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Application of kinase bypass strategies to nucleoside antivirals

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ABSTRACT

Nucleoside and nucleotide analogs have served as the cornerstones of antiviral therapy for many viruses. However, the requirement for intracellular activation and side-effects caused by distribution to off-target sites of toxicity still limit the efficacy of the current generation of drugs. Kinase bypass strategies, where phosphorylated nucleosides are delivered directly into cells, thereby, removing the requirement for enzyme catalyzed phosphorylation steps, have already changed the face of antiviral therapy in the form of the acyclic nucleoside phosphonates, cidofovir, adefovir (given orally as its dipivoxil prodrug) and tenofovir (given orally as its disoproxil prodrug), currently used clinically. These strategies hold further promise to advance the field of antiviral therapy with at least 10 kinase bypass and tissue targeted prodrugs, representing seven distinct prodrug classes, currently in clinical trials. This article reviews the history of kinase bypass strategies applied to nucleoside antivirals and the evolution of different tissue targeted prodrug strategies, highlighting clinically relevant examples.

1. Introduction

Nucleoside and nucleotide analogs have been a critical parts of the treatment of the human immunodeficiency virus (HIV), herpes viruses (including herpes simplex virus type-1 and -2 (HSV-1 and HSV-2, respectively), varicella-zoster virus (VZV), and cytomegalovirus (CMV)) and the hepatitis B and C viruses (HBV and HCV, respectively) with no less than 22 distinct agents approved by a regulatory agency for clinical use (Ray and Hitchcock, 2009). Nucleoside and nucleotide analogs have made a tremendous impact in the treatment of HIV where the current standard of care includes two nucleoside or nucleotide analogs in combination with a third agent from another class (the history of nucleoside and nucleotide analogs in HIV was recently reviewed in a special issue of Antiviral Research commemorating the 25 year anniversary of the approval of azidothymidine (AZT) (Broder, 2010; Cihlar and Ray, 2010; Martin et al., 2010).

Therapy with nucleoside and nucleotide analogs can be limited by the requirement for intracellular anabolism to their active nucleoside triphosphate (NTP) metabolites. The first step in phosphorylation of nucleoside analogs, formation of the nucleoside monophosphate (NMP), is catalyzed by a number of cytosolic and mitochondrial nucleoside kinases and phosphotransferases involved in nucleotide salvage pathways. Subsequent phosphorylation steps are catalyzed by NMP kinases (NMPKs; convert NMP to nucleoside diphosphate (NDP)) and NDP kinases (NDPKs; convert NDP to NTP). The first phosphorylation step is often slow or even absent in some cases, particularly when the nucleoside structure has been markedly altered. However, the location of the rate limiting step in anabolic phosphorylation is nucleoside-dependent and some nucleosides exhibit slow conversion at phosphorylation steps distal to the first kinase step. The anabolism of nucleoside and nucleotide analogs has been reviewed in great detail elsewhere (Ray and Hitchcock, 2009; Van Rompay et al., 2000; Welin and Nordlund, 2010).

Nucleoside and nucleotide therapy can be limited by unwanted side effects caused by distribution to off-target cells and tissues. For example, ribavirin is transported into erythrocytes by nucleoside transporters where its metabolites accumulate causing anemia (Jarvis et al., 1998). Once activated, nucleotide analogs serve as mimics of some of the fundamental building blocks of life and, therefore, can cause toxicity. For example, many 2′,3′-dideoxynucleotide analogs can inhibit the mitochondrial DNA polymerase gamma causing a wide range of side-effects (reviewed by White (2001)).

Seminal work in the area of kinase bypass, including the first reports of the antiviral activity of acyclic nucleoside phosphonates (ANP) (De Clercq et al., 1986), the ability to circumvent the first phosphorylation step in thymidine kinase (TK) deficient cells or to inhibit resistant herpes viruses with altered viral TK activity *in vitro* (Balzarini et al., 1996; Hostetler et al., 1993, 1992; McGuigan et al., 1996; Prisbe et al., 1986), the ability of kinase

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bypass strategies to impart cellular activity to inactive nucleosides (for example, see papers on 2′,3′-dideoxyuridine (ddU) and 4′-azidonucleosides (McGuigan et al., 1994; Perrone et al., 2007a,b)), *in vivo* proof of concept illustrated by the anti-HBV activity of a lipid prodrug of acyclovir-MP (ACV-MP) observed in woodchuck (Hostetler et al., 2000) and the clear establishment of the intracellular delivery of NMP analogs using stable isotope methods (Kim et al., 2004), have provided the foundation for further progress in the treatment of viral infections. While initial studies primarily established the antiviral activity of kinase bypass strategies *in vitro*, the clinical success of the ANPs has clearly validated the clinical relevance of these strategies (Lee and Martin, 2006). With at least 10 kinase bypass and cell targeted prodrugs currently in clinical trials, the field has now progressed to possibly better achieving the goal of site specific delivery (Stella and Himmelstein, 1980).

Kinase bypass strategies have been discussed in a number of prior reviews (Bobeck et al., 2010; Cahard et al., 2004; Calogeropoulou et al., 2003; De Clercq and Field, 2006; Gosselin et al., 1996; Hecker and Erion, 2008; Hostetler, 2009; Jessen et al., 2008; Jones and Bischofberger, 1995; Mehellou et al., 2009; Meier and Balzarini, 2006; Schultz, 2003; Tan et al., 1999). While this review is focused on strategies applied to antiviral nucleosides, a related body of primary literature also exists for anticancer agents. Rather than provide a comprehensive review of kinase bypass strategies, examples are used from different structural classes to highlight key concepts and, where appropriate, reference to more exhaustive reviews is given. While a historical overview of the evolution of prodrugs for kinase bypass is provided, this review primary focuses on strategies that are of potential clinical relevance, especially those where examples have recently advanced into clinical trials.

2. Initial application of kinase bypass strategies

The two major barriers to bypassing the need for kinase activation of nucleotide analogs are the presence of a large amount of extracellular phosphatase activity and poor cell membrane permeability due to the presence of negative charges at physiologic pH. Initial attempts at kinase bypass primarily focused on stabilizing the NMP to phosphatase activity by forming 3′,5′-cyclic phosphates (Section 2.1) and stable phosphonate linkages (Section 2.2; some structures are shown in Fig. 1).

2.1. 3′,5′-cyclic phosphates

In 1972 Long et al. synthesized 3′,5′-cyclic ara-cytidine-MP (ara-CMP) (Fig. 1) and found it to be active *in vitro* as an anti-HSV-1 and -proliferative agent (Long et al., 1972). 3′,5′-Cyclic ara-CMP was stable to phosphatase activity and, in addition to being more stable than the MP, 3′,5′-cyclic ara-CMP appeared to cross cell membranes more efficiently than ara-CMP. The *in vitro* antiproliferative activity was verified *in vivo* in a mouse leukemia model. Similarly, Roland Robins and coworkers synthesized 3′,5′-cyclic 3-deazaguanosine-MP in 1984 and reported anti HSV-1 and vaccinia virus activity roughly equal to 3-deazaguanosine (Revankar et al., 1984). While there was no evidence of improved antiviral activity, 3′,5′-cyclic 3-deazaguanosine-MP was about 1.5 logs more active than 3-deazaguanosine as an inhibitor of the growth of L1210 leukemia cells *in vitro*, providing some evidence of increased delivery of the active metabolite into cells. Prisbe and coworkers synthesized the cyclic phosphate of ganciclovir (GCV) in 1986 and found that it had antiviral activity against TK deficient and GCV resistant HSV-1, clearly establishing bypass of the first phosphorylation step (Prisbe et al., 1986).

As opposed to the successful early reports of the application of 3′,5′-cyclic phosphates to nucleoside analogs described above, a number of subsequent papers showed less compelling findings. A series of papers by Beres et al. (1986a,b,c) described the synthesis and antiviral evaluation of a series of 5-halo and 5-alkyl substituted 3′,5′-cyclic phosphates of C, U and 2′-deoxyU (dU). 3′,5′-Cyclic 5-fluoro-CMP (5-F-CMP) was 10- to 100-fold less active against HSV-1 and vaccinia virus than its corresponding nucleoside. The authors noted that the 3′,5′-cyclic analogs were not active in TK deficient cells, suggesting a failure to bypass the first phosphorylation step (Beres et al., 1986a). The 3′,5′-cyclic 5-halo-UMP series was inactive against HSV-1 (Beres et al., 1986b) while the 3′,5′-cyclic 5-halo-2′-dUMP series was found to be active (Beres et al., 1986c). A 3′,5′-cyclic phosphate of cyclopropavir, (Z)-9-([2,2-bis(hydroxymethyl)cyclopropylidene]-methyl)guanine, was synthesized by Zemlicka and coworkers and found to have antiviral activity against Epstein-Barr virus (EBV) in Daudi cells (Yan et al.,

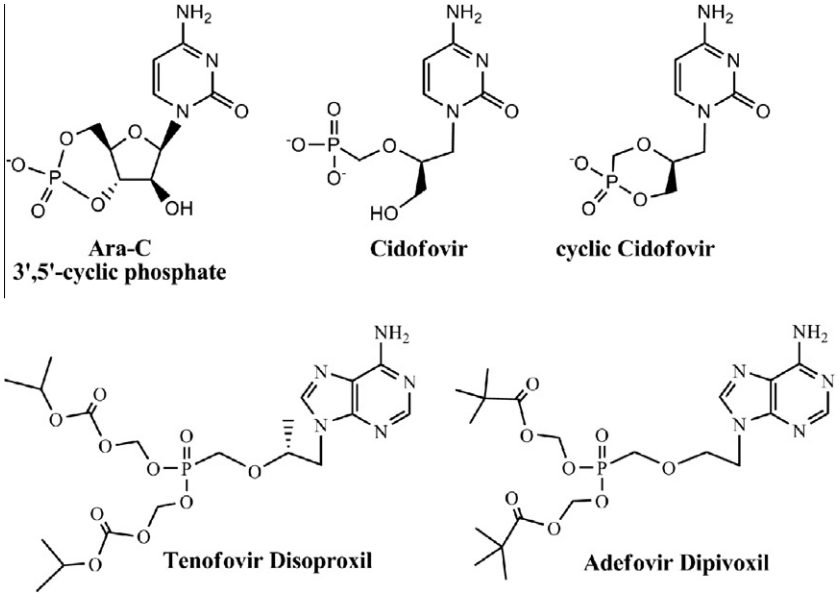


Fig. 1. Examples of initial kinase bypass strategies and the clinically approved prodrugs of the acyclic nucleoside phosphonates AFV and TFV.

2005). Curiously, the MP of this compound was inactive in Daudi cells but was substantially more active in H1 cells. The 3′,5′-cyclic phosphate was significantly less active than the MP against two CMV strains *in vitro*.

2.2. Phosphonates

Replacement of the labile 5′–CH_2O–P bond with a stable CH_2–P bond yields nucleotide analogs completely stable to phosphatase activity. ANP were the first and currently are the only kinase bypass strategy to yield approved antiviral drugs. The history of nucleoside phosphonates dating back to the 1960s and the clinical development of the antiviral ANPs has been reviewed by Lee and Martin (2006).

With the discovery of the therapeutic potential of the acyclic nucleoside analog ACV (Schaeffer et al., 1978) it was also quickly realized that the antiviral activity of nucleoside analogs were limited by an inefficient first phosphorylation step. In order to address this limitation and bypass the first phosphorylation step, Prisbe, Martin and coworkers described, in addition to the cyclic phosphate of GCV discussed in Section 2.1, a phosphonate analog of GCV-MP containing 5′-CH_2CH_2-P that displayed moderate to weak anti-HSV-1 and CMV activity (Prisbe et al., 1986). De Clercq, Holy and coworkers reported the potent broad spectrum activity of HPMPA in a 1986 letter to Nature (De Clercq et al., 1986). Subsequent work led to the discovery of the antiviral activity of HPMPC (cidofovir; CDV; Fig. 1), PMEA (adefovir; AFV) and, later, PMPA (tenofovir; TFV) (Balzarini et al., 1993; De Clercq et al., 1987). CDV would go onto be approved for the indication of CMV retinitis in HIV patients in parent form administered via intravenous infusion in 1996. While TFV was shown to have anti-HIV activity when administered to patients intravenously (Deeks et al., 1998), the orally administered bis-POM and bis-POC prodrugs of AFV (dipivoxil; ADV) and TFV (disoproxil fumarate; TDF), respectively, were ultimately pursued in the clinic for the treatment of HIV and HBV (prodrugs discussed further below, structures shown in Fig. 1).

While not subject to phosphatase cleavage, nucleoside phosphonates still carry two negative charges at physiologic pH resulting in poor oral bioavailability, inefficient cell entry and the potential for renal toxicity due to accumulation in the kidney. Akin to early attempts with NMPs, Bischofberger and coworkers attempted to improve the distribution of CDV by synthesizing cyclic CDV (Fig. 1) and found that the antiviral activity, cytotoxicity and selectivity index was similar for cyclic CDV to that of CDV in HSV-1 infected cells *in vitro* (Bischofberger et al., 1994). After administration of cyclic CDV, decreased systemic exposure to CDV was noted in patients, perhaps due to the clearance of cyclic CDV by both non-renal mechanisms as well as renal filtration and tubular secretion. Likely due to reduced systemic and renal exposure to CDV, cyclic CDV showed reduced potential for renal toxicity in three animal species (Hitchcock et al., 1995) and had the potential to reduce nephrotoxicity in the clinic (Cundy et al., 1999).

3. Evolution and structural diversity in prodrug strategies for kinase bypass

While phosphonate and 3′,5′-cyclic phosphate strategies serve to improve the extracellular stability of the phosphate linkage, neither strategy fully addresses the issue of cellular permeability. While both 3′,5′-cyclic phosphates and cyclic phosphonates took an intermediate step by removing one negative charge, further prodrug strategies are required to fully mask negative charge and optimize cellular permeability. In addition to simply masking negative charges with bioreversible protecting group, addition of prodrug moieties allows for attempts at achieving the more ambitious goal of site specific delivery to cells and tissues affected by viruses (general concept reviewed by Stella and Himmelstein (1980)). Section 3.1 discusses a number of examples of bioreversible protecting groups applied to nucleotide analogs and Tables 1–3, present the progression of kinase bypass strategies applied to NMPs, nucleoside phosphonates, and NDPs and NTPs, respectively. Structures of some early kinase bypass strategies are presented in Fig. 2 and clinically relevant NMPs and ANPs are presented in Figs. 3 and 4, respectively.

3.1. Bioreversible protecting groups

In early studies, various esters of 3′,5′-cyclic AMP (cAMP) were synthesized and some compounds such as the N^6-monooctanoyl, N^6-monobutyryl and the N^6-2′-*O*-dibutyryl esters were found to promote biological activity (Posternak et al., 1962). Another approach used by Farquhar et al. was to use bis-acyloxymethyl groups such as bis-pivaloyloxymethyl (POM) as phosphate-protective groups to mask the negative charges on NMPs in order to increase the ability of these compounds to traverse biological membranes (Farquhar et al., 1983). One of these NMPs was shown to be active *in vitro* as an antiproliferative agent.

3.1.1. Tri-esters

In an effort to target antiviral nucleotides to macrophages by utilizing the mannose receptors, Gouyette et al. synthesized phosphotriesters of AZT-MP, ddT-MP and 5-F-dUMP (F-dUMP) having a hexadecyl moiety and mannose or (hydroxymethyl)mannose residues esterified to the NMP (Gouyette et al., 1989). Subsequently a glucose analog was also synthesized (Henin et al., 1991). These triesters were found to interact with unilamellar phospholipid vesicles and were detected in the intravesicular space suggesting the spontaneous occurrence of flip-flop of the molecules across the phospholipid bilayer. The AZT-MP triesters were tested in CEM and U937 cells infected with HIV and found to be less active, more cytotoxic and less selective than unmodified AZT (Henin et al., 1991).

Simple dialkyl substituted triesters of ara-AMP (di-propyl shown in Fig. 2) showed less activity than ara-A *in vitro* (McGuigan et al., 1989). However, these studies did not establish if prodrugs successfully bypassed the first phosphorylation step, releasing ara-AMP into cells. A more complex approach included preparation of di-nucleoside phosphate salicylic acid esters of AZT and ddT (Khamnei and Torrence, 1996). These prodrugs rapidly released NMP in pig liver esterase and rat brain extract and were relatively stabile in human plasma but no cell based activity was reported.

Simple mono-alkyl, 3′,5′-cyclic phosphate triesters, first applied in an effort to release cAMP into cells (Cotton et al., 1975; Gohil et al., 1974) and to increase the antiviral and antitumor activity of analogs of dUMP (benzyl 3′,5′-cyclic 5-iodo-dUMP (IDU) shown in Fig. 2) (Beres et al., 1986a,b,c), have recently been established as a potentially clinically relevant class of prodrugs with the potent anti-HCV activity observed for PSI-352938 (Fig. 3) (Reddy et al., 2010). Many of the prodrug moieties further discussed below, include mono-amino acid ester, isopropyloxymethylcarbonyl (POC; Section 3.1.4), POM (Section 3.1.4) and *S*-acetylthioethanol (SATE; Section 3.1.5), have also been applied to mask the negative charge on the 3′-5′-cyclic phosphates of nucleoside analogs with anti-HCV activity (Gunic et al., 2007; Meppen et al., 2009). A mono-amino acid ester prodrug of cyclic CDV has also been described (Keith et al., 2003).

3.1.2. Phospholipids

Phospholipid prodrugs are attractive strategies for facilitating kinase bypass due to the presence of cellular metabolic machinery

Table 1
Evolution of antiviral prodrug kinase bypass strategies for nucleoside phosphates.

Class	Description	Nucleoside	References
3′,5′-Cyclic	Mono-acid	Ara-C	Long et al. (1972)
		GCV	Prisbe et al. (1986)
	Mono-alkyl	5-sub-dU	Beres et al. (1986a,b,c)
	Mono-POC/POM/SATE	2′-C-MePurine	Gunic et al. (2007)
	Mono-amidate	2′-C-MeC	Meppen et al. (2009)
HSA	Glycoprotein conjugate	Ara-A	Fiume et al. (1985)
Triesters	Carbohydrate	AZT	Gouyette et al. (1989)
	Bis-alkyl	Ara-A	McGuigan et al. (1989)
	Salicyl phosphate ester	AZT, ddT	Khamnei and Torrence (1996)
Phospholipid	Diacylglycerol	AZT	Calogeropoulou et al. (1995), Hostetler et al. (1990)
		ACV	Beadle et al. (2000), Hostetler et al. (2000, 1997)
	Dialkylthioglycerol	AZT, FLT	Bogner et al. (1997a,b), Girard et al. (2000), Venhoff et al. (2009)
Neo-glycoprotein	Macromolecule	AZT	Molema et al. (1990)
Amidate	Mono-ethanol, mono-amino acid ester	AZT	Devine et al. (1990)
	Mono-ethanol, mono-alkyl amine	AZT	Curley et al. (1990)
	Mono-trihaloethanol, mono-amino acid ester	AZT	McGuigan et al. (1991a)
	Bis-amino acid ester	AZT, FLT	Jones et al. (1991), McGuigan et al. (1991b)
	Mono-aryl, mono-amino acid ester (“protide”)	ddU	McGuigan et al. (1994)
		AZT	Wagner et al. (1995)
		4′-AzidoN	Perrone et al. (2007a,b)
		2′-C-MeG	McGuigan et al. (2010, 2009)
		2′-F-2′-C-MeU	Sofia et al. (2010)
		N6-subsituted 2′-F-2′-CMeG	Furman et al. (2011)
	Mono-acid, mono-amino acid ester	2′-C-MeC	Gardelli et al. (2009)
	Mono-aryl, mono-amino alcohol ester	2′-C-MeC	Donghi et al. (2009)
POM	Bis-POM	ddU	Sastry et al. (1992)
SATE	Bis-SATE	ddU	Perigaud et al. (1993)
	Mono-SATE, mono-aryl	AZT	Schlienger et al. (1998), Schlienger et al., 2000)
	Mono-SATE, mono-amidate	AZT	Egron et al. (2003, 2001), Zhou et al. (2011)
DTE	Bis-DTE	ddU, AZT	Perigaud et al. (1993), Puech et al. (1993)
BAB	Bis-AB	AZT	Routledge et al. (1995)
Cyclic phosphate	CycloSAL	d4T	Meier et al. (1998)
	Ester “lock-in”	d4T	Gisch et al. (2007), Vukadinovic et al. (2005)
	2 Nucleotides	d4T	Gisch et al. (2009)
Cyclic 1,3-propanyl esters	“HepDirect”	various	Erion et al. (2004)
		2′-C-MeA	Hecker et al. (2007)

Table 2
Evolution of antiviral prodrug kinase bypass strategies for nucleoside phosphonates.

Class	Description	Nucleotide analog	References
Phosphonate	5′-CH_2CH_2-P	GCV analog	Prisbe et al. (1986)
	5′-OCH_2-P	HPMPA	De Clercq et al. (1986)
		CDV, AFV, TFV	Balzarini et al. (1993), De Clercq et al. (1987), Shaw et al. (1997a)
POM	Bis-POM	AFV	Srinivas et al. (1993), Starrett et al. (1992)
POC	Bis-POC	TFV	Robbins et al. (1998), Shaw et al. (1997b)
DTE	Bis-DTE	AFV	Puech et al. (1993)
SATE	Bis-SATE	AFV	Benzaria et al. (1996)
Cyclic	Mono-acid	CDV	Bischofberger et al. (1994)
	Mono-amino acid ester	CDV	Keith et al. (2003)
Amidate	Mono-aryl, mono-amino acid ester	TFV	Eisenberg et al. (2001), Lee et al. (2005)
		GS-9148	Cihlar et al. (2008), Ray et al. (2008)
	Bis-amino acid ester	6-Substituted PMEG	Wolfgang et al. (2009)
Phospholipid	Hexadecyloxypropyl	CDV	Beadle et al. (2002)
		TFV	Painter et al. (2007)
		HPMPA	Wyles et al. (2009)
Cyclic 1,3-propanyl esters	“HepDirect”	AFV	Erion et al. (2004)
Cyclic phosphate	CycloSal	AFV	Meier et al. (2005)
Peptidomimetic	Macromolecule	CDV	Eriksson et al. (2008)

capable of supporting intracellular activation, prolonged cellular retention, plasma stability when applied to NMPs and the potential for tissue targeting. These properties have led phospholipid strategies to be broadly explored in the context of both nucleoside phosphates and phosphonates. A stearyl ester of cytaribine-MP (staracid) was approved for the treatment of leukemia in Japan in 1992 (prodrug strategies applied to cytaribine have recently been reviewed (Chhikara and Parang, 2010)) and two lipid prodrugs of

Table 3
Strategies for delivery or di- and tri-phopshate nucleoside analogs.

Class	Description	Nucleotide analog	References
Phospholipid	Diacylglycerol	AZT-DP	Hostetler et al. (1990)
		3′dT-DP	Hostetler et al. (1992)
		ACV-DP	Hostetler et al. (1993)
Phospholipid	Distearoylglycerol	AZT-TP	van Wijk et al. (1994)
Tri-ester	Acyl ester	AZT and d4T nucleotides (-MP, -DP and -TP)	Bonnaffe et al. (1996)
Triphosphate mimics	"P3 M"	AZT-TP	Wang et al. (2004)
Alkyloxy-benzyl	Bis-AB on β-phosphate	AZT-DP and d4T-DP	Meier et al. (2008)

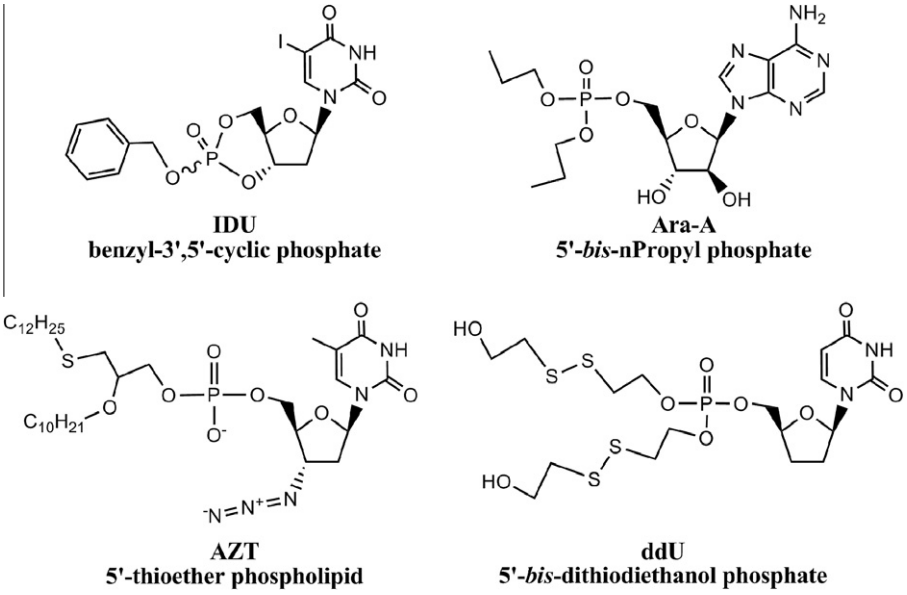


Fig. 2. Examples of kinase bypass prodrug strategies applied to deliver of nucleoside analog monophosphates.

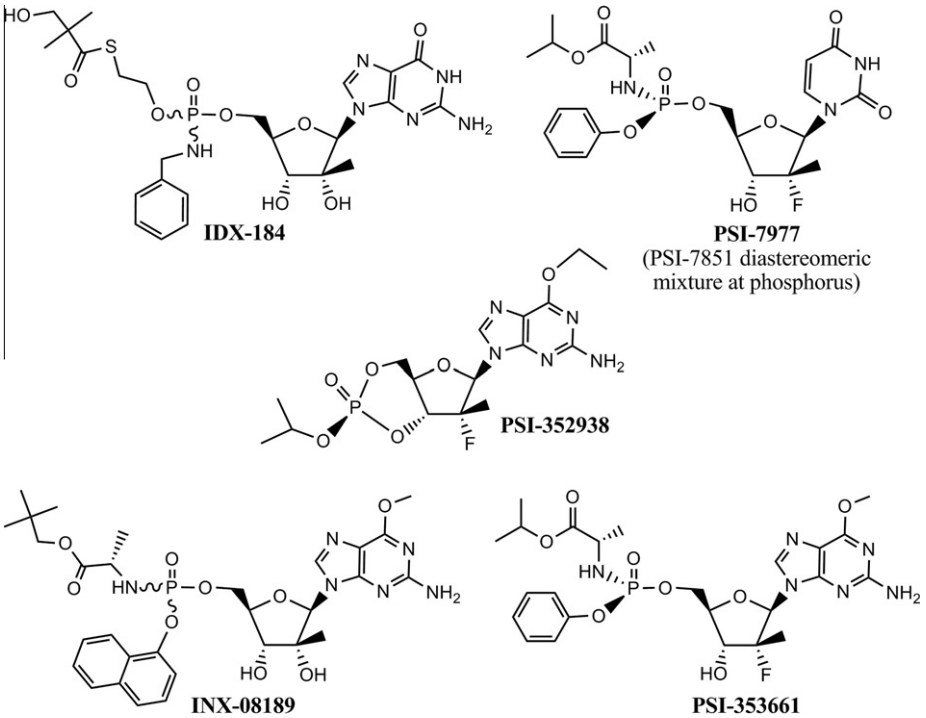


Fig. 3. Clinically relevant prodrugs of nucleoside analog monophosphates as liver targeted anti-HCV agents.

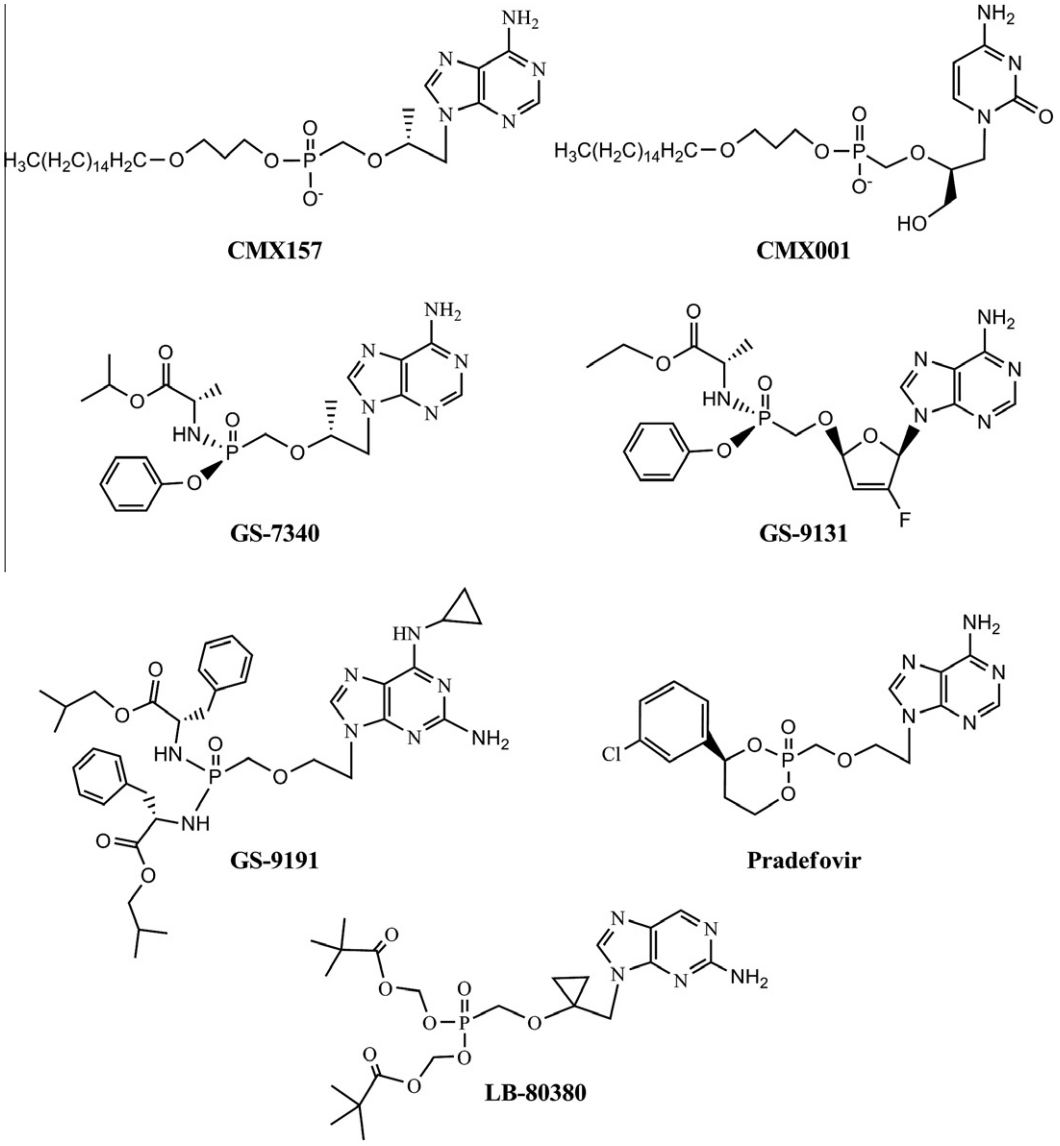


Fig. 4. Prodrugs of nucleoside phosphonates studied clinically as antiviral agents.

ANPs, CMX001 and CMX157, are currently in clinical development as antiviral agents (discussed further below in Section 3.1.2.2 and recently reviewed elsewhere (Hostetler, 2009)).

3.1.2.1. Applied to phosphates. An early approach to kinase bypass was made by esterifying AZT, ddT or ddC with diacyl glycerol phosphate (phosphatidic acid) to obtain phosphatidyl-AZT, phosphatidyl-ddT and phosphatidyl-ddC, analogs of the naturally occurring phospholipids like phosphatidylcholine and phosphatidylethanolamine. These compounds were active in HIV-infected CEM and U937 cells. Phosphatidyl-ddT was significantly more active than ddT itself and it was suggested that this could be due to direct conversion of phosphatidyl-ddT to ddT-MP by cellular enzymes (Hostetler et al., 1990). Further metabolic studies in CEM cells showed kinase bypass and direct formation of NMPs (Hostetler et al., 1991). No clinical evaluation of phosphatidyl-AZT or other phosphatidyl-dideoxynucleosides has been completed. However, a similar compound, fozivudine tidoxil, a thioether analog of phosphatidyl-AZT was synthesized and evaluated in human clinical trials (Bogner et al., 1997a,b; Girard et al., 2000). Proposed advantages of this form of AZT were kinase bypass, increased levels in lymphoid tissues and the potential for once daily dosing. In Phase I/II clinical trial in AIDS patients, the highest dose (600 mg twice daily) produced a 0.67 $\log_{10}$ drop in viral load after 28 days (Girard et al., 2000). A prodrug applying the same prodrug moiety to 3′-deoxy-3′-fluoro-thymidine (FLT), fosalvudine tidoxil, has also progressed as far as Phase I/II clinical studies (Venhoff et al., 2009). However, to date no further clinical studies with either prodrug have been reported.

Both ACV and AZT are not effectively converted to their MPs in liver (Hostetler et al., 1997) and are not active in patients with HBV (Berk et al., 1992) in spite of the fact that AZT-TP and ACV-TP are potent inhibitors of the HBV polymerase (Hantz et al., 1984). However, their 1-*O*-octadecylglycerol-3-phosphate esters are potent inhibitors of HBV replication in 2.2.15 cells *in vitro* suggesting conversion to AZT-MP and ACV-MP (Hostetler et al., 1997). 1-*O*-Octadecylglycero-3-phosphate-ACV is orally active in woodchuck hepatitis virus (WHV) infection; the compound inhibited viral replication with a 95% reduction in serum WHV DNA levels. In contrast, the maximal tolerated dose of ACV, a 5.3-fold-higher molar dosage, had no demonstrable activity. Oral 1-*O*-hexadecylpropanediol-3-phosphate-ACV appeared to be safe and effective in chronic WHV. It is active because the prodrug can be metabolized intracellularly in liver to ACV-MP, bypassing

TK activation of ACV. ACV-MP is then converted to ACV-TP, the active metabolite (Hostetler et al., 2000). Similarly, 1-*O*-hexadecylpropanediol-3-phosphate-ACV is orally active in experimental HSV-1 infections in mice by a similar mechanism (Beadle et al., 2000).

Piantadosi and coworkers synthesized a series of ether lipid conjugates of AZT-MP and other dideoxynucleosides (Piantadosi et al., 1991). The most active compound was the 3-octadecanamido-2-ethyloxypropyl conjugate of AZT-MP (AM 18-OEt) which had an EC_{50} of 0.03 μM in CEM cells and a selectivity index of 1800. The compound was less active than AZT itself but the selective index was greater than that of AZT and the authors proposed that AM 18-OEt ester of AZT-MP might be less toxic and reach the macrophage reservoir more effectively. It was not possible to determine if kinase bypass was achieved with this type of compound because there were no studies in TK negative cells.

Tsotinis and coworkers synthesized a series of alkoxy and aryloxy esters of AZT-MP (Tsotinis et al., 1996). Chiral octadecyloxypropyl and substituted phenyl or naphthyl esters of AZT-MP were prepared and evaluated. All prodrugs were less active than AZT itself against HIV-1 and HIV-2 and aromatic mono-esters were more active than the octadecyloxypropyl compounds with EC_{50} values of 0.013–0.020 μM versus 0.2–2.2 μM, respectively. Interestingly, none of the prodrugs were active in HIV-infected TK negative CEM cells indicating the absence of kinase bypass. This suggests that the AZT-MP 5′–O–P bond is more readily hydrolyzed than the phosphoester with the octadecyloxypropyl or naphthyl moieties.

3.1.2.2. Applied to phosphonates. ANPs such as CDV and TFV are stable analogs of dAMP and, therefore, do not require nucleoside kinases. However, compounds of this class are not orally bioavailable unless their negative charges are masked. One prodrug approach applied to ANPs has been to synthesize lysophospholipid analogs allowing for oral bioavailability, increased cell uptake and enhanced antiviral activity *in vitro* and reduced potential for nephrotoxicity *in vivo*. The approach is broadly applicable to ANP antivirals having a number of different structures including CDV, AFV, TFV and others and was recently the subject of a review (Hostetler, 2009). Some of the most interesting of these alkoxyalkyl esters are discussed below.

To improve oral bioavailability, Hostetler and Beadle synthesized hexadecyloxypropyl-CDV (HDP-CDV) and octadecyloxypropyl-CDV (ODE-CDV) (Beadle et al., 2002). These compounds were tested *in vitro* in cells infected with vaccinia virus and CMV and found to have antiviral activity 40- to 55-times greater than that observed with unmodified CDV (Beadle et al., 2002; Hostetler, 2009). The increased antiviral activity was due to greater cell uptake mediated by the HDP and ODE portions of the molecules which are cleaved by an intracellular phospholipase C, releasing CDV which is actively converted to CDV-DP, the active metabolite (Hostetler, 2009, 2010). HDP-CDV (CMX001; Fig. 4) has broad spectrum antiviral activity against double stranded DNA viruses including orthopoxviruses such as variola, monkeypox and vaccinia, herpes viruses including CMV, HSV-1, human herpes virus 8 (HHV-8), EBV, VZV, as well as adenoviruses, polyomaviruses and ORF virus (Hostetler, 2009). Recently CMX001 has also been reported to have *in vitro* activity against JC virus (Jiang et al., 2010). CMX001 is being developed as a potential countermeasure for biodefense against smallpox (Lanier et al., 2010) and is in Phase II clinical trials directed at CMV, BK and adenovirus infections. It has been especially useful in disseminated adenovirus infections in immunosuppressed patients where eradication of an adenovirus infection has been reported (Paolino et al., 2011).

HDP-TFV (CMX157; Fig. 4) is a second generation analog of TFV which exhibited antiviral activity against HIV >250-fold greater than unmodified TFV with EC_{50} values in the 1–3 nM range in human peripheral blood mononuclear cells (PBMCs). It was also found to be 4.5-fold more active than TFV against HBV *in vitro*. CMX157 was orally bioavailable in rats; in contrast to other types of prodrugs, the HDP ester survives the oral absorption process and is not a substrate for the renal proximal tubule organic anion transporter. The compound showed no toxicity in short term toxicology studies in rodents at doses up to 100 mg/kg/day for a week (Painter et al., 2007). CMX157 was active against all major subtypes of HIV-1 and HIV-2 in PBMCs and monocyte derived macrophages with EC_{50}s ranging from 0.2 to 7.2 nM. CMX157 retained low nanomolar activity against a panel of 30 multidrug resistant nucleotide reverse transcriptase inhibitor mutants and did not demonstrate antagonism when tested with marketed antiretroviral drugs (Lanier et al., 2011). CMX157 is currently in Phase I clinical trials in healthy volunteers.

9-(*S*)-[3-Hydroxy-2-(phosphono-methoxy)propyl]adenine ((*S*)-HPMPA) was the first ANP described in 1986 (De Clercq et al., 1986). HDP and ODE esters of (S)-HPMPA were recently found to have antiviral activity in HBV infected transgenic mice. Oral treatment of HBV transgenic mice with HDP-(*S*)-HPMPA, and ODE-(S)-HPMPA for 14 days reduced liver HBV DNA levels by roughly 1.5 log, a response equivalent to that of ADV (Morrey et al., 2009). As their alkoxyalkyl esters, ANPs have been reported to have activity against DNA viruses and viruses which utilize reverse transcription (HIV and HBV). Recently, however, alkoxyalkyl esters of (*S*)-HPMPA were found to have antiviral activity against HCV, an RNA virus (Wyles et al., 2009). ODE-(*S*)-HPMPA was the most active compound; HDP and ODE esters of (*R*)-HPMPA were several fold less active while unmodified (*S*)-HPMPA and (*R*)-HPMPA were inactive. In genotype 1B and 2A replicons analyzed by HCV RNA analysis, ODE-(*S*)-HPMPA was the most active compound, with EC_{50}s of 1.3 and 0.69 μM, respectively (Wyles et al., 2009).

A close analog of (*S*)-HPMPA is 9-(*S*)-[3-methoxy-2-(phosphonomethoxy)propyl]adenine ((*S*)-MPMPA) in which the 3-propyl hydroxyl is blocked with a methyl residue. Recently, it was found that the ODE ester of this compound, ODE-(*S*)-MPMPA, is also active against HCV. Interestingly, the antiviral activity against HCV was preserved, with EC_{50}s of 1–2 μM in genotype 1B and 2A replicons but it exhibits much lower cytotoxicity with a CC_{50} of >150 μM (Valiaeva et al., 2011). Oral studies in mice show no adverse effects after 7 days of treatment at 10 mg/kg, a dose which would be toxic if using ODE-(*S*)-HPMPA. Preliminary studies show a high barrier to development of resistance and prolonged antiviral suppression after the drug has been removed from the replicon culture medium (Wyles et al., 2011).

3.1.3. Amidate

Amidate prodrugs are perhaps the most extensively explored class of kinase bypass prodrugs and have been applied to a large number of antiviral NMP analogs. Only select examples are discussed here, for a more thorough review see Mehellou et al. (2009). In 1990 two papers described mono-ethanol and mono-amino acid ester or alkyl amine prodrugs of AZT-MP with potent anti-HIV activity *in vitro* (Devine et al., 1990; Molema et al., 1990). Further studies explored replacement of the mono-ethanol with mono-trihaloethanol, -aryl and a second amino acid ester (generating a bis-amino acid ester prodrug) (Jones et al., 1991; McGuigan et al., 1994, 1991a,b). More recently, amino alcohols have been explored as amino acid replacements (Donghi et al., 2009). The ability of mono-aryl, mono-amino acid esters to impart favorable anti-HIV activity to the otherwise inactive nucleoside analog ddU (McGuigan et al., 1994) and to improve the properties of AZT *in vitro* (Wagner et al., 1995) has been followed by these prodrug moieties being applied successfully to a number of nucleoside analogs (for example, see references from Perrone et al.

regarding 4′-azido containing nucleosides (Perrone et al., 2007a,b)). As discussed below, amidate prodrugs have also been explored as a prodrug strategy for nucleoside phosphonates leading to the discovery of the clinical candidates GS-7340 (Fig. 4), GS-9131 and GS-9191.

While often questioned in the past for their lack of established effectiveness *in vivo*, a number of phosphoramidate and phosphonamidate prodrugs have recently shown promise in clinical trials. As shown in Fig. 3, there are at least two mono-aryl, mono-amino ester prodrugs of NMPs being pursued clinically as liver targeted prodrugs for the treatment of HCV, PSI-7977 (the purified *S*-diastereoisomer of PSI-7851) and INX-08189 (Lam et al., 2010; McGuigan et al., 2010; Murakami et al., 2010). PSI-353661, an alternate prodrug strategy to that applied in the 3′,5′-cyclic prodrug PSI-352938, is a third prodrug from this class that has been characterized extensively in preclinical studies (Furman et al., 2011). Optimization of prodrugs of 2′-C-MeG-MP resulted in selection of a naphthyl moiety as the aryl group in INX-08189 in place of the phenyl applied to most ProTides (McGuigan et al., 2009).

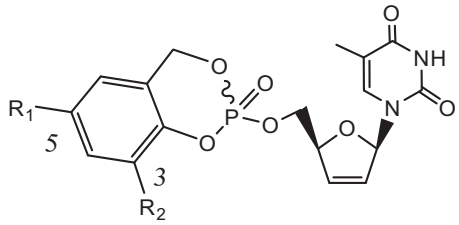
GS-7340 is the isopropylalaninyl monoamidate phenyl monoester prodrug of TFV (Fig. 4) currently in clinical development for the treatment of HIV. GS-7340 is up to 1000-fold more potent than TFV *in vitro*, is stable in plasma and distributes favorably to lymphoid cells and tissues following oral administration to dogs (Eisenberg et al., 2001; Lee et al., 2005). Monotherapy with GS-7340 in HIV-1 infected patients at doses of 50 and 150 mg for 14 days was associated with larger viral load drops and reduced TFV plasma exposure relative to TDF at 300 mg (Markowitz et al., 2011). The structure activity relationship for phosphonamidate prodrugs of a related cyclic nucleoside phosphonate analog, GS-9148, is presented in Table 5 and discussed further below in Section 4.3. While technically an anti-proliferative agent, the bis-phenylalanine isobutyl prodrug of a 6-substituted version of 9-(2-phosphonylmethoxyethyl)guanine (PMEG), GS-9191, is being evaluated clinically for the treatment of external genital warts caused by infection with the human papillomavirus (Wolfgang et al., 2009).

3.1.4. POM and POC

As discussed above in Section 2.2, ANPs have poor oral bioavailability and the first to be approved for clinical use, CDV, is administered *via* intravenous infusion. In order to simplify administration for the chronic treatment of HBV and HIV, prodrug strategies were needed to allow for oral delivery. The addition of bis-POM and -POC successfully increased the cell permeability and oral bioavailability of AFV and TFV in nonclinical studies (Robbins et al., 1998; Shaw et al., 1997b; Srinivas et al., 1993; Starrett et al., 1992) and both are now approved drugs (Fig. 1). Unlike POM, at higher doses the POC prodrug moiety does not deplete carnitine due to the lack of pivalate (Brass, 2002). The bis-POM prodrug of another ANP, LB-80380, is currently in clinical development for the treatment of HBV (structure shown in Fig. 4) (Choi et al., 2004). In addition to ANPs, bis-POM has also been applied to NMPs, including ddU-MP, and shown to improve anti-HIV activity *in vitro* (Sastry et al., 1992).

POM and POC prodrugs can be cleaved by many enzymes that are present at high levels in plasma and tissues (Liederer and Borchardt, 2006) and have, therefore, been generally thought of as oral prodrugs, only meant to facilitate absorption of a parent molecule across the intestinal wall followed by rapid degradation. However, studies in dogs and cynomolgous monkeys have shown higher lymphoid cell loading following oral TDF relative to subcutaneous

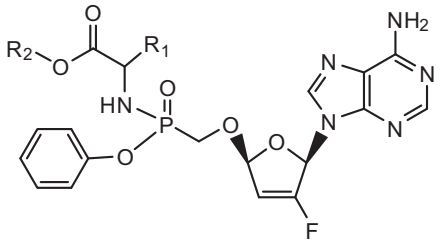
Table 4
Structure activity relationship of 3-substituted cycloSAL and 5-diacetoxymethyl(5-di-AM)-cycloSAL-d4TMP prodrugs for HIV-2 activity in CEM and CEM thymidine kinase deficient (TK−) cells and stability in phosphate buffered saline (PBS) and CEM cell extract.



R_1	R_2	HIV-2 EC_{50} (μM)		$t_{1/2}$ (h)	
		CEM	CEM TK−	PBS	Cell extract
D4T	0.63	47.5	NA	NA	
H	H	0.13	0.90	4.4	4.0
H	CH_3	0.07	0.05	17.5	14.5
H	*tert*-Butyl	0.65	0.33	73	ND
5-di-AM	H	0.40	10.5	1.2	0.08
5-di-AM	CH_3	0.70	6.3	2.3	0.08
5-di-AM	*tert*-Butyl	0.75	3.0	7.0	0.12

Results previously reported (Gisch et al., 2007).
NA, not applicable; ND, not determined.

Table 5
Structure activity relationship for mono-amino acid and mono-phenol prodrugs of the cyclic nucleoside phosphonate analog GS-9148.



R_1 (amino acid)	R_2 (ester)	*c* Log D	HIV EC_{50} (nM)	MT-2 CC_{50} (nM)
GS-9148 (diacid)	−5.70	10,600	>100,000	
H (glycine)	Iso-butyl	2.72	1120	22,000
CH_3 (alanine)	Ethyl	2.45	96	74,000
	CH_2-cyclo-propyl	2.82	59	>100,000
	n-Propyl	2.96	52	31,000
	Iso-propyl	2.98	728	NR
	Cyclo-butyl	3.05	36	57,000
	Iso-butyl	3.25	58	>100,000
	CH_2-cyclo-butyl	3.32	25	>100,000
	n-Butyl	3.47	92	91,000
	Cyclo-pentyl	3.55	63	>100,000
	n-Pentyl	3.98	33	27,000
	3-Pentyl	4.00	662	8350
CH_2CH_3 (amino-butyric acid)	Iso-butyl	3.76	103	>100,000
	n-Butyl	3.98	88	70,000
$CH_2(C_6H_5)$ (phenylalanine)	Eth	4.05	33	25,000
	CH_2-cyclo-propyl	4.42	7.5	>100,000
	n-Propyl	4.56	4.9	>100,000
	Iso-propyl	4.59	63	>100,000
	Cyclo-butyl	4.65	20	>100,000
	Iso-butyl	4.86	6.0	38,300
	CH_2.cyclo-Butyl	4.92	3.8	46,500
	(*R*)-sec-butyl	5.10	141	>100,000
	(*S*)-sec-butyl	5.10	199	>100,000

Results previously reported (Mackman et al., 2010).
NR, not reported.

TFV administration (Durand-Gasselin et al., 2009; Lee and Martin, 2006). Evidence for improved distribution to infected cells following oral administration of TDF can also be found by a cross study comparison of monotherapy studies of intravenous TFV (Deeks et al., 1998) and oral TDF in HIV-1 infected patients (Barditch-Crovo et al., 2001) where greater antiviral activity was observed at lower TFV plasma exposure following oral TDF administration (Lee and Martin, 2006). Combined, these results illustrate that even a short duration of exposure to a cell permeable prodrug can result in enhanced target tissue loading. Ongoing clinical trials with GS-7340 and CMX157, prodrugs with enhanced stability and measurable systemic exposure, look to further enrich target cells and tissues while minimizing plasma exposure to TFV (GS-7340 and CMX157 were discussed above in Sections 3.1.3 and 3.1.2, respectively).

3.1.5. DTE and SATE

The laboratory of Jean-Louis Imbach introduced SATE and dithiodiethanol (DTE) as intracellular targeted prodrug moieties in 1993 applying them to ddU-MP (bis-DTE ddUMP shown in Fig. 2) (Perigaud et al., 1993). The bis-SATE and bis-DTE prodrug moieties were later shown to be more generally applicable to other NMPs and ANPs through studies with AZT and AFV (Benzaria et al., 1996; Puech et al., 1993). Early applications of SATE prodrugs were reviewed by Gosselin et al. (Gosselin et al., 1996). Optimization of the rate of intracellular cleavage (discussed further below in Section 4.2) led to the study of mixed phosphotriesters containing mono-aryl (Schlienger et al., 1998, 2000) or mono-amidate (Egron et al., 2003, 2001) moieties in combination with a mono-SATE. A mono-SATE, mono-amidate prodrug of 2′-C-Me-GMP, IDX-184 (Fig. 3), was ultimately chosen as a clinical candidate for the treatment of chronic HCV (Zhou et al., 2011).

3.1.6. Cyclic phosphates (CycloSAL and HepDirect)

Cyclosaligenyl (CycloSAL) and cyclic 1-aryl-1,3-propanyl ester (HepDirect) prodrug strategies both mask the negative charges on nucleotide analogs by incorporating the phosphate or phosphonate into substituted six membered heterocycles. In 1998 Meier and colleagues reported lypophilic prodrugs of d4T-MP that through a chemical activation process showed potent anti-HIV activity in wild type and TK deficient T-cells (Meier et al., 1998). The CycloSAL strategy has subsequently been applied to the ANP AFV (Meier et al., 2005) and refined to favor intracellular delivery by utilizing an enzymatically catalyzed initial cleavage step (discussed further in Section 4.1) (Gisch et al., 2007; Vukadinovic et al., 2005). A CycloSAL di-nucleotide delivery system has also been reported to be effective *in vitro* (Gisch et al., 2009). The HepDirect prodrug strategy was developed as a broadly applicable way of delivering NMPs, ANPs and phosphate containing drug molecules to the liver (Erion et al., 2004). A HepDirect strategy was shown to successfully stabilize the anti-HCV nucleoside 2′-C-MeA from adenosine deaminase mediated catabolism and to allow for effective liver loading of the active TP following oral administration to rats (Hecker et al., 2007). While structurally similar, the activation of CycloSAL and HepDirect prodrugs are quite distinct and are discussed in Sections 4.1 and 4.4, respectively. CycloSAL and HepDirect prodrug strategies have been reviewed in greater detail elsewhere (Erion et al., 2006; Hecker and Erion, 2008; Meier and Balzarini, 2006).

Two HepDirect prodrugs have entered into clinical development for the respective treatment of HBV and HCV. Pradefovir is a liver targeted prodrug of AFV explored as a therapy for HBV (Erion et al., 2004) (Fig. 4). The HepDirect prodrug of a nucleoside analog, RG7348 (structure has not been disclosed), has also recently been reported to have entered clinical development for the treatment of chronic HCV (Ligand Pharmaceuticals, Inc., press release, April 20, 2010; available at: http://phx.corporate-ir.net/phoenix.zhtml?c=102955&p=irol-newsArticle&ID=1414925&highlight=).

3.1.7. Protein conjugates

Protein conjugates of the antiviral nucleotide ara-AMP were reported in 1985 (Fiume et al., 1985). Fiume et al. reported making glycoprotein conjugates with human serum albumin (HSA) in an attempt to obtain specific liver loading and increase the therapeutic index of ara-A for the treatment of HBV. In an effort to target T-lymphocytes neoglycoprotein carriers were designed for AZT-MP based on the presence of sugar-recognizing lectins on T-lymphocytes (Molema et al., 1990). The most active compounds *in vitro* were the mannose-HSA-AZT-MP conjugates which exhibited potencies per unit of AZT-MP which were 23- to 38-times greater than that of HSA-AZT-MP. However, antiviral activities were similar to unmodified AZT and AZT-MP and, while the authors suggested that it might occur, evidence for kinase bypass was not provided.

3.1.8. Diphosphate and triphosphate prodrugs

While the first phosphorylation step is often rate limiting, some nucleoside analogs' activity is limited by subsequent phosphorylation steps. For example, AZT is a classic example of a nucleoside analog that accumulates in cells as its MP due to a rate limiting second phosphorylation (Furman et al., 1986). In this context, more efficient delivery of AZT-MP may not be advantageous to antiviral activity. In principle, kinase bypass strategies to deliver NDPs or NTPs should be possible. However, this represents a more significant challenge because of the increased negative charge, the higher molecular weight of NDP or NTP analogs and the potential for adding a great deal of chiral complexity. Nevertheless, a number of prodrug strategies for NDP and NTP analogs have been reported including the use of phospholipid analogs, a cycloSAL NDP approach and synthesis of NTP mimics. Kinase bypass strategies for NDPs have been reviewed by Chris Meier and colleagues (Jessen et al., 2008).

In normal mammalian cellular metabolism, naturally occurring CDP diglyceride is involved in the synthesis of several important classes of phospholipids including phosphatidylinositol, phosphatidylglycerol and cardiolipin, giving rise to the phospholipid and CMP. For example, the following reaction scheme shows the biosynthesis of phosphatidylglycerol:

CDP-diglyceride + *sn*-Glycerol-3-P →

→ Phosphatidylglycerol + CMP

The biosynthetic process for phosphatidylglycerol, cardiolipin and phosphatidylinositol in all mammalian cells leads to CMP. Interestingly, it was shown that several other synthetic NDP diglycerides, including UDP diglyceride, ADP diglyceride and GDP diglyceride, have been shown to be able to substitute for CDP diglyceride leading to the formation of the phospholipid product generating UMP, AMP and GMP (Poorthuis and Hostetler, 1976). However, very little of these alternative NDP diglycerides are generally formed in significant amounts in mammalian cells. Building on this finding, Hostetler and coworkers synthesized ACV-DP diglyceride and showed that it was highly active *in vitro*, even in infections caused by TK deficient strains of HSV-1 and ACV-resistant HSV-2 clinical isolates where ACV itself is completely ineffective (Hostetler et al., 1993). This study effectively demonstrated kinase bypass by an antiviral NDP diglyceride. Another study examined ddT-DP (ddT-DP) dimyristoylglycerol (Hostetler et al., 1992). ddT-DP Dimyristoylglycerol had an EC_{50} of 1.6 μM against HIV compared with 29 μM for unmodified ddT. In TK deficient CEM cells, only ddT-DP dimyristoylglycerol was active against

HIV infection. Taken together these studies clearly demonstrated kinase bypass by NDP diglyceride and dimyristoylglycerol. However, while applied to NDPs, these prodrug strategies likely only deliver NMPs into cells.

An acylphospholipid nucleoside strategy was developed for antiviral NMP, NDP and NTPs by Bonnaffe et al. (1996). Myristoyl AZT nucleotides (AZT-MP-Myr; AZT-DP-Myr; AZT-TP-Myr), were synthesized and tested against HIV-1 *in vitro*. All compounds had EC_{50} values of 10–30 nM, similar to that observed for AZT. However, it was found that hydrolysis of the analogs in RPMI buffer was very rapid and the fact that AZT-MP-Myr, AZT-DP-Myr and AZT-TP-Myr all had antiviral activity similar to that of unmodified AZT may suggest that these compounds did not gain their antiviral activity by transmembrane diffusion of the intact nucleotide.

Van Wijk et al. synthesized AZT-TP distearoylglycerol, a phospholipid conjugate of AZT. AZT-TP distearoylglycerol was active against HIV-1 replication in CEM and HT4-C6 cells with EC_{50} values of 0.33 and 0.79 μM, respectively. When incubated with a rat liver mitochondrial preparation, both AZT and AZT-MP were liberated, demonstrating the ability to bypass TK by utilizing intracellular phospholipid metabolism pathways (van Wijk et al., 1994).

Chris Meier and coworkers attempted to apply the CycloSAL prodrug strategy to NDPs (Meier et al., 2008). However, the chemical breakdown of the prodrugs was found to release the NMP instead of the desired DP. Using an enzyme based prodrug strategy previously reported for AZT-MP (Routledge et al., 1995), a set of alkyloxybenzyl prodrugs of 2′,3′-dideoxy-2′3′-didehydro-T-DP (d4T-DP) and AZT-DP were synthesized. In particular, the bis-(4-benzoyloxybenzyl) prodrug of d4T-DP showed anti-HIV activity comparable to that of d4T in wild type cells and maintained most of it activity in TK minus cells suggesting successful kinase bypass of at least the first phosphorylation step.

The group of Dan Cook synthesized a series of AZT-TP mimics (AZT P3Ms) by making substitutions of S or CF_2 for the pyrophosphate oxygens and replacing the α hydroxyl with boron (B) and the β,γ hydroxyls with NH_2, NHMe, N_3, F, OMe, Phenol and other groups (Wang et al., 2004). The compounds were tested for their ability to inhibit HIV-1 reverse transcriptase (RT) *in vitro*. The most active AZT P3M was AZT 5′-α-R_p-borano-β,γ-(difluoromethylene)triphosphate (AZT 5′-αB-βγCF_2TP) which had similar activity to AZT-TP in its inhibitory effect on RT. AZT 5′- αB-βγCF_2TP was stable in serum for >48 h versus only 2 h for AZT-TP. Results with these types of agents in cells infected with HIV have not been reported to date. Given their high molecular weight and lack of a transmembrane facilitator moiety, it seems unlikely that these compounds would be taken up in sufficient amounts to inhibit viral replication.

4. Prodrug activation pathways and key concepts

Reflecting the structural diversity of prodrug moieties applied to facilitate the delivery of charged nucleotide analogs, different enzymatic and chemical steps are involved in the activation of prodrugs employed in kinase bypass strategies. The first clinically successful prodrug approaches of adding POM and POC moieties to AFV and TFV, respectively, were applied with the primary goal of enhancing oral bioavailability of the nucleotide analog into plasma and, thus, utilized prodrug moieties that are unstable in plasma. Second generation prodrug strategies attempt to not only improve oral bioavailability but also target cell loading. For these advanced prodrug strategies to work, selective activation pathways present in target tissues are required. Below in Sections 4.1 through 4.6 specific prodrug series are used to illustrate key concepts in prodrug design.

4.1. CycloSAL and intracellular activation

The evolution of the cycloSAL series of prodrugs can be used to illustrate the importance of selective intracellular activation in targeted prodrug strategies. In their earliest form, cycloSAL prodrugs successfully masked charge and increased membrane permeability but their entirely chemical breakdown pathway did not offer selectivity for intracellular versus extracellular activation. This made cycloSAL prodrugs useful tools for *in vitro* studies but made them unlikely to achieve the ultimate goal of target tissue enrichment *in vivo*. To overcome this limitation, Meier and colleagues used a "Lock-In" strategy applying moieties that stabilize the chemical degradation until after an initial carboxyesterase enzymatic cleavage step, favoring intracellular activation (Gisch et al., 2007). Results summarized in Table 4 show how modifications at the 3- and 5-positions were used to tune chemical stability and obtain more selective intracellular activation.

4.2. SATE and DTE and targeting specific intracellular pathways

Different intracellular enzymatic processes can be the targets of prodrug strategies. For example, a paper from the laboratory of Imbach reported strategies for targeting intracellular esterase or reductase cleavage using bis-SATE or bis-DTE prodrug moieties, respectively (Perigaud et al., 1993). Similar to the activation pathway of "Lock-In" cycloSAL prodrugs described above, the intracellular activation of SATE prodrugs involves an initial esterase cleavage step followed by rapid chemical degradation (Fig. 5). While represented equivalently in Fig. 5, the cleavage of both SATE moieties of the bis-SATE prodrug presumably occurs sequentially. The observation that the intracellular cleavage of the mono-SATE/mono-acid prodrug intermediate is rate limiting subsequently led to the development of mono-SATE prodrugs with either a mono-aryl (Schlienger et al., 1998, 2000) or a mono-amidate (Egron et al., 2003, 2001) masking the other negative charge of the NMP to enhance the rate of intracellular activation.

4.3. Mono-amino acid ester, mono-aryl and prodrug optimization

Sequential enzymatic and chemical steps have been shown to be responsible for the activation of mono-amino acid ester, mono-aryl NMP and phosphonate prodrugs (Fig. 6). While many hydrolases appear to be able to cleave the ester promoiety (Birkus et al., 2008; Murakami et al., 2010), highly efficient cleavage has

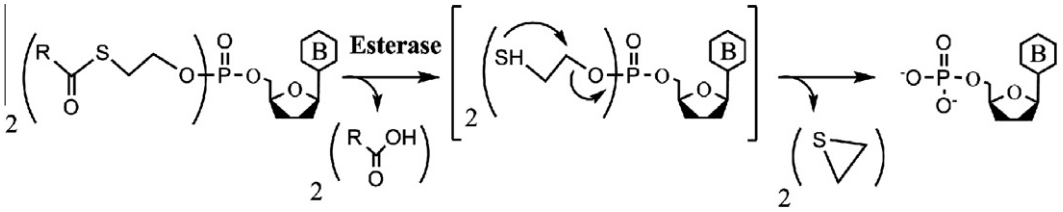


Fig. 5. Generic pathway for activation of bis-SATE prodrugs.

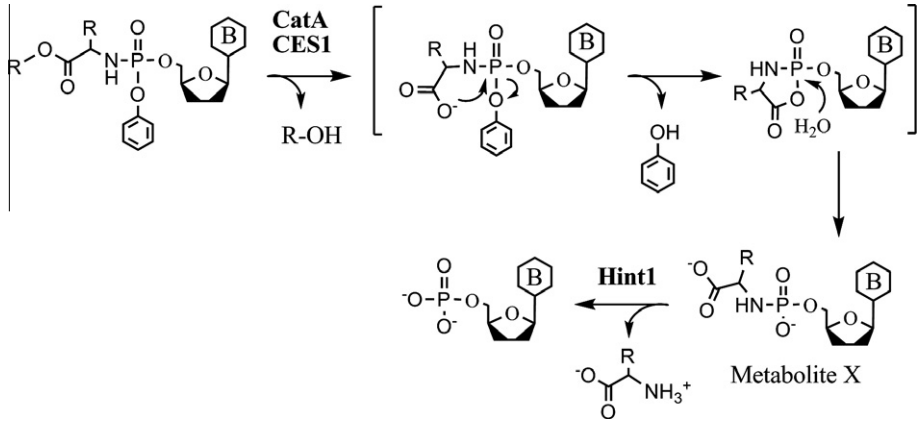


Fig. 6. Generic pathway for activation of mono-aryl, mono-amino acid ester prodrugs.

been observed with the lysosomal carboxypeptidase cathepsin A (CatA) and this enzyme was shown to play a critical role in the activation of the anti-HIV clinical candidate GS-7340 in fibroblasts (Birkus et al., 2007). Following a chemical step resulting in elimination of the aryl functional group, a diacid metabolite is formed consisting of the NMP analog and amino acid. While potentially chemical labile under acidic conditions in the lysosome, an early report found evidence for a phosphoramidase responsible for cleaving the P–N linkage (Saboulard et al., 1999), and this activity was later found to be the histidine triad nucleotide binding protein 1 (hINT1) (Chou et al., 2007). The presence of ester and amino acid prodrug moieties cleaved by distinct enzymatic process allows for the optimization of the biological activity. Table 5 shows the effects of different amino acids and esters on the activity of the nucleoside phosphonate analog GS-9148 (Mackman et al., 2010). Interestingly, the cleavage of the ester in the context of this particular nucleotide analog was sensitive to branching at the alpha position, with isopropyl and secbutyl performing relatively poorly, while an isopropyl ester has been applied successful to monoamidate prodrugs of TFV, 2′-F-2′-C-MeU-MP and 2′-F-2′-C-MeG-MP to generate the clinical candidates GS-7340 (HIV), PSI-7977 and PSI-353661 (HCV), respectively (Chang et al., 2011; Lee et al., 2005; Sofia et al., 2010). Stereochemistry was also found to be important for the efficiency of ester cleavage by the enzyme cathepsin A, *in vitro* antiviral activity and *in vivo* cell loading leading to selection of the *S*-diastereomer (at phosphorus) of the ethylalaninyl monoamidate of GS-9148, GS-9131, to be chosen as the clinical candidate for the treatment of HIV (Cihlar et al., 2008; Ray et al., 2008). Similarly, GS-7340, PSI-7977 and PSI-353661 are also the *S*-diastereomer at phosphorus (INX-08189 and IDX-184 are currently being developed as diastereomeric mixtures at phosphorus).

4.4. HepDirect and targeted tissue loading

For prodrugs targeting tissues loaded from the systemic circulation, like the lymphoid cells and tissues targeted by GS-9131, the liver's anatomical position (receiving all of portal blood flow) and metabolic capacity make it a barrier to successful nucleotide delivery. However, these factors make the liver an amendable target for the treatment of chronic viral infections with HBV and HCV with nucleotide prodrugs. While orally administered prodrugs will often unavoidably be taken up by the liver, targeted liver activation can be applied to limit off target distribution and unwanted toxicity. The HepDirect prodrug strategy was conceived of to use hepatic cytochrome P450 enzymes to selectively activate prodrugs in the liver (Erion et al., 2004). Initial cytochrome P450 mediated oxidation of the benzylic carbon results in spontaneous ring opening and release of an aryl vinyl ketone byproduct and the desired NMP or phosphonate (Fig. 7). The dependence on cytochrome P450 for activation makes *in vitro* characterization of the activity of HepDirect prodrugs problematic because hepatic cell lines typically used for antiviral assays have minimal cytochrome P450 activity. HepDirect prodrugs have therefore often been applied to nucleotides with well established pharmacologic activity and the prodrugs optimized based on liver triphosphate levels in animal studies.

4.5. Less well understood activation pathways

The mechanisms of activation for many prodrug strategies have not been fully elucidated. Alternative pathways likely exist for the activation of even the relatively well characterized prodrugs discussed in Sections 4.1 through 4.4. The activation of one of the oldest kinase bypass strategies of synthesizing 3′,5′-cyclic NMPs and their alkyl triesters is not fully understood. Reddy et al. reports that preliminary work on the activation pathway of PSI-352938 suggests cleavage of the isopropyl triester followed by hydrolysis of the 3′-phosphoester to form the desired 5′-MPs (Reddy et al., 2010). While the identity of the phosphotriesterase catalyzing the proposed first step of hydrolysis has not been reported, the cleavage of 3′-phosphoester bond is likely catalyzed by a phosphodiesterase (PDE). Indeed, cyclic CDV has been reported to be

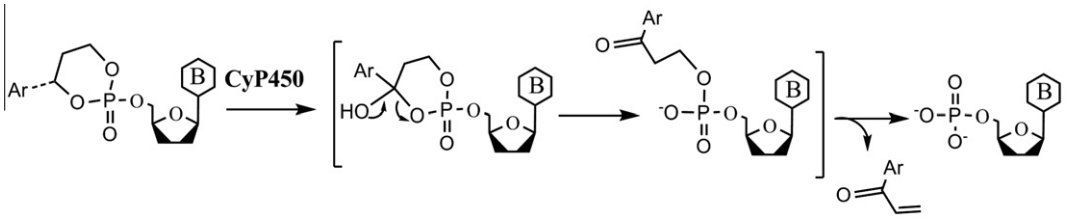


Fig. 7. Generic pathway for activation of HepDirect prodrugs.

converted to CDV by an intracellular 3′,5′-cyclic CMP PDE (Mendel et al., 1997). There is a large family of PDEs primarily responsible for the cleavage of the second messangers cAMP and 3′,5′-cyclic GMP (cGMP; reviewed by Ke and Wang (Ke and Wang, 2007)). Similarly, while a role for phospholipases is likely in the activation of many phospholipid prodrugs, the activation and degradation of these prodrugs may be catalyzed by diverse pathways. Phospholipase C has been proposed as the enzyme catalyzing the activation of lysophospholipid prodrugs like CMX001, while phospholipase D may represent a catabolic pathway for lipid prodrugs of NMPs due to its cleavage of the bond 5′ to the phosphate, causing release of the free nucleoside (lipid prodrug activation discussed further elsewhere (Hostetler, 2009)).

4.6. Potential for toxic byproduct formation

The toxicity of released prodrug moieties, in addition to the potential toxicity risks of the nucleotide analogs themselves, is an important consideration when choosing a prodrug strategy. While potentially compensated for by the reduction in dose made possible by efficient delivery, the kinase bypass prodrug strategies described in this manuscript can release various aryl groups and alcohols and some prodrugs have the potential to form reactive species. For example, the first clinically validated kinase bypass promoieties POM and POC both release formaldehyde. Many of activation pathways of prodrugs described above in Section 4 also release potentially toxic species. The putative activation pathways for SATE and HepDirect prodrugs include formation of the alkylating agents episulfide and aryl vinyl ketone, respectively. Mono-amino acid ester, mono-aryl prodrugs release an aryl group (typically phenol). Gardelli et al. was able to eliminate the potentially toxic aryl moiety from ProTides, identifying mono-acid mono-amino acid ester prodrugs of 2′-C-MeC-MP that efficiently generated high liver levels following subcutaneous administration to rodents (Gardelli et al., 2009).

5. Conclusions

The application of kinase bypass strategies to antiviral nucleosides has a long history dating back to the modulation of activity *in vitro* by adding 3′-5′-cyclic phosphate to nucleoside analogs in the 1970s to the clinical proof of concept provided by the ANPs starting with the approval of CDV in 1996. The application of bioreversible protecting groups allows for the more efficient loading of target tissues with the active TP metabolites with the goal of limiting exposure to sites of toxicity. The tissue targeted prodrugs of NMPs and ANPs currently in clinical trials hold the promise to significantly improve response rates and reduce the duration of therapy for HCV, to address safety concerns accompanying the life long therapy currently required for HBV and HIV and to allow for the broader application of nucleotide based therapies to other viruses including a variety of double stranded DNA viruses.

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