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Mini-review

Perspectives on the development of acyclic nucleotide analogs as antiviral drugs

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

The development of Viread® (tenofovir disoproxil) for HIV and Hepsera® (adefovir dipivoxil) for HBV presented many unique challenges. Unlike nucleosides and most conventional drugs, the parent acyclic nucleotide analogs are charged at physiologic pH and not suitable for oral administration which is highly desired in chronic therapies. Physicochemical properties, cellular permeation, renal toxicity, and bioavailability all had to be addressed during the development of these compounds. As a class, the acyclic nucleotides have long intracellular half-lives, allowing once-daily dosing, which provided the initial rationale for treatment of chronic viral diseases such as HIV and HBV. Prodrugs originally designed to deliver the parent acyclic nucleotide analog to the systemic circulation, also function to increase the tissue distribution and intracellular concentrations of the acyclic nucleotide diphosphate inside cells.

Keywords: Tenofovir; Adefovir; Antiviral drug; Nucleotide; Prodrug; Intracellular; Acyclic nucleotide analog

Contents

> "Nucleotide analogs will never be drugs"—anonymous quote from late 1980s.

From the perspective of physicochemical properties, plasma pharmacokinetics, oral bioavailability, potential toxicity and competition with nucleoside analogs, the author of the above quote was almost correct. The magnitude of the challenges associated with making a drug out of an acyclic nucleotide analog has caused many to pause and has delayed the development of this class of molecules. From early conception to the first marketed acyclic nucleotide analog (cidofovir, Vistide®), took over 25 years. Vistide® is administered by IV infusion and is indicated for the treatment of CMV retinitis in HIV patients. In light of these "issues", why expend resources overcoming challenges not inherent in nucleosides or other traditional drug classes. The answer is that, acyclic nucleotide analogs exhibit a unique and favorable intracellular pharmacology which can translate into significant in vivo potency, infrequent dosing and enhanced safety relative to the nucleoside analogs. Using two marketed acyclic nucleotide analogs, tenofovir disoproxil fumarate (TDF, Viread®) and adefovir dipivoxil (ADV, Hepsera®), which are administered orally for the chronic treatment of HIV and HBV,

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respectively, we will review the developmental challenges, solutions and opportunities presented by these drugs.

1. Background

It was recognized early that replacement of the labile 5′ CH_2O–P bond of the nucleoside monophosphate with a CH_2–P linkage might provide advantages with respect to metabolic activation and biochemical stability. The first syntheses of nucleotide analogs where the 5′ CH_2O–P linkage of a natural nucleotide was replaced by a 5′ CH_2CH_2–P linkage date back to the 1960s when Yengoyan and Rammler (1966), Holy (1967) and Jones and Moffatt (1968) independently reported the syntheses of the uridine and adenosine analogs. These early nucleotide analogs were used extensively in enzymatic studies, however, there were few reports of useful biological activity. Work on cyclic nucleotide analogs continues to date with activity reported against HCV (Koh et al., 2005), HIV (Cihlar et al., 2006) and multiple tumor cell lines (Jung et al., 2000). In an analogous approach, but using acyclic nucleotide analogs as a starting point, Prisbe and Martin (Prisbe et al., 1986) synthesized the phosphonic acid analog of the mono phosphate of the acyclic nucleoside analog, 9-[1,3-dihydroxy-2-propoxy)methyl]guanine (ganciclovir, Cytovene®) (Fig. 1a). The authors hypothesized that the activity of ganciclovir against CMV and EBV might be improved if the initial phosphorylation could be bypassed. The compound was weakly active against HCMV, consistent with the activity of its triphosphate analog against the HCMV DNA polymerase.

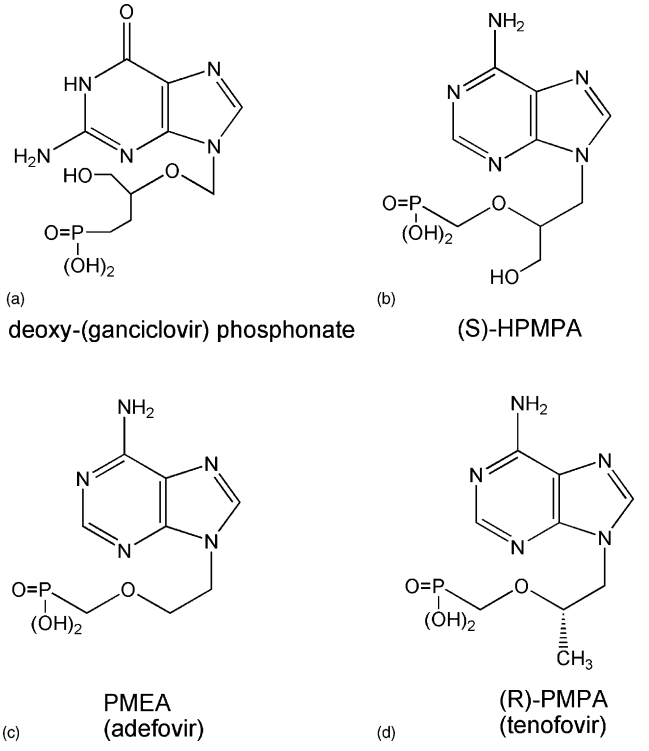

Fig. 1. (a–d) Chemical structures of the acyclic nucleotides.

In a 1986 Letter to Nature, Erik De Clercq and Antonin Holy described the broad spectrum antiviral activities of (*S*)-9-(3-hydroxy-2-phosphonomethoxy)propyladenine (HPMPA) and 9-(2-phosphonomethoxy)ethyladenine (PMEA) (Fig. 1b and c) (De Clercq et al., 1986). In both acyclic nucleotide analogs, the pseudo 5′ CH_2O–P bond of the acyclic nucleoside phosphate was replaced by the isoteric OCH_2–P linkage. The inversion of the O and C was critical for the observed biological activity. In a prescient comment, the authors wrote "acyclic adenylate derivatives in general are potent anti-retrovirus agents and should be explored further for their inhibitory effects on the AIDS virus". This work led quickly to a proliferation of related analogs and widespread antiviral screening. From these studies, two structurally related analogs, PMEA and PMPA (*R*(2-phosphonomethoxy)propyladenine (Fig. 1d) (Balzarini et al., 1993) were chosen for further development against HIV and subsequently HBV. The discovery and evolution of the acyclic nucleotide analogs has been recently reviewed by De Clercq and Holy (2005).

Although PMEA (generic name: adefovir) and PMPA (generic name: tenofovir) differ by only a methyl group, they exhibit overlapping but distinct antiviral spectrum. Adefovir is more broad spectrum, with activity against DNA and RNA viruses. Tenofovir is devoid of anti-DNA virus activity, but active against retroviruses, including HIV and SIV (De Clercq and Holy, 2005). The addition of the methyl group also differentiates the molecules with respect to their safety profile (below), intracellular half-lives, choice of prodrugs and their clinical indications.

2. Intracellular pharmacology

The antiviral activities of most nucleoside analogs are due to their metabolic activation to the triphosphate (or diphosphate in the case of the nucleotide analogs) and subsequent inhibition of viral polymerases. The eventual potency of a nucleoside is a combination of its permeability into a cell, ease of phosphorylation by viral or host polymerases, metabolic stability of the triphosphate, efflux of the nucleoside from the cell, intrinsic activity against the viral polymerase, and selectivity versus the host cell polymerases.

The original rationale for introducing a phosphonic acid into a nucleotide analog was to bypass an often sluggish initial phosphorylation by host or viral kinases. This is especially significant for acyclovir (Zovirax®) and ganciclovir resistant HSV or VZV mutants which are deficient in the viral thymidine kinase required for the initial phosphorylation (Cheng et al., 1983). Since the phosphonic acid linkage is chemically and metabolically stable, hydrolysis back to the nucleoside cannot occur as in the case of the nucleoside monophosphates. Additionally, the acyclic nucleotide analogs, adefovir and tenofovir, are not subject to deamination or deglycosylation. Subsequent phosphorylation of the acyclic nucleotide analogs to the mono- and diphosphates is readily accomplished by host cellular kinases (Robbins et al., 1995). As diphosphates, adefovir and tenofovir, are potent inhibitors of selected viral polymerases with significantly reduced activity against the host cell

Table 1
Antiviral activity of selected NRTIs in MT2 cells (HIV1 IIIb)

Inhibitor	EC_{50} (μM)	CC_{50} (μM)
Tenofovir	1.2 ± 0.3	>1000[a]
TDF	0.017 ± 0.006	50 ± 28[a]
Adefovir[b]	16 ± 6	225 ± 25[c]
ADV[b]	0.5 ± 0.2	n.d.
AZT	0.15 ± 0.4	75 ± 35[c]
FTC	0.51 ± 0.25	>1000[d]
ddI	2.6 ± 0.1	>500[c]
d4T	5.4 ± 1.8	>100[c]

[a] Lee et al. (2005).
[b] Srinivas et al. (1993).
[c] Mulato and Cherrington (1997).
[d] Gilead (unpublished).

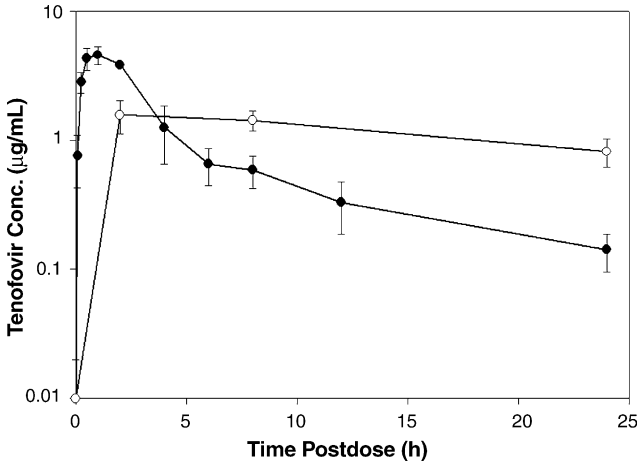

Fig. 2. Mean ± S.D. concentrations of tenofovir in dog plasma and in PBMC after a single subcutaneous dose of tenofovir at 10 mg/kg ($n = 4$) [(●) plasma; (○) PBMC].

DNA polymerases (Cherrington et al., 1995). Both molecules are efficiently incorporated into viral DNA, however, lacking a 3′ OH site, further elongation of the DNA chain is not possible.

Under the conditions of the in vitro HIV antiviral assay, the activities of the acyclic nucleotide analogs are modest compared to nucleoside analogs. Table 1 compares the activity of adefovir and tenofovir to several nucleoside analogs. The limited cellular permeability of the acyclic nucleotide analogs limits the relative amount of drug inside the cells, whereas, with nucleosides, which show greater cellular permeability, the conditions of the in vitro assay are more equivalent to a steady state. When the permeability of the adefovir and tenofovir are increased by masking the charge with a prodrug moeity, the in vitro potency increases by ∼30 to 100-fold. The reduced in vitro antiviral activity of the acyclic nucleotide analogs relative to the nucleoside analogs initially did call into question the wisdom of moving these compounds into development. What is masked by the conditions of the in vitro antiviral assay are the prolonged intracellular half-lives of the acyclic nucleotide analogs. Adefovir diphosphate has an intracellular half-life in MT-4 cells of 16–18 h (Balzarini et al., 1991). The intracellular half-life of tenofovir diphosphate is between 12 and 15 h in activated lymphocytes and ∼50 h in resting lymphocytes (Robbins et al., 1998). These long intracellular half-lives allow the acyclic nucleotide analogs, tenofovir and adefovir, to be dosed once-daily in spite of a terminal plasma half-life of 4–8 h (Deeks et al., 1998) for tenofovir and 2–4 h for adefovir (Cundy et al., 1995). In the dog there is a clear dissociation of the observable tenofovir plasma levels and intracellular tenofovir levels in PBMCs after a single subcutaneous dose of tenofovir in dogs (Fig. 2). At 24 h, total tenofovir levels in dog PBMCs are greater than 10-fold the plasma levels. Since PBMCs are a surrogate marker for lymphatic tissue, the site of HIV replication, these results have significant therapeutic implications. Foremost, plasma levels of parent acyclic nucleotide analog are not an indicator of intracellular levels and therefore do not predict efficacy. Also, the intracellular half-lives are sufficiently long such that missing an occasional dose or slightly extending the dosing interval will not likely have a significant effect on the efficacy of the drug.

3. Prodrugs

The absolute oral bioavailability of adefovir in rats (Starrett et al., 1994) (19), monkeys (Cundy et al., 1994b) and humans (Cundy et al., 1995) is low. The poor bioavailabilities observed for adefovir are due to its low passive permeability across the intestinal membrane. Similarly, the absolute bioavailabilities of oral tenofovir in preclinical studies are low. Because of their limited oral bioavailabilities, the metabolic and pharmacological advantages derived from the acyclic nucleotide analogs are of limited value in the treatment of chronic diseases such as HIV and HBV where oral therapies are highly desired. In order to realize the potential of the acyclic nucleotide analogs in these diseases, we initiated an extensive chemistry and metabolism effort around the discovery of suitable acyclic nucleotide prodrugs. Prodruging the acyclic nucleotide analogs presented multiple challenges beyond those encountered with traditional prodrug approaches. The phosphonic acid moiety on the nucleotide analog requires a double prodrug to create a neutral molecule. This requirement increases molecular weight of final compound, adds to the complexity of the synthesis and importantly, increases the difficulty of maintaining the proper balance of chemical and metabolic stability. Another important consideration in the design, is the potential for direct enzymatic hydrolysis of the prodrug at the phosphorus, potentially resulting in the irreversible phosphorylation of the catalytic residues in an enzyme active site. The initial prodrug approach with adefovir explored a series of diakyl ester prodrugs. Oral absorption in rats could be improved, however, there was limited conversion to the parent drug and the major metabolites observed in plasma were the di- and monoalkyl esters. The optimization of the bis(acyloxymethyl) ester prodrugs used previously for phosphate prodrugs led to the selection of bis(pivaloyloxymethyl) PMEA (adefovir dipivoxil) as the development candidate (Shaw et al., 1997a; Starrett et al., 1992, 1994). The pivaloyloxymethyl prodrug of adefovir provided an advantageous balance of metabolic stability and lability, enabling the parent drug to be delivered to the plasma after

oral administration. In dogs and monkeys the bioavailability of adefovir after delivery of the prodrug was approximately 30% (Cundy et al., 1994a, 1997). The metabolism of the pivaloyloxymethyl prodrugs releases 2 equivalents of pivalic acid which can form an ester bond with endogenous plasma carnitine, leading to net carnitine depletion. In the early clinical studies with adefovir dipivoxil at high doses, carnitine depletion was observed after 30 days of dosing. This has been previously observed with pivampicillin, an antibiotic that can be administered for periods up to 12 months (Holme et al., 1992). During the chronic dosing of ADV, HIV patients were supplemented with 500 mg of oral carnitine.

In an attempt to avoid the release of pivalic acid, a series of alkoxycarbonyloxy methyl prodrug esters of tenofovir were evaluated in dogs for oral bioavailability. The oral bioavailabilities ranged from 16% for the neo-pentoxy to 30% for the isopropyloxy (TDF) prodrugs (Shaw et al., 1997b). There was a positive correlation between the dog intestinal homogenate stability and the bioavailability, indicating that degradation of the prodrug in the intestinal lumen was a limiting factor for oral absorption. Interestingly, all of the alkoxy carbonyl prodrugs were rapidly degraded in liver extract (except *t*-butoxy). The in vitro plasma half-life of TDF was 20 min., suggesting that any TDF which escaped first pass metabolism in the liver would have ample time to circulate as the prodrug. Subsequently, it has been shown that the bioavailability of tenofovir disoproxil in humans was 45% (Barditch-Crovo et al., 2001).

4. Safety

Acyclic nucleotide analogs are eliminated primarily through the kidney. They are cleared through a combination of glomerular filtration and active secretion by the proximal tubular cells. In preclinical safety studies, the target organ for toxicity is typically the kidney, resulting from an accumulation of the parent nucleotide and its metabolites in the proximal tubular cells. Cidofovir, an acyclic nucleotide analog, approved for the treatment of CMV retinitis in HIV infected patients is administered intravenously with the concombinant oral administration of probenecid which blocks its uptake by the organic anion transporter, thus minimizing renal toxicity (Ho et al., 2000). The realization from preclinical studies that the kidney was the target organ for toxicity was one of the reasons that this class of molecules was not pursued aggressively in the early stages of their evaluation. Clinically, studies to evaluate the efficacy of these acyclic nucleotide analogs have included extensive monitoring of renal function. In the case of ADV, first tested clinically for the treatment of HIV, renal monitoring did not reveal any abnormalities until approximately 6 months of dosing at which time serum creatinine levels began to increase. The magnitude of the effect was dose dependent, but the time to onset was similar in all dose groups. The delayed onset was not observed in preclinical studies. As a consequence, ADV was not granted market approval for the treatment of HIV. However, at the lower dose of 10 mg, ADV proved safe and effective for the treatment of chronic HBV infection and was approved for this indication in 2002.

During the clinical development of ADV for HIV, we initiated a parallel development of TDF for HIV. Preclinical safety studies and in vitro mitochondrial function assays indicated that the safety margin for TDF was significantly better than ADV. Furthermore, the preclinical efficacy for tenofovir in the SIV infection model clearly showed that tenofovir was unique in its ability to both prevent and treat SIV in the macaque (Tsai et al., 1995; Van Rompay et al., 1999). In macaques treated subcutaneously with tenofovir either 48 h before, or 4 or 24 h after virus inoculation, infection was completely prevented. Although tenofovir differs from adefovir by only a methyl group, the lower intrinsic cytotoxicity (Table 1) leads to a substantial increase in therapeutic index of tenofovir. TDF was approved for the treatment of HIV in 2001.

5. Intracellular delivery

In a randomized double-blind placebo controlled study in patients with HIV infection, the safety, pharmacokinetics and antiviral activity of escalating daily doses of tenofovir administered by IV infusion for 7 days was examined (Deeks et al., 1998). It was shown in this study that systemically administered tenofovir produced dose related decreases in HIV RNA with a median decrease of 1.1 $\log_{10}$ copies/mL at 3 mg/kg after seven doses. This study was followed by a 28 days study in HIV patients with increasing daily oral doses of TDF (Barditch-Crovo et al., 2001). The median decrease in HIV RNA of the 7 daily doses TDF at 300 mg was 0.83 $\log_{10}$ copies/mL. The antiviral response to oral TDF was proportional to plasma tenofovir AUC. In a retrospective comparison of the individual patient data, it was apparent that for a given plasma AUC of tenofovir, the viral load drop observed after the oral administration of TDF was greater than that observed with IV tenofovir (Fig. 3). The reasons for this were not immediately obvious, since the only species observed in plasma was tenofovir. A possible explanation was that the prodrug was absorbed intact into the systemic circulation, however, it was cleared to levels below the detection limit by the first plasma sampling time point at 5 min. Circulat-

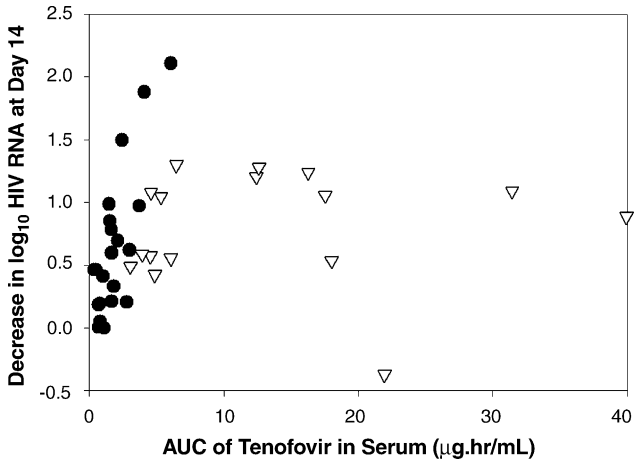

Fig. 3. Relationship between plasma exposure to tenofovir and decrease in $\log_{10}$ HIV RNA after seven daily doses of intravenous tenofovir or oral TDF in individual HIV-infected adults [(●) oral TDF; (▽) IV tenofovir].

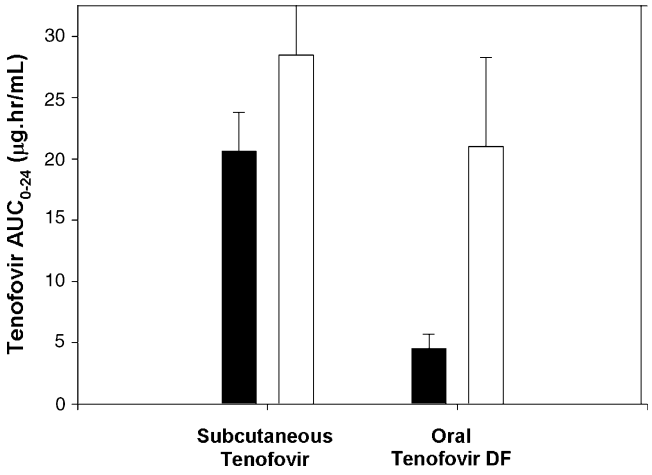

Fig. 4. Mean ± S.D. AUC values for total tenofovir in dog plasma or PBMCs after a single oral dose of subcutaneous tenofovir or oral TDF at 10 mg eq. tenofovir/kg ($n = 4$) [black—plasma; white—PBMC].

ing TDF would be expected to load cells quickly and to a greater extent than systemic tenofovir by passively diffusing into tissue where it would be rapidly metabolized to tenofovir and then to the diphosphate. We decided to test this hypothesis in dogs by measuring the amount of tenofovir delivered to PBMCs after oral TDF as compared to subcutaneous tenofovir. As seen in Fig. 4, the AUC ratio of tenofovir in PBMCs compared to plasma is 4.5 after oral delivery of TDF and it is ∼1.3 after subcutaneous delivery of tenofovir in the dog. Therefore, for the same tenofovir AUC, oral TDF will deliver more active drug to PBMCs than IV tenofovir. These data are consistent with our hypothesis of increased intracellular delivery with TDF and provide an explanation for the enhanced antiviral effect observed.

In two separate studies, in HBeAg negative (Hadziyannis et al., 2003) and HBeAg positive (Marcellin et al., 2003) chronic hepatitis B patients, the administration of 10 mg of ADV resulted in a median viral load drop of 3.91 and 3.52 logs, respectively, at 48 weeks. Systemic levels of adefovir observed after oral administration of 10 mg ADV are very low and cannot explain the potent antiviral effect observed. Furthermore, adefovir itself is cleared exclusively by the kidney and would not be expected to accumulate in the liver after systemic administration. As with TDF, it is highly likely that significant amounts of the intact prodrug, ADV, do get absorbed across the intestinal tract and are then metabolized to adefovir in the hepatocytes during first pass metabolism. ADV is rapidly metabolized to adefovir in liver homogenates (Shaw and Cundy, 1993). Radiolabelled distribution studies in dogs comparing IV administered adefovir to oral ADV demonstrate a greater liver exposure from the latter (Cundy and Shaw, 1995).

6. Summary

Gilead Sciences is the only company which currently markets acyclic nucleotide analogs. Since its launch in 2001 for the treatment of HIV, Viread has become the leading NRTI in the US. In large controlled clinical trials in naïve and treatment experienced patients, Viread, in multiple combinations with other retroviral drugs, has demonstrated a potent and durable antiviral response. The long intracellular half-life observed for tenofovir diphosphate translates into a once-a-day 300 mg dose of Viread. The combination of Viread and Emtriva, a nucleoside which also has a long intracellular half-life, into a once-daily combination tablet known as Truvada, has greatly simplified the therapeutic options available to HIV patents. Hepsera was the second drug approved for chronic HBV infection following lamivudine and is active in lamivudine resistant patients. The emergence of resistance is greatly delayed with adefovir relative to lamivudine and the signature resistant mutants are distinct.

Both Viread and Hepsera are important new drugs against life-threatening viral diseases. From the initial phases of evaluation to registration, they presented unique and difficult challenges relative to nucleosides and other conventional drug candidates.

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