

# **An overview of ProTide technology and its implications to drug discovery**

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## **Abstract**

### **Introduction**

The ProTide technology is a phosphate (or phosphonate) prodrug method devised to deliver nucleoside monophosphate (or monophosphonate) intracellularly bypassing the key challenges of antiviral and anticancer nucleoside analogues. Three new antiviral drugs, exploiting this technology, have been approved by the FDA while others are in clinical studies as anticancer agents.

### **Areas covered**

The authors describe the origin and development of this technology and its incredible success in transforming the drug discovery of antiviral and anticancer nucleoside analogues. As evidence, discussion on the antiviral ProTides on the market, and those currently in clinical development is included. The authors focus on how the proven capacity of this technology to generate new drug candidates has stimulated its application to non-nucleoside-based molecules.

### **Expert opinion**

The ProTide approach has been extremely successful in delivering blockbuster antiviral medicines and it seems promising in oncology. Its application to non-nucleoside-based small molecules is recently emerging and proving effective in other therapeutic areas. However, investigations to explain the lack of activity of certain ProTide series and comprehensive structure activity relationship studies to identify the appropriate phosphoramidate motifs depending on the parent molecule are in our opinion mandatory for the future development of these compounds.

## Keywords

ProTide, Phosphor(n)amidate, prodrugs, pro-nucleotide, nucleoside analogues, anticancer, antiviral.

## Article highlights box

- Among different phosphate prodrug approaches, the proTide technology has shown to be superior in the delivery of the monophosphate species of therapeutic nucleoside analogues.
- Extensive preclinical studies in the antiviral and anticancer fields produced three FDA-approved antiviral drugs and three clinical candidates currently investigated as anticancer agents.
- More investigations are needed to explain why the ProTide approach was not so effective when applied to certain nucleoside analogues as it was on others.
- There is strong evidence supporting the dependence of ProTide intracellular metabolism by the phosphorus stereochemistry. Therefore, the development of a robust and wide applicability diastereoselective synthetic methodologies is strongly needed.
- Although *L*-alanine is the amino acid of choice for ProTide of nucleoside analogues when this technology is applied to non-nucleoside substrates, other amino acids have given better results in terms of activity, activation, and stability.
- Phosphoramidates of non-nucleoside substrates share the same activation pathway of their nucleoside analogues counterparts. This suggests the potential application of this technology to a wider range of substrates with other therapeutic applications.

The box summarizes the key points contained in the article.

## 1. Introduction

The application of a prodrug methodology to drug discovery has been known since it was formally introduced in 1958,[1] although earlier examples were previously reported.[2]

It is a strategy by which a pharmacologically active molecule is masked with a cleavable group (promoiety) that under specific conditions in the human body falls off releasing the active species. Prodrug design aims to optimize the physicochemical and pharmacological properties of drugs to improve their solubility and pharmacokinetic features and decrease their toxicity. Overall,

this approach proved quite successful in generating new medicines: about 20% of all small molecular drugs approved between 2000 to 2008 were prodrugs. To further highlight the importance of prodrugs, since 2008, at least 30 prodrugs have been approved by the US Food & Drug Administration (FDA).[3]

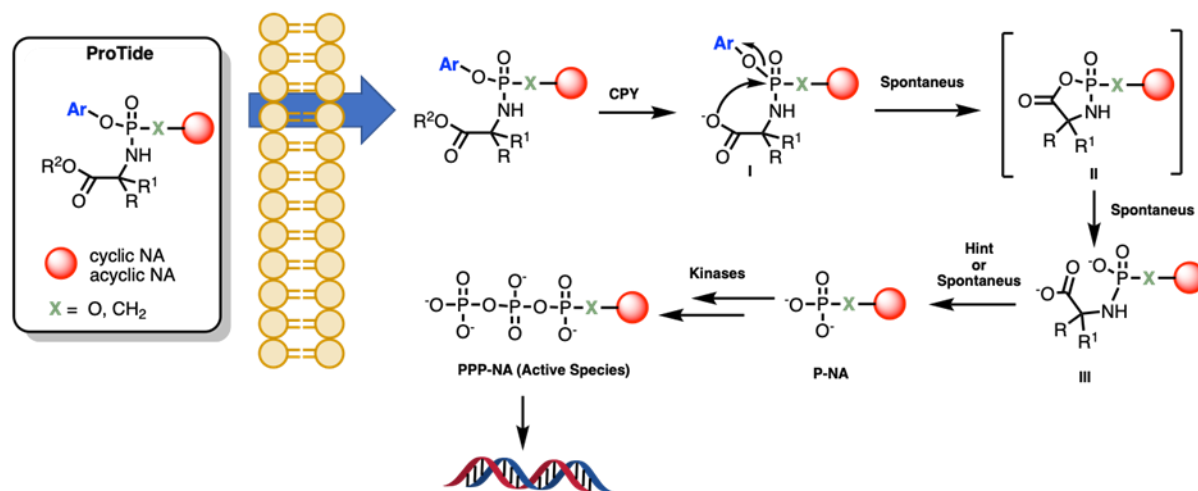
A key class of biological substrates that have been subjected to prodrug approaches are nucleosides analogues (NAs). NAs, which resemble naturally occurring nucleosides, are an important class of antiviral and anticancer agents commonly used in the therapy of many different viral infections and cancer conditions.[4] To exert their biological activity, NAs must be phosphorylated (*in vivo*) via three consecutive phosphorylations to generate the triphosphate form, which is generally the biologically active molecule.[5] These compounds act by interfering with viral or human enzymes as competitive inhibitors of their natural substrates or by being incorporated into newly synthesized viral DNA and RNA strands.

Unfortunately, NAs suffer of many drawbacks such as poor cellular uptake because insufficient expression of membrane transporters, premature breakdown, and slow conversion to triphosphate due to the rate-limiting first phosphorylation step.[4]

To overcome these limitations, NA prodrugs carrying a phosphate group or an isosteric and isoelectronic phosphonate moiety have been investigated. [6-11] Some of these approaches have been successful and include prodrugs that have reached the clinic as antiviral therapy. Among these is the phosphoramidate technology called ProTide (Pro Nucleotide), invented more than 20 years ago by Professor Chris McGuigan and his team at Cardiff University. Following the first report on the ProTide in 1993, [12] this technology has been investigated in many laboratories both from academia and industry. This platform has been successfully applied to a vast number of NAs with antiviral and anticancer activity, leading to three FDA-approved medicines and several preclinical and clinical candidates.[13] Beside the accomplishment obtained with NAs, many reports have recently suggested that this technology can be effectively applied to non-nucleosides substrates. [14] The preliminary results gained so far are pointing toward a successful extension of this approach to other therapeutic areas. In this account, after a brief discussion of the principles of the ProTide approach to introduce the reader to the topic, we will describe how this technology is so successful in the antiviral and anticancer fields. We will then highlight how it is extended to other substrates and why, in our opinion, it will succeed in the treatment of many other diseases.

## **2. The phosphor(n)amidate (proTide) technology**

A ProTide is a cyclic or acyclic nucleoside aryl phosphate (or phosphonate) masked with an amino acid ester promoity linked via P–N bond (Figure 1). Such prodrug is capable to cross the cell membrane by passive diffusion and therefore it does not require a membrane transporter.[15] Intracellularly, the ProTide is activated [16-18] (Figure 1) by a carboxylic-ester hydrolase or carboxypeptidase-type enzyme which mediated the hydrolysis of the carboxylic ester of the amino acid leading to intermediate (I). The ester cleavage is followed by an internal nucleophilic attack of the acid residue on the phosphorus atom, displacing the aryloxy group and giving the transient formation of the putative five-membered cyclic intermediate (II). The first experimental evidence of the formation of this cyclic intermediate has been recently reported.[19] This cyclic anhydride is rapidly hydrolysed to the corresponding aminoacyl phosphor(n)amidate (III) which undergoes P–N bond cleavage, either mediated by an enzyme with phosphoramidase activity or spontaneous hydrolysis to eventually release the phosphorylated parent drug (P-NA). The nucleoside monophosph(on)ate generated then undergoes two successive phosphorylations to generate the active species (PPP-NA) (Figure 1). In some cases, the monophosphate or the diphosphate species might have as well biological activity.[20] [21]



**Figure 1. Mechanism of activation of ProTides**

The amino acid motif is normally selected from a range of natural and unnatural  $\alpha$ -amino acids. *L*-alanine is the preferred one and indeed present in all the drugs that have reached the clinic. Short, linear (methyl, ethyl, pentyl) or branched alkyls (isopropyl, 2-ethyl-butyl, neopentyl) and benzyl esters are usually employed. Although phenol- and 1-naphthol- are commonly used as aryl components, the 5,6,7,8-tetrahydro-1-naphtol has more recently appeared as valid and effective moiety.[22] Although ProTides potency varies with all the individual components of

the phosphoramidate core, the nature of the amino acid ester has proved to predominantly drive the activity of the prodrugs, as it is closely connected with their stability and metabolic activation. Therefore, an SAR study of amino acid ester and aryl moieties is generally performed to find an optimal combination of the masking group for biological activity.

Synthetic procedures used to prepare phosphoramidate of NAs, involve the coupling of a phosphorylating agent to a NA in presence of either *N*-methylimidazole (NMI) or *tert*-butyl magnesium chloride (Grignard reagent, *t*-BuMgCl).[23] The “Grignard methodology” is not selective thus formation of undesired regioisomers and/or bis-phosphoramidates are usually observed. Therefore, to avoid the formation of undesired regioisomers or multiple phosphorylations, selective protection of the free hydroxyl groups of NAs prior to the coupling reaction with a phosphorylating reagent is often required. Coupling mediated by NMI favours instead the selective phosphorylation of primary hydroxyl group of the nucleoside (5'-position). Recently, a highly regioselective synthesis of phosphoramidates via a direct 5'-phosphorylation of unprotected nucleoside, mediated by dimethylaluminum chloride, was reported by Simmons *et al.* [24] A microwave enhanced synthesis of ProTide analogues was also recently described. [25]

Usually, the phosphorylating agents are used as a pair of diastereoisomers at the phosphorus centre. This leads to the formation of two diastereoisomeric aryloxyphosphoramidates in a 1:1 ratio  $R_P$  and  $S_P$ . Such mixtures are very difficult to separate by standard chromatographic methods, or by crystallization. Considerable efforts were then directed to the development of diastereoselective strategies toward phosphoramidates. The demand for efficient diastereoselective methods raised after the discovery of a significant difference in the antiviral activity between  $S_P$  and  $R_P$  isomers of clinical candidates [26-28]. The superior activity observed with one isomer was proved to correlate with its ability to produce higher level of the triphosphate species compared to the other [28]. Evidence that the two phosphoramidates (and phosphonamidates) diastereoisomers can be processed at different rates in enzymatic assay by carboxypeptidase were also reported. [20,29,30]

A multistep approach to obtain ProTides in a diastereoselective fashion was developed by the Meier group, who adopted a chiral auxiliary on the phosphoramidating reagent as stereocontrolling tool.[31] Later, Ross and co-workers developed a very practical method in which a diastereomerically pure phosphoramidating agent, bearing a *p*-nitrophenyl- or pentafluorophenyl leaving groups was used.[32] The first catalytic, diastereoselective methodology for the synthesis of *P*-chirogenic phosphoramidate prodrugs was developed by Pertusati *et al.*[33] Later, a more efficient, metal-free method that attains high selectivity for

nucleoside phosphoramidation was reported. In this methodology, a complex chiral catalyst enabled the phosphoramidation of different 2'-modified nucleosides with high stereoselectivity at the phosphorus center, exclusively towards  $R_P$  configuration.[34]

The preparation of phosphoramidate (P-C bond) prodrugs of nucleotide analogues is generally accomplished from the corresponding phosphonic acid either via phosphorodichloridate [35] or phosphonic silyl ester. [22,30] A diastereoselective synthesis for phosphoramidates have not been reported yet. A one-pot, two-steps synthesis of alkenyl acyclic nucleoside phosphonate prodrugs using cross-metathesis reactions was reported by Pileggi *et al* [36] and independently, by Agrofoglio.[37]

### 3. ProTides of NAs

#### 3.1 Antiviral application

The design of the phosphoramidate prodrugs dated back in 90's when McGuigan and his team evaluated a series of phosphate triester derivatives of azidothymidine (AZT; zidovudine) against human immunodeficiency virus (HIV-1).[38] [39] Simple AZT and 2',3'-dideoxythymidine (d4T, stavudine) dialkyl phosphates were inactive as anti-HIV agents, whereas substituted dialkyl phosphates were active. These data supported the idea that opportunely modified phosphates were able to exert their biological effects via intracellular release of their nucleotide form. This prompted McGuigan, in collaboration with Professor Jan Balzarini, the modification of the prodrug's structure to achieve optimal activation and potency. After several modifications, aryloxyphosphoramidates of AZT were designed.[12] These compounds had a pronounced, selective anti-HIV activity in CEM cells; the magnitude of the biological effect varied considerably in function of the nature of the phosphate-masking groups. Moreover, several of the compounds retained marked antiviral activity in thymidine kinase-deficient (TK<sup>-</sup>) mutant CEM cells in which AZT was virtually inactive. AZT aryloxyphosphoramidates account for the earliest examples of ProTides reported in the literature, granting to the McGuigan's group the inventorship of this class of prodrugs.

The collaboration between McGuigan's and Balzarini's laboratories continued along the years and it was of crucial importance for the development of the ProTide technology. The AZT studies were followed by the successful application of the ProTide technology to many more antiviral NAs. Representative examples are 2',3'-didehydro-2',3'-dideoxythymidine (d4T, Stavudine), [40-42] 2',3'-dideoxyadenosine and 2',3'-didehydro-2',3'-dideoxyadenosine,[43] 2',3'-dideoxyadenosine-3'-fluoroadenosine,[44] 2',3'-dideoxydouridine and 2',3'-didehydro-

2'3'dideoxyuridine [45] and also to the carbocyclic nucleoside Abacavir (ABC, Ziagen),[46] carbocyclic adenosine derivatives [47] and 2',3'-dideoxy-2'3'-didehydro-7-deazaadenosine, [48] to treat different viral infections. A particular case is Stampidine, the stavudine 4-bromophenoxy *L*-alanine methyl ester phosphoramidate, developed as anti-HIV agent to overcome the dependence of stavudine from intracellular nucleoside kinase-mediated activation to the nucleoside 5'-monophosphate. Despite the encouraging data from phase I study of Stampidine,[49] in development by the Parker Hughes Institute, no further progression of this agent in clinical trials has been reported.

Attempts to target influenza virus using the proTide technology were also pursued although with modest results. [50-52] Collaborations between McGuigan group and other academic researchers started as for example with Van Calenbergh's laboratories at Ghent University.[53] A boost in the ProTide research occurred when Hepatitis C became an important research topic. To target this disease McGuigan's group applied the ProTide strategy first to the ribonucleoside analogues 4'-azido nucleosides,[54-57] and then to the  $\beta$ -2'-C-methyl-guanosine.[58] The last class of compounds showed an excellent antiviral activity and served as scaffold to further SAR studies that led to the discovery of the double-prodrug **1**, (INX189, Figure 2). This *O*-6-methyl-2'-C-methyl-guanosine prodrug **1**, showed nanomolar activity in hepatitis C virus (HCV) replicon assay.[59,60] It was then licensed by Cardiff to Inhibitex, and it took only 18 months from the first synthesis in Cardiff to the first dose in human volunteer. This compound showed efficacy both in phase I and II studies. This rapid success triggered in 2012, the Inhibitex acquisition by Bristol Meyer Squibb to continue the clinical investigation of INX189 renamed then BMS-986094. Tragically, the combination of BMS-986094 and daclatasvir (BMS-790052), caused the death of one patient, and severe heart and kidney damage on other eight patients. For this, the BMS-986094 trial was closed. The toxicity was attributed to the parent nucleoside rather than to the proTide motif,[61] but not further investigation was done on this matter. This event raised questions about the adequacy of prior safety trials and the potential pitfalls of accelerating drug development, but it did not stop the application of the ProTide technology.

The antiviral research in the nucleoside phosphate prodrug area was, at the time, very exciting and competitive. While McGuigan's group and Inhibitex were working at the development of INX189, Pharmasset, was working to the development of PSI-7977 (**2**), another nucleoside phosphoramidate, which was then advanced by Gilead and approved in December 2013 by the FDA as Sofosbuvir (Sovaldi®, **2**) (Figure 2), for the treatment of HCV infections.[28] Sofosbuvir undergoes liver metabolism into the antiviral agent 2'-deoxy-2'- $\alpha$ -fluoro- $\beta$ -C-



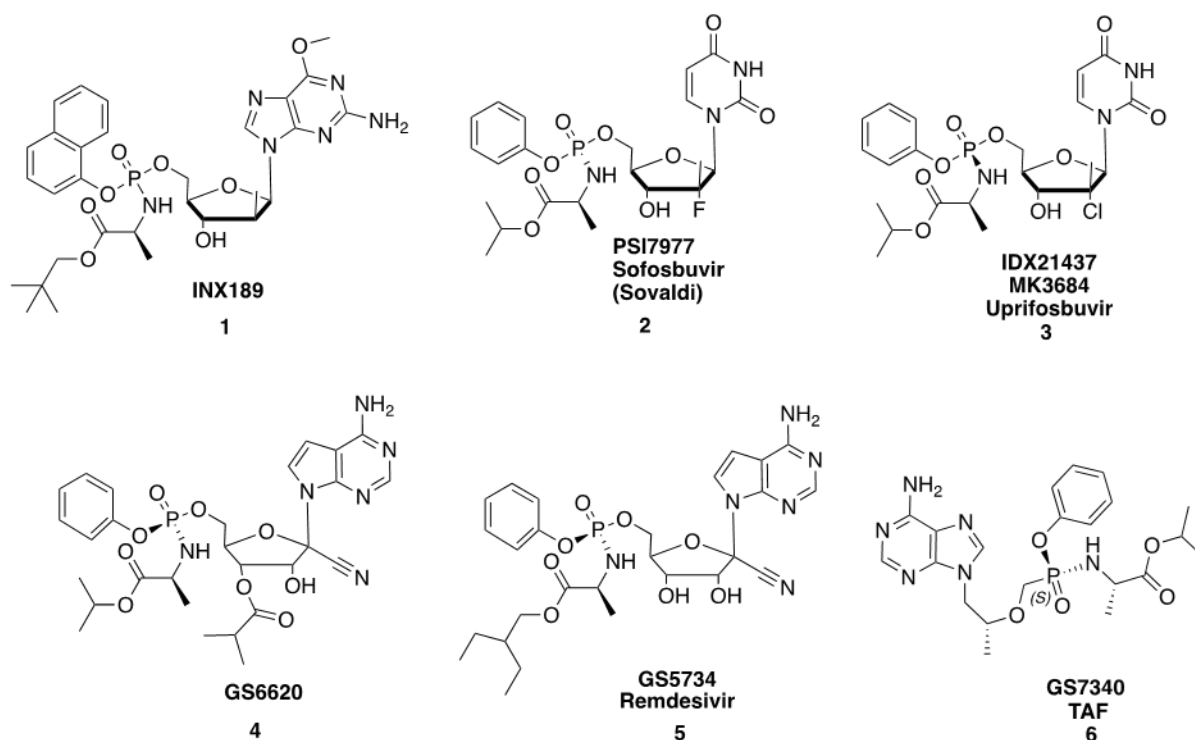
methyluridine-5'-monophosphate whose triphosphate specie is a strong inhibitor of the HCV ribonucleic acid- (RNA) dependent RNA polymerase (NS5B). Sofosbuvir's phase I trials were performed with the diastereoisomeric mixture, while phases II and III were carried out with the pure *Sp* diastereoisomer. This was found 8 times more potent than the *Rp* isomer. [28] Sofosbuvir eventually became the world's fastest selling drug ever. Its efficacy has been established in patients with genotype 1, 2, 3 or 4 infections, including those with hepatocellular carcinoma. [62,63] Beside Cardiff university, among Pharmasset's competitors was Merck & Co with its anti-HCV ProTide MK3682 (Uprifosbuvir, **3**) (Figure 2).[34] This compound, identified first at Idenix pharmaceutical (IDX21437),[64] is the only ProTide reported so far in which the *Rp*-diastereoisomer has superior biological properties to the *Sp* isomer. Uprifosbuvir was evaluated in the clinic but although the positive results obtained in these investigation [65] eventually its development was discontinued in consideration of the evolving marketplace and the growing number of treatment options available for patients with chronic HCV infection. Along with Sofosbuvir, Gilead worked at the development of another anti-HCV proTide, GS-6620 (**4**) (Figure 2).[66] Although in a phase I clinical study this compound showed potent anti-HCV activity, its high intra- and interpatient pharmacokinetic and pharmacodynamic variability hampered its further development.[67] Despite this, it continued to be researched as a potential treatment for other viral diseases leading to the discovery of ProTide GS5734 (Remdesivir, **5**) (Figure 2),[68] evaluated in clinical trials for the treatment of Ebola virus infections. Despite the promising preclinical results, Remdesivir did not meet efficacy endpoints in a randomized trial,[69] showing that not always excellent preclinical data are mirrored in clinical trials. Beside Ebola virus, Remdesivir has demonstrated activity against other RNA viruses such as coronaviruses [70] including Severe acute respiratory syndrome coronavirus (SARS-CoV-1) [71] and the Middle East respiratory syndrome coronavirus (MERS-CoV) [72]. For this reason, it was identified as a promising therapeutic candidate for SARS-CoV-2 infection (Covid-19 disease) [73]. When tested in the early stages of the pandemic outbreak, Remdesivir was shown to shorten recovery times for severely ill Covid-19 patients, which had evidence of lower respiratory tract infection. [74,75] These results led to an emergency use authorisation by the FDA in May 2020. However, data from a wide international study involving thousands of patients showed that the drug has no significant impact on mortality, length of hospital stays, or need for ventilation among hospitalised patients. [76,77] Therefore, the world health organization (WHO) has issued a conditional recommendation against the use of Remdesivir in hospitalized patients, regardless of disease severity.[78]



The McGuigan's group extended the application of its prodrug technology to acyclic nucleosides analogues. Acyclovir (ACV), an antiviral medication primarily used for the treatment of herpes simplex virus infections (HSV), varicella zoster virus (VZV) (chickenpox and shingles) was the first example investigated. [79-82] Also in this case, the technology was capable to enhance the antiviral activity of the parent compound but, unfortunately, it was associated with some toxicity impeding further progress of these prodrugs.

Replacement of the phosphate moiety with an isosteric and isoelectronic phosphonate group, results in a nucleoside phosphonate, (NP) a chemically and enzymatically more stable compound than the corresponding phosphate.[83] Differently from the O-P linkage, the CH<sub>2</sub>-P-bond, due to its chemical nature, is not susceptible to the hydrolytic action of phosphodiesterase and phosphatase. Like their monophosphate counterpart, a nucleoside phosphonate analogue needs to be further phosphorylated by cellular nucleotide kinases. These stable phosphonate analogues, mimicking the nucleoside monophosphates, can bypass the initial (slow) enzymatic phosphorylation and be more effective antiviral agents. This approach has led to the discovery of acyclic nucleoside phosphonates (ANPs), one of the most successful class of antivirals molecules ever discovered.[84] ANPs, pioneered by the Holý group in the 1980s, were found to exhibit a broad spectrum of antiviral activities, particularly against DNA viruses and retroviruses.[85,86] Two ANPs prodrugs, the bis (pivaloyloxymethyl) ester of adefovir (bis-(POM)-PMEA; Hepsera®), and the diisopropylloxycarbonyloxymethyl ester of tenofovir fumarate (bis-(POC)-(R)-PMPA fumarate; Viread®), were approved respectively for the treatment of HBV and HIV infections. However, their toxicity, caused by the release of decomposition products, generated some concern. This prompted more research devoted to identifying a better and safer ANP prodrug.[10] In this context, McGuigan's laboratory was the first to apply the ProTide technology to ANPs.[35] In this study, the phenyloxy phosphonamidate (with *L*-alanine methyl ester) prodrugs of adefovir and tenofovir showed to have a superior antiviral activity when compared to the parent drugs.

Gilead was also investigating the application of the ProTide technology to tenofovir [87]. Eventually, the *Sp* isomer of tenofovir phenyloxy isopropyl-(*L*)-alaninyl phosphonamidate (TAF, GS-7340, **6**) (Figure 2) established itself as a lead compound for the treatment of HIV. In 2015, the TAF-based regimen (elvitegravir/cobicistat/emtricitabine/TAF, Genvoya) was FDA-approved for treatment of HIV-1.[88] In addition, a year later TAF (Vemlidy) received FDA-approval for the treatment of chronic HBV infections in adults with compensated liver disease.[89] It is worth to highlight here that the *Sp*-diastereoisomer was found to be much more active than the *Rp*-diastereoisomer.



**Figure 2.** Antiviral ProTides that have reached the clinic.

### 3.2 Anticancer Application

Soon after the McGuigan's laboratory showed that the ProTide technology was a powerful tool to enhance activity of antiviral NAs, its applicability in the oncology area was explored. The first account of the ProTide technology in this field was its application to the anticancer nucleoside brivudine (BVDU) reported in 2001 by Lackey *et al.* [90]

The application of the ProTide technology to BVDU, described by McGuigan *et al* [91] aimed to improve the anti-VZV properties of this nucleoside. However, BVDU phosphoramidates were found 5–25-fold less potent than the parent compound against VZV in tissue culture. This was interpreted as the consequences of poor intracellular delivery of BVDU monophosphate, its rapid degradation to BVDU or poor onward phosphorylation of BVDUMP to the bio-active triphosphate. In parallel to this investigation, the New Biotics group had independently prepared and found that the phenyloxy *L*-alanine methyl ester phosphoramidate of BVDU (NB10, Thymectacin, **8**) (Figure 3) was a potent and effective anti-cancer agent.[90] Further studies on Thymectacin revealed that it is selectively toxic to tumour cells expressing elevated levels of thymidylate synthase (TS), a key enzyme in DNA synthesis.[21] Thymectacin entered clinical evaluation against colon cancer and phase I data indicated that the compound is well tolerated.[92] Based on the wide-ranging knowledge of ProTides as anti-viral agents, McGuigan and his team embarked in a program to improve Thymectacin. They first modify

the ester and amino acid regions [93] followed by variation of the aromatic moiety.[94] A substantial enhancement in *in vitro* potency versus colon and prostate cancer cell lines was observed with the naphthyloxy- *L*-alanine benzyl ester derivative when compared to the parent nucleoside. In this study the two diastereoisomers of this prodrug were separated. For the first time, through computational and NMR studies, the absolute stereochemistry of the phosphorus center of these two diastereoisomers was tentatively assigned. When tested individually the two diastereoisomers showed a different cytostatic activity suggesting the importance of developing diastereoselective synthetic methodology. A further SAR study exploring other BVDU ProTides have been recently reported.[95] However no better compounds emerged from these investigations.

After this first report on BVDU prodrugs, multiple studies of the application of the ProTide approach toward other anticancer NAs followed. Phosphoramidate of 2'-deoxy-zebularine, an anticancer nucleoside with DNA methyl transferase inhibition mechanism, provided evidence for an increase in anticancer activity with respect to the parent nucleoside.[96] Several of these prodrugs were identified as more potent inhibitors of DNA methylation and stronger inducers of *p16* tumor suppressor gene in a pancreatic cell line than zebularine. However, their activity was dependent on the administration of thymidine to overcome the potent inhibition of deaminase enzymes. This has hampered their further development.

A significant breakthrough was the application of the ProTide technology to 5-fluoro-2'-deoxyuridine (FUdR), which led to substantial increases in *in vitro* cancer cell cytotoxic in comparison to the parent drug.[29] Among several FUdR ProTides the naphthyloxy *L*-alanine benzyl ester derivatives (NUC3373, **9**) (Figure 3) was identified as lead candidate by NuCana plc. Whereas FUdR substantially lost its cytostatic potential in thymidine kinase (TK)-deficient cell cultures, NUC3373 markedly kept its antiproliferative activity in TK-deficient tumour cells. The prodrug is largely independent from the intracellular TK activity to exert its cytostatic action. NUC3373 was found to inhibit TS in the TK-deficient and wild-type cell lines at drug concentrations that correlated well with its cytostatic activity in these cells. NUC3373 does not seem to be susceptible to inactivation by catabolic enzymes such as thymidine phosphorylase (TP) and uridine phosphorylase (UP). NUC3373 is currently evaluated in a phase I study in patients with advanced solid tumours and in a phase Ib in combination with other agents typically administered with fluorouracil (5-FU; drug used as standard therapy) in patients with advanced colorectal cancer.[97] The preliminary results of phase I study showed that NUC3373 has a favourable pharmacokinetic and safety profiles with respect to 5-FU.[98]

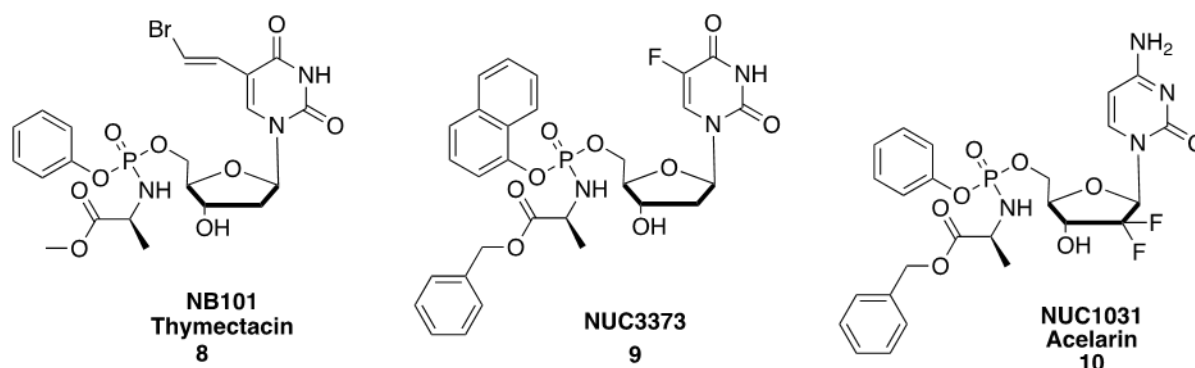
On the contrary, the ProTides approach was not so successful when applied to different 6-substituted-5-fluorouridine nucleoside analogues.[99] All the prodrugs tested showed poor *in vitro* biological activity, most probably due to a compromised substrate recognition of the Hint phosphoramidase-type enzyme.

In 2014 the application of ProTide technology to the anti-cancer nucleoside gemcitabine, in use for pancreatic and other solid tumours, was reported. The results obtained in a panel of *in vitro* cancer cell lines demonstrated that the ProTide technology is able to overcome key cancer resistance mechanisms, associated with gemcitabine's lack of clinical efficacy. [29] Among the synthesized compounds, one in particular, NUC1031 (Acelarin **10**) (Figure 3) was shown to be the most potent anticancer derivative *in vitro*. Importantly, compared with gemcitabine, its activation was significantly less dependent on deoxycytidine kinase, on nucleoside transporters, and it was resistant to cytidine deaminase-mediated degradation. Moreover, it showed a significant reduction in tumour volumes in *in vivo* in pancreatic cancer xenografts. Acelarin, has been evaluated in three clinical studies across several solid tumor indications, including ovarian, biliary and pancreatic cancer. A phase I, dose-ranging study in patients with advanced metastatic solid tumours,[100] showed that Acelarin was well tolerated, with a 78% disease control rate. Intracellular levels of active anti-cancer metabolite was over 200 times higher than those reported for gemcitabine.[101] Based on these disease control rates and its tolerability profile, a phase II study in patients with platinum-resistant ovarian cancer has been launched in June 2017.[102] Acelarin was also evaluated in another phase Ib study in patients with metastatic or advanced biliary tract cancer to determine its optimal dose in combination with cisplatin.[103] In this study, it was observed an approximate double increase of the response rate with Acelarin/cisplatin compared to previously reported data for gemcitabine/cisplatin combination[104]. On the basis of this results, a phase III study for the first-line treatment of patients with biliary tract cancer started.[105] In June 2019, FDA has granted orphan drug designation for Acelarin for the treatment of biliary tract cancer. To further demonstrate the success of the ProTide technology, in March 2019, the cordycepin phosphoramidate, NUC-7738, designed and prepared at Cardiff University,[106] entered phase I dose-escalation study in patients with advanced solid tumours.[107]

Preliminary results of the application of the ProTide approach to C-nucleosides were also reported, showing that the chemistry for the preparation of these derivatives needs to be tuned due to the peculiar behaviour of C-nucleosides compared to their nucleoside analogues. Although no biological evaluation was reported, it was demonstrated that the first enzymatic

activation step for these prodrugs can be mediated by carboxypeptidase as for ProTides of common nucleoside analogues.[108]

Since  $^{18}\text{F}$ -labelled model ProTides could directly address key mechanistic questions and predict response to ProTide therapy, efforts directed to prepare 18 fluorinated phosphoramidates of NAs were reported.[109]. Table 1 includes all the ProTides that have reached the clinic or are in clinical development.



**Figure 3.** Anticancer ProTides that have reached the clinic

**Table 1**

Compound	Parent Nucleoside	Amino acid	Ester (R <sup>2</sup> )	Aryl moiety (Ar)	Therapeutic Indication	Status
Stampidine	Stavudine	L-Ala	Methyl	Phenyl	Antiviral (HIV)	Phase I
Sofosbuvir	PSI-6206	L-Ala	<i>iso</i> Propyl	Phenyl	Antiviral (HCV)	Approved
TAF	Tenofovir	L-Ala	<i>iso</i> Propyl	Phenyl	Antiviral (HIV & HBV)	Approved
Remdesivir	GS441524	L-Ala	2-Ethyl-butyl	Phenyl	COVID-19	Approved
Uprifosbuvir	2'-C-Methyl-2'-Chloro-Uridine	L-Ala	<i>iso</i> Propyl	Phenyl	Antiviral (HCV)	Halted in Phase III
Thymectacin	Brivudine	L-Ala	Methyl	Phenyl	Anticancer	Phase I
Acelarin	Gemcitabine	L-Ala	Benzyl	Phenyl	Anticancer	Phase III
NUC3373	2'-Deoxy-5-Fuorouridine	L-Ala	Benzyl	Naphthyl	Anticancer	Phase II
NUC7738	3'-Deoxy-Adenosine		Not disclosed yet		Anticancer	Phase I

### 3.3 Other therapeutic applications

NAs represent unequivocally the cornerstone of antiviral and anticancer treatment, but they also have shown potential for the treatment of other pathological conditions. [110-113] For this reason, other therapeutic applications have witnessed the use of the ProTide technology.

The ProTide approach was exploited to deliver the monophosphate nucleoside of a NA, known as inhibitor of the flavin-dependent thymidylate synthase X (ThyX) an enzyme crucial for thymidine metabolism in several pathogens but completely absent in humans.[114] This was the first example of the application of such technology to a nucleoside endowed with antibacterial activity. Some of these derivatives showed increased bioactivity against mycobacteria (with respect to the parent drug). This was attributed by the authors to a better permeation through the mycobacterial cell wall due to well-optimized balance between their lipophilicity and molecular size, made possible by the ProTide features.

ProTide technology was also used to elaborate nucleoside-based molecules that activate a protein kinase, PINK1, whose loss of function is linked to early onset of Parkinson's disease. In this study unfortunately the parent nucleoside showed comparable PINK1 activation to the prodrugs suggesting, according to the authors, that the first phosphorylation step bypassed by the ProTides, is not the rate limiting step for the activation of the parent nucleoside.[115]

Promising results of ProTide prodrugs were shown in the treatment of mitochondrial DNA (mtDNA) depletion syndrome (MDS), a group of rare autosomal-recessive diseases, characterized by reduction of mitochondrial DNA.[116] In this study, a novel ProTide of 2'-deoxy guanosine nucleoside (CERC-913, structure not disclosed), designed to bypass defective mitochondrial deoxyguanosine kinase (DGUOK), showed to restore, in a dose-dependent fashion, mtDNA content in a primary hepatocyte culture model of DGUOK deficiency.

#### **4. Aryloxy phosphoramidates of Non-NAs**

The ProTide technology, as its name indicate, has been invented for the delivery of nucleoside monophosphates (nucleotides). However, many other non-nucleoside compounds are in needs of phosphorylation to play important role as endogenous molecules in the human body or as therapeutic agents in many different pathological conditions. It was soon recognized that such technology has the potential to be applied in other therapeutic area. Although the prodrugs generated are sometimes called ProTides we believe that it would be more appropriate to refer to them as aryloxy phosphor(n)amidates.

##### **4.1 Carbohydrates Aryloxy phosphoramidate**

Phosphor(n)amidate technology was applied to carbohydrates, a class of very hydrophilic compounds. McGuigan *et al* reported the conversion of *N*-acetylglucosamine to a series of *O*-6,[117] *O*-3 and *O*-4 aryloxy phosphoramidate prodrugs [118], evaluated for their potential

chondroprotective activity against osteoarthritis. By comparison to the parent drug, some of the analogues showed a significant enhancement in the inhibition of inflammatory cytokine-induced proteoglycan degradation. Specifically, the *O*-3 and *O*-4 (*L*)-proline- prodrugs proved to be the most active of the series, and well processed in chondrocytes. Data on human cartilage supported the notion that these novel *O*-3 and *O*-4 regioisomers may represent novel promising leads for osteoarthritis treatment. These findings showed that amino acids other than *L*-alanine, can be effective promoieties in this technology.

Inspired by the success of this work, phosphoramidate technology was transferred to 1,3,4-*O*-acetyl-*N*-acetyl mannosamine to deliver *N*-acetyl-mannosamine-6-phosphate, a critical intermediate in sialic acid biosynthesis. Deficiency of this carbohydrate on cell surface is a hallmark of GNE myopathy, a rare congenital disorder of glycosylation (CDG).[119] Among a small library of phosphoramidates, the prodrug bearing *L*-leucine ethyl ester was the most effective at increasing sialic acid levels in GNE cell lines. This correlate well with its rapid activation in a carboxylesterase (CPY) enzymatic assay. Favourable ADME properties were also achieved. This compound was suggested to be a potential lead for optimization to address substrate deficiencies in GNE myopathy and other CDGs.

As 2-deoxy- $\alpha$ -D-ribose-1-phosphate is involved in the biosynthesis and/or catabolic degradation of several NAs of biological and therapeutic relevance, it was identified as a good substrate for the ProTide approach. When a novel series of phenoxy C1-phosphoramidate derivatives of 2-deoxy- $\alpha$ -D-ribose was assayed as anticancer agents, some inhibitory effects on the proliferation of a panel of different cancer cell lines were observed for the *L*-valine methyl ester prodrug.[120] On the contrary, the phosphoramidate series of 2-deoxy-D-ribose 2-fluoro-2-deoxyarabinose and 2,2-difluoro-2-deoxyribose-1-phosphoramidates showed no *in vitro* inhibitory activity against a variety of viruses and cancer cell proliferation.[121]

#### **4.2 Aryloxy phosphoramidates of other small molecules**

The first example of the proTide technology applied to non-nucleoside compounds was reported by Ruda *et al* in 2007.[122] In this study, ProTides of hydroxymethyl dioxolane were investigated along with other phosphate prodrugs against the *Trypanosoma brucei*, the organism responsible for human African trypanosomiasis. The phosphoramidate tested was among the most active prodrugs, showing a drastically improvement of the *in vitro* activity of the parent compound.

The application of the phosphoramidation approach was also investigated to address the dephosphorylation and the charged nature at physiological pH of anticancer phosphoserine-



[123] and phosphotyrosine-containing molecules.[124] . In these studies, it was found that a small library of prodrugs (with only ester variation of *L*-alanine) of both phosphoserine and phosphotyrosine-containing molecules significantly improved the pharmacological efficacy of the parent compounds in cancer cells.

The phosphoramidate platform was also exploited to design anticancer or antiviral phosphoantigen analogues with enhanced pharmacokinetic properties relative to the natural ligand. Structural modifications, including replacement of an O–P bond with a CH<sub>2</sub>-P bond, and/or application of different phosphate prodrug approaches have been reported.[125] [126] Among these the phosphor(n)amidate technology emerged as the most promising one. Collectively, these studies highlight the potential of this prodrug technology in the discovery of novel immunotherapeutic molecules.

A molecule that did not benefit from the phosphoramidate approach was the antibacterial agent fosfoxacin.[127] No growth inhibition was observed for any of the bacterial species on which such prodrugs were tested. This could be due to the lack of formation of active compounds in the bacterial cells. The authors also suggested that prodrugs instability, presumably linked to the hydroxamate structural features, (premature hydrolysis or/and side reactions of the prodrug before entering the bacteria) could be the cause of this lack of activity.

With the aim of developing kinase independent SIP receptor modulators, phosphoramidates of benzyl ether derivatives were also elaborated.[128] These prodrugs were shown to be activated *in vitro* following the same pathway of ProTides suggesting their potential future application as therapeutic agents for the treatment of neurodegenerative diseases.

Another study reports on the application of the phosphoramidate technology for caging the phosphate group of a non-nucleoside-based inhibitor of the prolyl isomerase Pin1, an enzyme highly expressed in tumours and whose knockdown has shown strong antitumor effects. The result obtained showed that the prodrugs have increasing permeability when compared to the parent drug.[129]

## 5. Expert opinion

Almost 30 years of ProTide investigations have shown that such technology is unequivocally very powerful when applied to NAs, as evidenced by the approval of Sofosbuvir and TAF, the two most successful drugs launched in the history of medicine, and further supported by the promising clinical results seen with anticancer ProTides.

In most of the preclinical studies, in which different class of phosphate prodrugs have been applied to antiviral and anticancer NAs, the ProTide was found to be superior to other prodrug approaches. The synthetic methodologies used for their preparation are robust and afford, in most cases, the prodrugs with excellent yields. However, although the synthetic methodologies available for the preparation of diastereomerically pure  $S_p$  and  $R_p$  diastereoisomers have made excellent progresses, they are far from being ideal. Some methodologies are high-priced, time consuming and hard to scale up and/or limited by low yields. [31-33] Unfortunately, the most advanced stereoselective methodology can afford exclusively the  $R_p$  isomer, which in only one case is reported to have superior biological activity.[34] In addition, while for the preparation of single isomer phosphoramidate there are different methods, phosphoramidates (P-C bond) still lack of such diastereoselective synthesis. Therefore, we still believe that further synthetic efforts should be made to fill the gap in this area. Investigation should be directed to elaborate a broadly applicable synthetic methodology, less dependent from the nature of the phosphoramidating agent, which is often the critical component rather than the parent molecule. Unique feature of the ProTide technology is the specific combinations of aryl, ester and amino acid groups (phosphoramidate motif) that protect the activated nucleotide analogue. SAR studies have been carried out to identify the optimal phosphoramidate motif for each underlying nucleoside analogue. Although the amino acid ester is reported to be the moiety generally driving the activity of the ProTide, only few reports include a wide, although still not comprehensive, number of amino acids. Moreover, while the SAR studies of antiviral and anticancer ProTides point to the *L*-alanine as the preferred amino acid there are now evidence that other amino acids are more effective than *L*-alanine for other substrates. [130-132] We therefore not only encourage scientists to include a good range of amino acids in their study, but we believe that the use of all-natural amino acids should be revisited for both NA and non-NA based compounds.

Preclinical studies also suggest that when the ProTide approach works, the parent drug activity is boosted because the NAs drawbacks, (premature break-down, slow activation and low permeability) are bypassed. This was certainly demonstrated for the approved ProTides for which the efficacy was shown to correlate with an increased concentration of the active species (usually the triphosphate NA). Lack of activation to the monophosphate NA was instead used as argument to explain the absence of *in vitro* activity for certain family of ProTides.[131,133] We believe that solid experimental evidence to corroborate this theory are still lacking and deeper investigations are needed to address completely this aspect. The elucidation of the observed lack of activity for certain ProTides series will provide useful information for the

future rational drug design of phosphoramidates of both NAs and non-nucleoside-based molecules, which are current under development in other therapeutic areas.

We also believe that not always excellent preclinical results, regardless the robustness in the preclinical science behind them, can be translated into the clinical settings. When compounds are finally tested in human, the wide-ranging age of patients, the different genotype of human populations, and the complexities of the diseases to be treated provide challenges which were never faced in the *in vitro* or *in vivo* animal models. This is for example the case of Remdesivir. Although it was able to inhibit EBOV replication in preclinical studies [68] it did not show significant efficacy in a randomized trial conducted during an Ebola outbreak.[69] Highly controversial it is also its approval for the treatment of COVID-19, which sees diverse views from scientists and clinicians worldwide. We believe that this pandemic has shown how COVID-19 affects people in such different way regardless their age and pre-existing health conditions, that in our opinion it is difficult to establish the efficacy of the Remdesivir with the existing data and that additional clinical studies must be performed.

In terms of potential therapeutic areas, we believe that the development of phosphoramidates of antibacterial, antifungal and antiparasitic NAs is an obvious space to investigate. In addition, preliminary *in vitro* results are showing that phosphoramidates do not require a nucleoside structure to be activated, suggesting the great potential of this technologies to be expanded in the delivery of various monophosphate small molecules, endowed with different biological activities. Rare diseases associated with phosphorylated carbohydrate deficiency as for example in the case of glycosylation disorders (CDG)[134] or with lack of endogenous nucleosides as in mitochondrial DNA (mtDNA) depletion syndrome [135] represent new avenues for the application of such technology.

In conclusion, we have showed how ProTide (phosphoramidate) technology has been extremely successful in delivering blockbuster antiviral medicines and how the clinical results obtained with such prodrugs to date have been highly promising in the oncology field. We have also provided evidence how preclinical investigations of the application of this prodrug approach to non-nucleoside small molecules have now started. We firmly believe that all together, these findings will drive the scientific community to expedite the application of such exquisite technology in many therapeutic areas.

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### **Declaration of Interest:**

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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