive tool regularly used to assess potential activity of phosphoramidate prodrugs of other NAs. To overcome the cytotoxicity observed with these prodrugs, very recently a virtual screening on a library of ACV derivatives was reported.[168] Docking experiments with a database of 3600 compounds against three different enzymes encompassing HIV reverse transcriptase, adenylate or guanylate kinase, and a model of DNA polymerase γ resulted in the selection of five NAs as potentially strong RT inhibitors and weak cellular DNA polymerase inhibitors including GCV, PCV, 6-Cl-PCV, 6-OMe-PCV and 2′-SH-GCV. Several phosphoramidate prodrugs of the selected NAs were synthesized and assessed for their potency against HIV, HSV, VZV, and HCMV. Most of the compounds exhibited inhibitory activity against HIV with activity in the low micromolar range, but again some toxicity was observed.

As reported before, over the last 20 years, ANPs have emerged as a novel class of clinically effective antiviral agents.[168] Explorations of various types of nucleoside phosphonate prodrugs have also led to the design and development of their aryloxyamidate prodrugs. ProTides originally designed to deliver nucleoside monophosphates, have also been successfully applied to nucleoside phosphonates. McGuigan's group was the first to report the synthesis and biological evaluation of ProTides based on adefovir and tenofovir.[119] Results of these studies indicate similar SARs for such prodrugs as earlier noted for NAs like d4T (L-alanine containing ProTides being the most

potent compounds).[142] In vitro enzymatic studies and structure–activity relationships indicate that the activation mechanism of phosphonamidate prodrugs may be the same as that described for the phosphoramidate triesters of NAs.

This early work was followed by investigation into the synthesis and antiviral evaluation of a broad family of phosphonamidates of adefovir and tenofovir.[66] ProTides, synthesized with a more efficient methodology, showed improved in vitro anti-HIV activity, compared to previously reported data for the parent ANPs. Phosphonamidates bearing 5,6,7,8-tetrahydro-1-naphthol unit which was for the first time introduced as hydrolysable aryl unit in the ProTide motif displayed an improved antiviral activity compared to the "common"-naphthyl and phenyl ProTide units. Enzymatic studies showed that this novel aryloxy group was processed through the "standard" metabolic pathway. In this view 5,6,7,8-tetrahydro-1-naphthol was considered as a good aryl group for future improvement of the ProTide motif and thus was subsequently used by the same authors to design phosphonamidates of C5-substituted pyrimidine acyclic nucleosides functionalized with but-2-enyl-chain.[67]

In the search of anti-HCV agents, McGuigan's group started a project (funded by Roche Palo Alto), involving application of the ProTide technology to the ribonucleoside analogues 4′-azidouridine,[169] 4′-azidoadenosine,[170] and 4′-azidocytidine.[171] Although 4′-azidouridine and 4′-azidoadenosine did not inhibit HCV, their triphosphate forms showed potent inhibitory activity against HCV RNA polymerase. Several phosphoramidates of these NAs were prepared, including variations in the aryl, ester, and amino acid regions. Among a number of 4′-azidouridine and -adenosine prodrugs with sub-micromolar inhibition of HCV replication in cell culture, the 1-naphthyl L-alanine benzyl ester phosphoramidates of both of the two NAs were the most active compounds in the replicon assay without detectable cytotoxicity. On the contrary, no significant improvement in the activity was observed with the 4′-azido cytidine family versus the parent nucleoside. Phosphoramidate ProTides derived from 4′-azidoinosine were also reported to be active at low micromolar levels in the replicon assay against HCV, whereas the parent NA was inactive in this assay.[172] These results confirmed that the ProTide technology allows delivery of ribonucleoside 5′- monophosphate, suggesting a potential path to the generation of novel antiviral agents against HCV infections. As a continuation of this anti-HCV programme, the ProTide approach was also applied by McGuigan's team to β-2′-*C*-methylpurine.[98] For this type of NAs, the antiviral activity is usually expressed through their corresponding intracellular triphosphate forms that are potent and competitive inhibitors of NS5B viral RNA polymerase. Although, the phosphoramidate of β-2′-methylguanosine containing L-alanine benzyl ester and 1-naphthyl group was found to be the most active, it suffered from rodent plasma instability. The variation of amino acids with the lead benzyl ester moiety led to enhanced stability in rodents, however this resulted in significant reduction in HCV replicon activity. Extensive modification of the ester functionality demonstrated no significant improvement in HCV potency.[97] Following these results modifications at the C-6 of the purine base as a means of potentially affecting potency without changing the inherent plasma stability of phosphoramidates were carried out.[173] These investigations led to the discovery of the aryloxyphosphoramidate double-prodrug of *O*-6-methyl-2′-*C*-methyl-guanosine bearing L-alanine neopentyl and 1-naphthyl as an amino acid ester and aryloxy moiety (INX-08189, BMS-986094, **[33]**, Figure 6). This prodrug was licensed out to Inhibitex, the start-up company in Atlanta, and later acquired by Bristol Myers Squibb to continue the development of this compound and its analogues.

INX-08189 exhibited nanomolar activity in vitro in HCV replicon assay, with EC_{50}s of 10 nM against genotype 1b, 12 nM against genotype 1a, and 0.9 nM against genotype 2a after 72 h of exposure.[174] It was also tested in the S282T mutant replicon and showed reduced activity with a 10-fold change in the EC_{50} yet still being capable to complete inhibition of HCV replication with EC_{90} value of 344 nM. In the replicon inhibition studies in which INX-08189 was used in combination with RBV, a high degree of synergy against the wild-type (WT) and S282T mutant replicons was observed. Intracellular metabolism of INX-08189 and its conversion to the active 5′-triphosphate form was investigated in the HCV genotype 1b replicon assay, showing an intracellular concentration of the triphosphate form (2′-*C*-MeGTP) of 0.84 ± 0.36 and 2.43 ± 0.42 pmol/1×10^6 cells were able to achieve 50% and 90% inhibition of viral application, respectively. The assessment of the mitochondrial toxicity in 14-day tissue culture studies demonstrated INX-08189 to be devoid of any mitochondria-specific toxicity in a liver-derived HepG2 and lymphocyte CEM human cell lines. Based on these advantageous properties, INX-08189 was advanced into in vivo studies supporting its further selection as a clinical candidate for the treatment of HCV infections. Pharmacokinetics and pharmacodynamics properties of INX-08189 were investigated in rats and cynomolgus monkeys by measuring the generation of the parent nucleoside 2′-MeG, along with the active triphosphate form (2′-*C*-MeGTP). The data for rats and monkeys were consistent in the linear relationship between the 2′-MeG

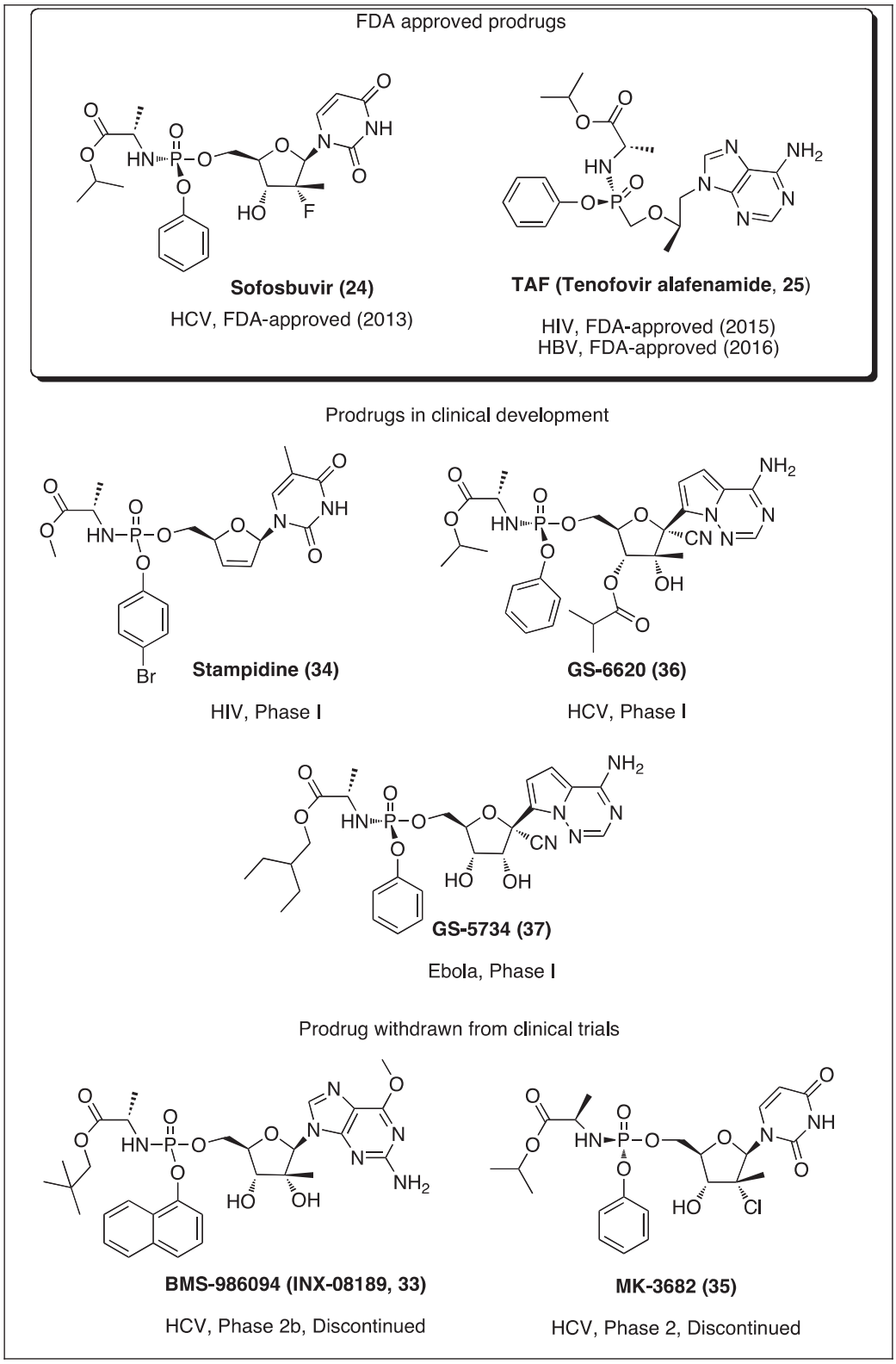


Figure 6. Antiviral phosphor(n)amidates in clinical use or in clinical development.

AUC_{0-24} values and the concentration of 2′-*C*-MeGTP in the liver at 24 h. In addition, in vivo results indicated also that 2′-*C*-MeGTP concentrations in the liver equivalent to the EC_{90} could be reached after a single dose of 3 mg/kg and 25 mg/kg in rats and monkeys, respectively.[174] The overall in vitro and in vivo data led to further progression and clinical development of INX-08189 as a highly potent HCV inhibitor. A safety and pharmacokinetics Phase Ia study in healthy volunteers, revealed INX-08189 administrated in a range of doses (3–100 mg) to be well tolerated at all doses with a lack of drug-associated serious adverse events.[175,176] In a following Phase Ib study in the treatment-naive genotype 1 HCV patients, a mean HCV RNA reduction of −0.71 and −1.03 $\log_{10}$ IU/mL was observed when patients were dosed once daily with 9 mg or 25 mg, respectively. However, due to severe adverse events including heart failure and acute kidney injury, a further clinical development of INX-08189 was halted.[177] A broad bioanalysis assays for the active nucleoside triphosphate, the prodrug INX-08189 and its metabolites in multiple target (diaphragm, heart, kidney, liver), and nontarget (lung) tissue were performed in order to evaluate the potential mechanism of the toxicity observed for INX-08189 in a Phase II clinical study. The triphosphate form (2′-*C*-MeGTP) was persistent in the heart and kidney in high levels after the treatment-free period (week 6 in 3 week-+3 week recover study) and thus appeared to be correlated with potential toxicities in these two organs.[178]

ProTides in clinical use or in clinical development as antiviral agents

The research undertaken in McGuigan's laboratories during the last 25 years was of great inspiration for many scientists all over the world. Nowadays ProTide technology is recognized as a prodrug strategy with proven capacity to generate new drug candidates for nucleoside-based antiviral indications. In fact, a potential of this prodrug approach was confirmed with the discovery of agents that are currently in clinical use (sofosbuvir and TAF), or in clinical development as antiviral drugs including stampidine **[34]**, MK-3682 **[35]**, GS-6620 **[36]**, and GS-5734 **[37]** (Figure 6). Furthermore, the indisputable potential of the ProTide approach in the antiviral field continuously encourages many research groups to design and develop novel phosphoramidate-type agents based on various structurally modified NAs. Currently, several academic groups and pharmaceutical companies have more ProTide compounds in the pipeline undergoing preclinical studies for the treatment of viral infections.[55,179–185]

Sofosbuvir

During the time of McGuigan's research on HCV, phosphoramidate prodrugs of the 5′-phosphate β-D-2′-deoxy-2′-α-fluoro-2′-β-C-methyluridine nucleoside were developed by Sofia et al., (Pharmasset)[62] leading to the discovery of the clinical antiviral agent sofosbuvir, which was launched in the market by Gilead Sciences. Sofosbuvir (Sovaldi®, GS-7977, PSI-7977) represents the first-in-class phosphoramidate-type inhibitor of NS5B RNA polymerase with FDA approval for the treatment of chronic HCV in patients infected with multiple HCV genotypes. Sofosbuvir (in 2009 entry to first-in-man Phase I trial) expresses high barrier of resistance[186] and currently is recognized as the gold standard of care for HCV-infected patients. Moreover, sofosbuvir is also FDA-approved for HIV coinfected patients and those awaiting liver transplant. Although originally synthesized via conventional method as a 1:1 diastereomeric mixture (PSI-7851), sofosbuvir was further clinically developed as a single isomer with S_P stereochemistry (PSI-7977) at the phosphorus atom as confirmed by X-ray structure determination. Biological evaluation of two individual isomers following separation by high-performance liquid chromatography (HPLC chromatography), was reported in the WT replicon cells and shown ∼18-fold difference in anti-HCV activity between PSI-7977 (S_p isomer EC_{90}: 0.42 μM) and PSI-7976 (R_p isomer, EC_{90}: 7.5 μM). When the two isomers were tested against replicons containing known nucleoside resistant mutants S282T and S96T, the ∼13-fold activity difference was observed for PSI-7977 (EC_{90} 7.8 μM in S282T) versus PSI-7976 (EC_{90}: >100 μM in S282T). In the replicon cells containing S96T NS5B polymerase mutation, the two inhibitors PSI-7977 and PSI-7976 showed similar antiviral activity (EC_{90} 0.23 μM vs. 3.3 μM in WT and 0.11 μM vs. 1.3 μM in S96T), with no cross-resistance detected for both isomers.[62] Additional studies for the ability to generate intracellular levels of the active 5′-triphosphate shown that S_P isomer (PSI-7977) produced 10-fold greater levels of triphosphate form in comparison with R_P isomer (PSI-7976) in clone A replicon cell lines, and 1.1-fold in human hepatocytes, respectively. An in vitro study confirmed that sofosbuvir undergoes the common metabolic pathway suggested for other phosphoramidate prodrugs to form the 5′-monophosphate metabolite which is further phosphorylated subsequently to the corresponding 5′-triphosphate active form first by uridine-monophosphate-cytidine-monophosphate kinase (UMP-CMP) and second by nucleoside diphosphate kinase. Mimicking the natural substrate of NS5B RNA-dependent RNA polymerase, sofosbuvir induces a chain termination process by being incorporated into

the growing RNA. In vivo preclinical pharmacokinetics study shown that sofosbuvir was well-absorbed and metabolized to its intermediate metabolite (corresponding to the achiral intermediate **[C]** in Figure 5) and NA.[187] A comprehensive characterization of pharmacokinetic, pharmacodynamics, and drug-interaction profile of sofosbuvir was reported by Gilead Sciences.[188] The pharmacokinetics of single and multiple ascending doses of this agent administrated to HCV-infected patients revealed, similarly to single-doses studies in healthy subjects, that sofosbuvir and its parent nucleoside exhibited time-independent, near-linear pharmacokinetics within all evaluated doses.[188] Following several clinical trials,[186] a global Phase III clinical trial study with sofosbuvir/RBV therapy was initiated. This trial indicated, that 12 weeks treatment was efficient for patients with HCV genotype 2 and 3-infection (without previous interferon-based therapy) and resulted in high rates of sustained virologic response (>90% and >60% for patients infected with genotype-2 and 3, respectively). However, for patients with HCV genotype 3-infection (associated with possibly a greater risk of hepatocellular carcinoma), treatment extended up to 16 weeks was more beneficial than 12 weeks.[189–191] On the basis of these results, an additional descriptive study was designed in which 250 patients with genotype 3-infection underwent treatment with sofosbuvir/RBV regimen for 24 weeks. The response rates in this subgroup were 91% and 68% among those patients without and with cirrhosis, respectively. Among total of 73 patients with genotype 2-infection treated for 12 weeks with sofosbuvir/RBV, 68 patients had a sustained virologic response 12 weeks after closure of study. Although with some limitations, the overall results of this study shown that the oral sofosbuvir/RBV regimen can be effective for both genotype 2 and 3-infected patients and also can offer an alternative to a pegylated interferon-based regimen for HCV patients.[192] Following these results sofosbuvir was approved in December 2013 to treat chronic hepatitis C[193] and in April 2017 for the same infections in paediatric patients 12 years and older.[194]

Tenofovir alafenamide (TAF)

As mentioned above, one of the most successful example of ANP prodrug is represented by TDF (**[23]**, Figure 3), sold under the trade name Viread, and used in the treatment of chronic hepatitis B and to prevent and treat HIV/AIDS. Viread was found to have considerably improved cell permeability and anti-HIV activity in in vitro screening,[195] increased oral bioavailability in animals,[196] and more efficient loading of peripheral blood mononuclear cell (PBMC) relative to parenteral tenofovir observed in vivo.[197] However, while TDF therapy is generally well tolerated it has negative effects on the renal function and on the bone mineral density. Different studies have demonstrated greater loss of kidney function and a higher risk of acute renal failure in patients receiving TDF-based therapies versus non-TDF regimens.[198] Renal adverse events have been associated with the higher levels of tenofovir in the plasma observed when TDF was given with HIV protease inhibitors or pharmaco-enhancers, inhibitors of the intestinal efflux of TDF[199,200] suggesting a link between plasma tenofovir exposure and the effect on proximal tubule function.[201] In addition to renal disfunction patients receiving TDF, antiretroviral therapy showed a larger reduction in bone mineral density than regimens without TDF.[202]

To circumvent these major problems, Gilead Sciences developed TAF (TAF, **[25]**) fumarate, the isopropylalaninyl monoamidate phenyl monoester prodrug of tenofovir.[203] TAF was initially synthesized as 1:1 mixture of diastereoisomers (GS-7171), which were then separated by chromatography. The antiviral activities of the diastereomeric mixture (GS-7171), as well as those of the individual diastereomers (R_P isomer GS-7339 and S_P isomer GS-7340), were evaluated in vitro in MT-2 cells infected with HIV-1 virus. When compared to the activity of tenofovir (EC_{50}: 5.0 ± 2.6 μM) in the same test, the two single isomers GS-7339 (R_P isomer, EC_{50}: 0.06 ± 004 μM) and GS-7340 (S_P isomer, EC_{50}: 0.005 ± 002 μM) were found to be 83- and 1000-fold more active. Again, also in this case it is worth to highlight that the S_P-diastereoisomer is much more active that the R_p-diastereoisomer suggesting that intracellular metabolism is sensitive to stereochemistry at the phosphorus.[203] In addition, when the D-alanine was incorporated as promoiety, the resulting prodrug (GS-7485), showed similar activity to tenofovir. Given that, the two prodrugs (GS-7171 and GS-7485) release the same pharmacologically active metabolite, the considerably reduced activity of the D-alaninyl analogue (GS-7485), versus the L-alaninyl analogue (GS-7171) might be explained by a strong metabolic preference inside the cells for the natural amino acid. TAF (GS-7340) was also found to show a greater selectivity index than TDF. The higher initial intracellular concentration of tenofovir achieved with TAF, relative to TDF, was considered as to be able to differentially affect antiviral potency and cytotoxicity.[203] The key properties that made TAF so successful were its stability in biological matrices, including plasma, and its selective intracellular cleavage. In vitro metabolism and accumulation in PBMCs studied in tissue culture showed that in MT-2 cell extract, GS-7340 was metabolized three times faster than in plasma, whereas TDF was metabolized 170-fold faster in plasma.[203] This

stability accounts for prolonged systemic exposure to intact prodrug and the accumulation of higher intracellular levels of the pharmacologically active metabolite tenofovir diphosphate relative to TDF. In MT-2 cells incubated with 10 μM GS-7340, the formation of the diphosphate metabolite was linear for 24 h with a concentration inside the MT-2 cells at 24 h exceeding the initial extracellular concentration of GS-7340 by 250-fold.[203]

In preclinical animal studies, TAF exhibits enhanced distribution of tenofovir into PBMCs and the lymphatic organs after oral administration, in comparison to tenofovir disoproxil fumarate. Twenty-four hours after a single dose of TAF in dogs, the concentration of tenofovir in lymphatic organs is between 5- and 15-fold greater than an equivalent dose of TDF. Intracellular tenofovir concentrations, measured by AUC_{0-24}, in PBMCs after a single oral dose of TAF in dogs are ~38-fold greater after an equivalent oral dose of TDF and ~100-fold greater than those observed after subcutaneous administration of tenofovir. These in vivo pharmacokinetics studies showed also that both (GS-7340 and GS-7339) were rapidly eliminated in plasma relative to tenofovir with the S_P-isomer cleared more rapidly than its R_p-counterpart.[203] Pharmacokinetic study in dogs demonstrated that TAF is taken up efficiently by the liver. Further in vitro studies showed that TAF is a substrate for the hepatic transporters OATP1B1 and OATP1B3. Although this might explain the high concentration of TAF in the liver, it is more likely that the high passive permeability of the phosphonamidate prodrug is the major vehicle of the drug into the liver.[204]

Moreover, intracellular activation and antiviral activity of TAF are adversely non-affected by other medications (often administered in combination) such as HIV and HCV protease inhibitors,[205] except for telaprevir and boceprevir that non-specifically inhibit cathepsin A, the key enzyme responsible for the activation of the prodrug.[127]

Furthermore, the rapid intracellular cleavage step catalyzed by cathepsin A in HIV- and HBV target cells, coupled with the formation of poorly permeable metabolites (the charged phosphates) effectively trapped in cells, accounts for the substantial accumulation of the pharmacologically active metabolite tenofovir diphosphate and increased therapeutic efficacy of TAF with respect to other prodrugs. Because of this accumulation inside the infected cells, TAF can be administered in lower therapeutic doses in comparison to TDF. Phase I/II clinical study to asses the pharmacokinetics, safety and anti-HIV activity of TAF showed that administration of 40 mg of TAF for 14 days in HIV-infected patient resulted in lower tenofovir C_{max} and lower systemic exposures compared with subjects who received TDF. Higher intracellular concentrations of tenofovir in PBMCs were detected with two doses (40 mg and 120 mg) of TAF then with 300 mg TDF. TAF had the same resistance profile as tenofovir and TDF (in vitro) with no resistance mutations detected for both of them.[206] Phase III studies were then undertaken to confirm the following observations and to further define the safety and efficacy profile of TAF. Results of these studies revealed TAF to be as effective as TDF in much lower dose and with lower occurrence of adverse side effects such as impaired kidney function.[207] In November 2015, the TAF-based regimen (elvitegravir/cobicistat/emtricitabine/TAF) was FDA-approved for treatment of HIV-1.[208]

TAF was also investigated for the treatment of HBV infections. In particular, Phase III clinical trials studies evaluating investigational use of once-daily 25 mg dose of TAF in treatment-naive and treatment-experienced adults with HBeAg-negative and HBeAg-positive chronic HBV infections, demonstrated again that TAF was non-inferior to TDF based on the percentage of patients having low HBV DNA levels after 48 weeks of therapy. In addition to high efficacy, the results of these studies reflect improved renal and bone safety parameters similar to those seen in clinical studies evaluating TAF-based regimens for HIV. In November 2016, TAF received FDA approval for the treatment of chronic HBV.[209]

Stampidine

Stampidine **[34]** is a rationally designed phosphoramidate derivative of stavudine (d4T, **[8]**). developed as an anti-HIV agent to overcome the dependence of **[8]** on intracellular nucleoside kinase-mediated activation to the nucleoside 5′-monophosphate. Stampidine was shown to inhibit the replication of HIV-1 strain human T-lymphotrophic virus (HTLV)-IIIB in human PBMCs at nanomolar 50% inhibitory concentration with IC_{50} value being 20 times lower than its parent nucleoside **[8]** and selectivity index >100,000. Stampidine was found to be active also against phenotypically and/or genotypically NRTI-resistant HIV strains, including 20 primary clinical HIV-1 isolates resistant to AZT. A similar nanomolar inhibitory activity was demonstrated by stampidine against primary clinical HIV-1 isolates with non-nucleoside RT inhibitor (NNRTI) binding site mutations and/or phenotypic NNRTI-resistant profiles. Moreover, among 12 stavudine substituted-phenyl phosphoramidates, stampidine was found to be the most effective in the inhibition of adenovirus-induced plaque formation in human foreskin fibroblast with no cytotoxicity up to 100 mM and selectivity index >4000.[210] In the in vivo pharmacokinetics and metabolism studies reported by Chen

et al.,[211] stampidine demonstrated favorable pharmacokinetics and rapid hydrolysis to L-ala-d4T-MP (the key precursor of the d4T triphosphate metabolite) as well as its parent nucleoside d4T following i.v. injection and oral administration in mice, rats, dogs, and cats.[212] In fact, the unique ability of stampidine to quick hydrolysis is correlated with the presence of *p*-Br substituted-phenyl part of the phosphoramidate prodrug, which in turn translates into rapid formation of L-ala-d4T-MP.[212,213] L-Ala-d4T-MP is considered as an intra- and/or extracellular source of d4T and/or d4T-MP, which might explain the superior antiretroviral activity of stampidine over d4T in cell culture.[214] In the postulated activation pathway for ProTide **[34]** (as well as other R_P or S_P phosphoramidates of stavudine), lipases are also involved in the hydrolysis of ester moiety in the amino acid side chain showing clear preference for the L-amino acid configuration as reported in the experimental and modelling studies.[215–217] Phase I randomized, placebo controlled study performed with the aim to investigate early safety, tolerability and activity data in 30 therapy-naive HIV-infected patients showed that stampidine did not cause dose-limiting toxicity at single dose levels ranging from 5 to 25 mg/kg.[218] Pre-exposure prophylactic therapy studies for stampidine are ongoing.[219] Despite the encouraging data, there are no reports available on further progression of this agent in clinical trials.

Uprifosbuvir

Uprifosbuvir (MK-3682, IDX-21437, **[35]**) is the R_P-isomer of D-amino acid–based phosphoramidate prodrug of 2′-*C*-methyl-2′-chloro-uridine-based NA[117] that reached clinical trial as anti-HCV agent. In the in vitro Huh-7 cell based HCV replicon assay, MK-3682 demonstrated weak activity, however when tested in vivo very high levels of nucleoside triphosphate were observed in mouse liver (6200 and 3750 pmol h/g for MK-3682 and its S_P counterpart isomer, respectively). Interestingly, this may indicate that MK-3682 undergoes different metabolic activation or differentiated kinetics of the ester hydrolysis between two diastereoisomers as suggested for the 2′-*β*-*C*-Me-2′-*α*-C-F-uridine series.[134] In fact, in vitro metabolic study performed ffor MK-3682 and its S_P analogue and sofosbuvir by purified CES1 and cathepsin A (CatA) showed clear substrate preference of CES1 for R_P isomer (94% of MK-3682 hydrolysed whereas only 4.5% of S_P isomer hydrolysed over 21 h). Incubation of these three compounds with CatA for 18 h resulted with no hydrolysis of MK-3682 and S_P isomer and complete metabolism of sofosbuvir.[220] The most recent data from the two Phase II studies on efficacy and safety of uprifosbuvir together with grazoprevir and ruzasvir, a HCV nonstructural protein 5A inhibitor, either with or without RBV for 16 and 24 weeks, respectively in patients who had previously failed an NS5A inhibitor-containing treatment, showed that this triple-combination therapy was safe and highly effective.[221] Several other clinical trials Phase I/II studies for MK-3682 were on-going in a combination therapy with RBV or non-nucleoside agents such as grazoprevir, elbasvir, and ruzasvir.[222–224] In 2017, further clinical evaluation of uprifosbuvir in a combination therapy was discontinued.[225]

GS-6620

A single S_P-isomer GS-6620 **[36]** represents a family of phosphoramidate prodrugs based on 2′-*C*-methyl-7,9-dideaza-4-aza-adenosine containing 1′-cyano and 2′-*C*-Me groups with inhibitory activity against NS5B polymerase.[226] The presence of the 2′-*C*-Me group in natural ribonucleoside analogues once incorporated to the primer position was reported to arrest an incoming nucleoside triphosphate from binding to active site of NS5B polymerase and therefore causing inhibition of further RNA elongation and viral replication.[227] GS-6620 is a double-prodrug with L-alanine-isopropyl and phenyl moieties attached at the 5′-hydroxyl as a phosphoramidate part, and isobutyryl ester added into the 3′-postion on the ribose ring with the aim to improve its permeability and oral bioavailability. Like other phosphoramidate agents, GS-6620 was initially synthesized via conventional method and obtained as a part of diastereomeric mixture further separated by chiral column chromatography.[226] The preliminary in vitro results revealed the S_P-diastereoisomer to be 6-fold more potent, and two times more efficient in the formation of triphosphate in primary human hepatocytes than its R_P analogue. The antiviral activity in the panel of HCV replicon assay including stable replicons (GT1b, 1a, 2a) and chimeric replicons (2b, 3a, 4a, 5a, and 6a) was also demonstrated by GS-6620 with EC_{50} ranging between 0.068 and 0.43 μM. In the additional cytotoxicity studies, GS-6620 showed detectable toxicity in erythroid marrow progenitor cells (CC_{50} 15 μM), a minimal cytotoxicity in cell lines such as Huh-7 (CC_{50} 67 μM), HepG2 (CC_{50} 66 μM), and PC-3 (CC_{50} 40 μM), and no cytotoxicity in the primary hepatocytes, PBMCs (quiescent and stimulated) at the highest concentration tested (100 μM).[228] In the mitochondrial toxicity study, no inhibition of mitochondrial DNA level at the highest concentration tested (20 μM) in HepG2 and PC-3 cells, as well as no inhibition of mitochondrial DNA content was observed. Moreover, no specific inhibition of mitochondrial protein synthesis and no inhibition of host DNA or RNA polymerases were detected at the highest concentration (up to 500 μM). In the cross-resistance studies GS-6620 showed reduced

Table 1. Prodrugs in clinic and clinical development.

Drug name	Originator/developer	Phase	Disease	Viral Target Mechanism of action	Ref
Sofosbuvir (Sovaldi, **[24]**)	Pharmasset/ Gilead Sciences	Approved 2013	HCV	NS5B RNA-dependent RNA polymerase inhibition	62
Tenofovir alafenamide (TAF, **[25]**)	Gilead Sciences	Approved 2016 Approved 2015	HBV HIV	HBV reverse transcriptase inhibition HIV reverse transcriptase inhibition	63
INX-08189 **[33]**	Inhibitex/BMS	Discontinued	HCV	NS5B RNA-dependent RNA polymerase inhibition	175
Stampidine **[34]**	Paradigm Pharmaceuticals, Parker Hughes Institute	I	HIV	RNA-directed DNA polymerase inhibition	213
MK-3682 **[35]**	Merck	Discontinued	HCV	NS5B RNA-dependent RNA polymerase inhibition	223
GS-6620 **[36]**	Gilead Sciences	I	HCV	NS5B RNA-dependent RNA polymerase inhibition	231
GS-5734 **[37]**	Gilead Sciences	II	Ebola V	Viral RNA-dependent RNA polymerase inhibition	235

activity only against genotype 1b (GT1b) replicons with the S282T NS5B mutation. In order to inhibit HCV RNA polymerase in hepatocytes, GS-6620 needs to be first activated to the free 5′-monophosphate that is subsequently phosphorylated to its 5′-triphosphate derivative. The postulated activation pathway is aligned with the conventional metabolic activation route for ProTides (Figure 5) and consists of an additional 3′-isobutyryl ester hydrolysis catalysed by CES2 at first, followed by the hydrolysis of the isopropyl ester group on the 5′-ProTide moiety catalysed by CatA or CES1.[229] The compound was clinically developed and evaluated for its antiviral activity, safety and tolerability in first-in-human Phase I clinical study in the treatment-naive patients chronically infected with HCV genotype 1.[230] GS-6620 was well tolerated up to 900 mg when given twice a day for five days and shown the greatest antiviral activity with median viral load reductions of 1.73 and 1.63 $\log_{10}$ IU/mL from baseline with 900 mg (administrated as a tablet) and 450 mg (administrated as a solution), when given twice daily, respectively. Although, GS-6620 demonstrated potent antiviral activity, its clinical efficacy and utility turned to be limited due to observed high levels of substantial intra- and intersubject pharmacokinetic and pharmacodynamic variability.[231]

GS-5734

GS-5734 **[37]** is the single S_P isomer of the 2-ethylbutyl-L-alaninate monophosphate prodrug of 1′-cyano-substituted adenosine *C*-NA with enhanced activity against a number of filoviruses including Marburg virus and several variant of ebola virus EBOV in cell-based assay.[232] A comprehensive report on structure–activity relationship, lead optimization, and robust diastereoselective synthesis of GS-5734 was recently published by Gilead Sciences following their focused library screening of ~1000 diverse nucleos(t)ide analogues with antiviral properties.[233] The outcome of this work and the discovery of GS-5734 coincided with the most recent Ebola outbreak in West Africa. The prodrug GS-5734 expressed high selectivity for the viral polymerase as compared to host polymerases. In vivo efficacy evaluation of GS-5734 (conducted in rhesus monkeys as the most relevant animal model of EVD) demonstrated its rapid elimination with appearance of parent nucleoside in systemic circulation. The triphosphate levels in PBMCs were elevated to a maximum within 2 h. A maximally efficacious dose of the prodrug was determined in the preclinical in vivo efficacy study performed on an EBOV-infected rhesus challenge model.[232] Further safety and pharmacokinetics study of GS-5734 (Phase I, once-daily i.v. infusion in single and multiple dose) have shown no serious adverse effects associated with the drug. GS-5734 was administered under compassionate use to two Ebola patients including a newborn infant with ebola virus disease (EVD).[234] Currently, Phase II clinical study (PREVAIL IV) is ongoing.[235]

Conclusion

The idea of phosph(on)ates prodrugs with aryloxyphosphoramidates (ProTides) in particular, was extensively investigated over the last three decades. Designed and pioneered by McGuigan and his group, the ProTide approach was successfully applied to a great variety of modified NAs with antiviral and anticancer activity. In this review, we presented a summary of the "ProTide technology" development commencing from its discovery to the most recent applications in the antiviral field. We outlined a discovery and clinical development of recently FDA-approved sofosbuvir (Sovaldi®), one of the best-selling drugs for treatment of hepatitis C infection. We highlighted all synthetic methodologies towards

aryloxphosphor(n)amidates including the latest, more sophisticated regio- and stereoselective procedures. Given its increasing interest in the scientific community, as well as a significant commercial impact, the ProTide technology will continue to be used as a strategy to improve the efficacy profile of nucleoside and also non-nucleoside existing and/or novel therapeutics. Certainly, McGuigan's discovery and extensive research on the phosphoramidate approach has provided an inspiration and the solid foundation for bringing the ProTide concept into the clinical success.

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