Antiviral Research 85 (2010) 39–58

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine

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ARTICLE INFO

Article history:
Received 31 July 2009
Received in revised form
19 September 2009
Accepted 23 September 2009

Keywords:
NRTI
HIV reverse transcriptase
Nucleoside
Nucleotide
NRTI resistance

ABSTRACT

Twenty-five years ago, nucleoside analog 3′-azidothymidine (AZT) was shown to efficiently block the replication of HIV in cell culture. Subsequent studies demonstrated that AZT acts via the selective inhibition of HIV reverse transcriptase (RT) by its triphosphate metabolite. These discoveries have established the first class of antiretroviral agents: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs). Over the years that followed, NRTIs evolved into the main component of antiretroviral drug combinations that are now used for the treatment of all populations of HIV infected patients. A total of thirteen NRTI drug products are now available for clinical application: eight individual NRTIs, four fixed-dose combinations of two or three NRTIs, and one complete fixed-dose regimen containing two NRTIs and one non-nucleoside RT inhibitor. Multiple NRTIs or their prodrugs are in various stages of clinical development and new potent NRTIs are still being identified through drug discovery efforts. This article will review basic principles of the in vitro and in vivo pharmacology of NRTIs, discuss their clinical use including limitations associated with long-term NRTI therapy, and describe newly identified NRTIs with promising pharmacological profiles highlighting those in the development pipeline.

This article forms part of a special issue of Antiviral Research marking the 25th anniversary of antiretroviral drug discovery and development, volume 85, issue 1, 2010.

Contents

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doi:10.1016/j.antiviral.2009.09.014

1. Introduction

In 1985, two years after the identification of human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983) and one year after the initial evidence about its etiological link to AIDS was reported (Gallo et al., 1984), Mitsuya et al. (1985) in Samuel Broder's group at the National Cancer Institute together with collaborators from Burroughs-Welcome company identified 3′-azidothymidine (AZT, zidovudine) as the first nucleoside inhibitor with in vitro anti-HIV activity. As described by Samuel Broder in the introductory chapter of this issue of Antiviral Research (Broder, 2010), the discovery of the anti-HIV activity of AZT was a defining moment, providing the first proof of concept that the replication of HIV could be controlled by chemotherapy and thereby establishing the foundation of antiretroviral drug discovery research. Furman et al. (1986) from Burroughs-Welcome first showed that AZT acts through its triphosphate metabolite by inhibiting reverse transcriptase (RT), the key enzyme of HIV responsible for the synthesis of proviral DNA. Thus, AZT became the first nucleoside HIV reverse transcriptase inhibitor (NRTI). Over the course of 25 years that followed after this seminal discovery, seven nucleosides and one nucleotide have been approved by the United States Food and Drug Administration for the treatment of HIV infection starting with the approval of AZT in 1987 and followed by didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), tenofovir disoproxil fumarate [TDF; prodrug for the oral delivery of the nucleotide analog tenofovir (TFV)] and, most recently in 2003, emtricitabine (FTC). A historical perspective on the discovery and development of the first generation NRTIs that became available in clinic within few years after the approval of zidovudine and played crucial role in the management of HIV/AIDS patients especially during the first decade of antiretroviral therapy is presented elsewhere in this issue (Martin et al., 2010).

Like in the case of other antiviral therapies such as those against herpes and hepatitis B viruses, nucleoside and nucleotide analogs have become the cornerstone of successful treatment of HIV infection. The goal of this review is to summarize basic principles of the in vitro and in vivo pharmacology of NRTIs together with their current role in HIV therapy including some of the main challenges associated with their long-term clinical application. Together with profiles of inhibitors that are currently in development as well as some recently identified NRTIs and their prodrugs, this article will also discuss prospects and potential roles of NRTIs in future antiretroviral therapy.

2. Molecular pharmacology of NRTIs

NRTIs are analogs of endogenous 2′-deoxy-nucleosides and -nucleotides. They are inactive in their parent forms and require successive phosphorylation steps by host cell kinases and phosphotransferases to form deoxynucleoside triphosphate (dNTP) analogs capable of viral inhibition. In their respective triphosphate (TP) forms, NRTIs compete with their corresponding endogenous dNTPs for incorporation by HIV RT. Once incorporated, they serve as chain-terminators of viral reverse transcripts, thus, acting early in the viral replication cycle by inhibiting a critical step of proviral DNA synthesis prior to integration into the host cell genome.

2.1. Structures of the approved NRTIs

There is structural diversity in the eight NRTIs that have been approved by regulatory agencies for HIV treatment (Fig. 1) and they are metabolized to analogs of all four natural dNTPs used during DNA synthesis. All currently approved NRTIs lack a 3′-hydroxyl and are obligate chain-terminators of DNA elongation. Besides simply removing the 3′-hydroxyl, as is the case for the 2′,3′-dideoxy nucleosides ddI and ddC, AZT contains a replacement of the 3′-hydroxyl with a 3′-azido functionality. Both d4T and ABC have unsaturation introduced into their ribose moieties resulting in 2′,3′-dideoxy-2′,3′-didehydro ribose ring analogs. Abacavir also replaces O4′ with a carbon resulting in a carbocyclic ring and 3TC and FTC replace C3′ with a sulfur. The most marked ribose modifications are present in 3TC, FTC, and TFV. In addition to the oxathiolane ring, 3TC and FTC have the unnatural L-enantiomeric ribose form. TFV, the only nucleotide analog among approved NRTIs, has an acyclic linker attached to a modified phosphate moiety where a C–P phosphonate linkage replaces the normal O5′–P phosphate linkage. In contrast to the diverse chemical modifications of the ribose ring, very few base modifications are present in currently approved NRTIs. FTC contains a fluorine at the 5-position of its cytosine ring and ABC has a 6-modified diaminopurine ring that serves as a prodrug to a guanine base; all the other approved NRTIs contain unmodified purine or pyrimidine bases.

2.2. Metabolism

The absolute dependence on host cell enzymatic processes for activation is a unique element in the pharmacology of NRTIs. The metabolism of NRTIs has been reviewed in detail previously (Balzarini, 1994; Ray and Hitchcock, 2009; Stein and Moore, 2001) and therefore, general concepts will only be illustrated by select examples here. A general scheme for the reversible activation of NRTIs is presented in Fig. 2 and some of the metabolic and pharmacologic properties of NRTIs are summarized in Table 1.

2.2.1. Cellular permeation

Activation of NRTIs is first dependent on cellular entry by passive diffusion or carrier-mediated transport. Transport plays an impor-

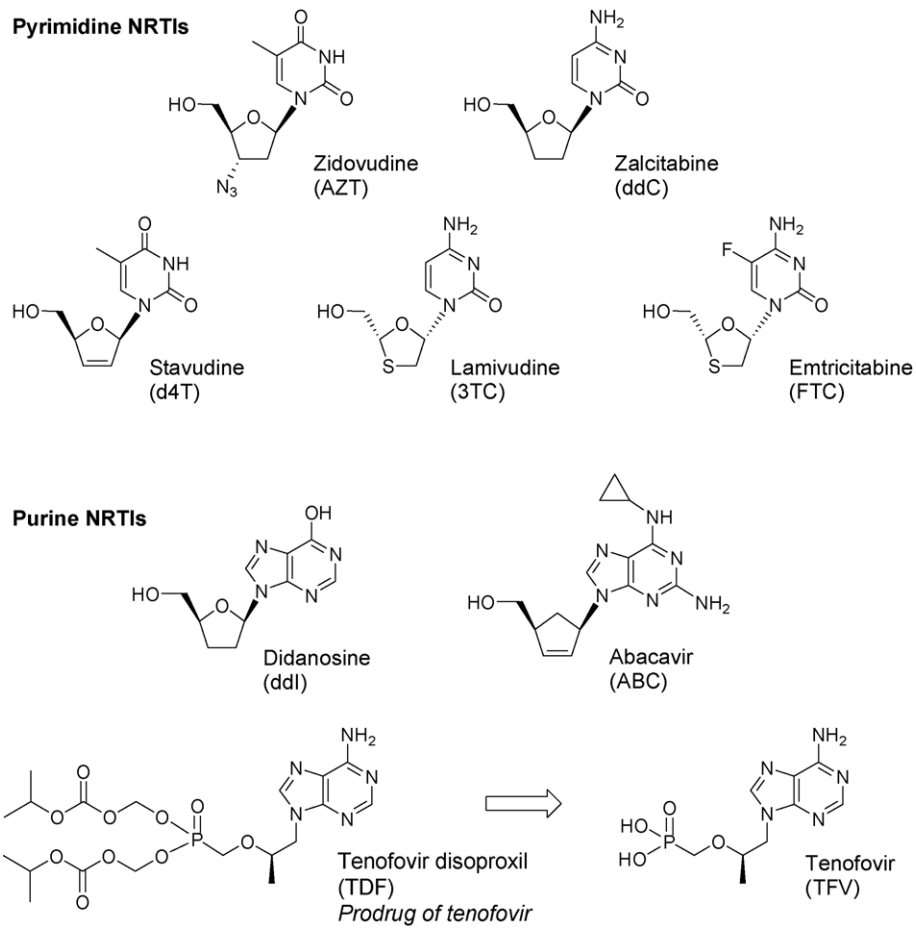

Fig. 1. Structures of approved NRTIs.

tant role in the disposition of NRTIs due to their hydrophilicity and somewhat limited membrane permeability. Multiple members of the solute carrier superfamily, containing over 350 transporters (Hediger et al., 2004), can transport NRTIs. Of particular interest for nucleoside analogs are the equilibrative and concentrative families of nucleoside transporters (Cass et al., 1999). Conversely, members of the ATP binding cassette family have been shown to efflux nucleoside and nucleotide analogs out of cells. A surprising result was obtained when it was found that the multi-drug resistance related protein 4 (MRP4) could be a resistance factor for the

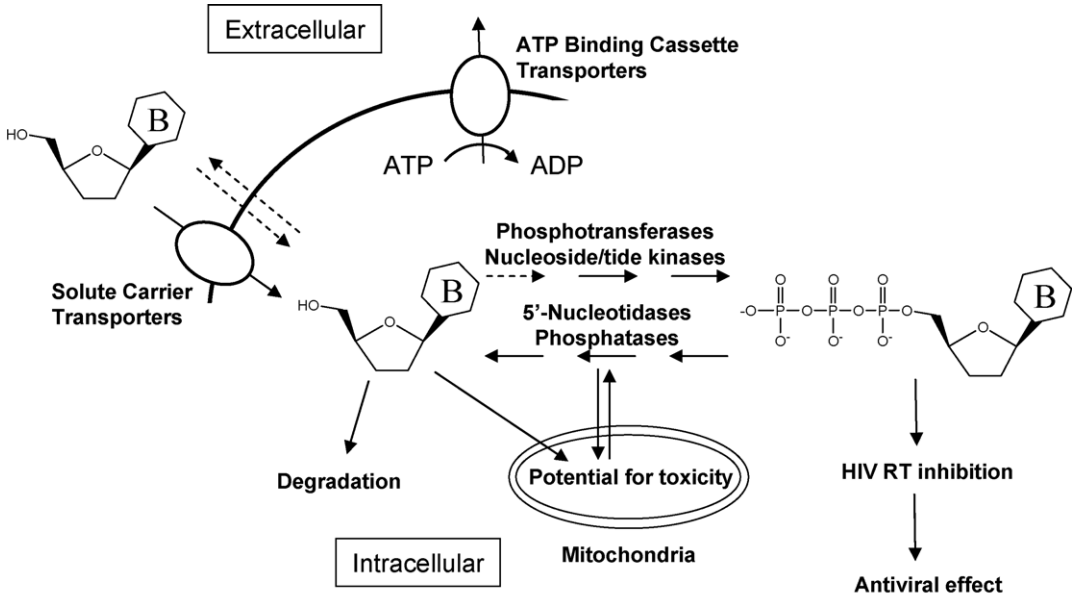

Fig. 2. General scheme for the reversible activation of NRTIs.

Table 1
Select metabolic and pharmacokinetic information for approved NRTIs.

NRTI	Nucleoside kinase or phosphotransferase	Terminal half-life (h)		Urinary recovery[a] (%)	References
		Plasma (NRTI)[a]	PBMC (dNTP analog)		
AZT	Thymidine kinase 1	0.5–3	7	14	Anderson et al. (2003), Furman et al. (1986)
ddI	IMP phosphotransferase	1.5	24	18	Becher et al. (2004), Johnson and Fridland (1989), Pruvost et al. (2005)
ddC	Deoxycytidine kinase	1–3		80	Balzarini et al. (1987)
d4T	Thymidine kinase 1	1.2	7	39	Becher et al. (2004), Ho and Hitchcock (1989)
3TC	Deoxycytidine kinase	5–7	22	71	Anderson et al. (2003), Shewach et al. (1993)
ABC	AMP phosphotransferase	1.45	12–19; 20.6	1.2	Faletto et al. (1997), Harris et al. (2002), Hawkins et al. (2005)
TFV	Not applicable	~17	>60	70–80	Hawkins et al. (2005), Pruvost et al. (2005)
FTC	Deoxycytidine kinase	~10	39	86	Shewach et al. (1993), Wang et al. (2004)

[a] Plasma terminal half-life and urinary recovery (%) taken from manufacturers' prescribing information.

acyclic dAMP analog adefovir, currently used as an anti-HBV agent (Schuetz et al., 1999). Since this initial discovery, monophosphate forms of several NRTIs have been shown to be transported by MRP4 and the related transporters MRP5 and MRP8 (Borst et al., 2007; Guo et al., 2003). Unphosphorylated nucleoside analogs have also been found to be transported by the ATP binding cassette family transporters breast cancer resistant protein and P-glycoprotein (Pan et al., 2007; Shaik et al., 2007). In addition to affecting distribution to sites of viral replication, the combined action of solute carrier-mediated influx and ATP binding cassette-mediated efflux in kidney proximal tubules can result in the elimination of NRTIs from the body. TFV has been shown to be subject to a low affinity, high capacity active tubular secretion pathway where it is taken up by the organic anion transporters 1 and 3 and effluxed into the urine by MRP4 (Cihlar et al., 2001; Imaoka et al., 2007; Ray et al., 2006).

2.2.2. Phosphorylation

Once inside of cells, the distribution of phosphorylated metabolites of NRTIs is dictated by the balance between phosphorylating and dephosphorylating enzymes. The first, and often rate limiting, phosphorylation step for nucleoside analogs are most commonly catalyzed by the four deoxynucleoside kinases involved in salvage of endogenous nucleosides, cytosolic deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1), and mitochondrial deoxyguanosine kinase and thymidine kinase 2 (Arner and Eriksson, 1995; Balzarini et al., 1987; Furman et al., 1986; Ho and Hitchcock, 1989; Johansson and Eriksson, 1996; Shewach et al., 1993). Likely reflecting the requirement of a constant supply of dNTPs to support DNA repair and mitochondrial DNA replication, many of the enzymes involved in deoxynucleoside salvage pathways are active throughout the cell cycle. One notable exception is the S-phase specific expression of TK1. The dependence of d4T and AZT on TK1 for activation results in markedly reduced anti-HIV activity in resting cells (Gao et al., 1993). TFV is an analog of dAMP and, therefore, is not dependent on nucleoside phosphorylating activity. The activity of nucleoside kinases are balanced by 5′-nucleotidases, a diverse set of enzymes that dephosphorylate NMPs (Hunsucker et al., 2005; Zimmermann, 1992). Interestingly, 5′-nucleotidases have been found to display phosphotransferase activity and can catalyze the monophosphorylation of ddI and ABC with IMP and AMP, respectively, serving as phosphate donors (Faletto et al., 1997; Johnson and Fridland, 1989). Addition of the second phosphate group to nucleoside monophosphate analogs is completed by the NMP kinases thymidylate kinase, uridylate-cytidylate kinase, adenylate kinases 1–5 and the guanylate kinase (Van Rompay et al., 2000). A variety of enzymes are able to catalyze the final phosphorylation step for NRTIs, including NDP kinase, phosphoglycerate kinase, pyruvate kinase and creatine kinase, resulting in formation of respective antivirally active triphosphate analogs (Bourdais et al., 1996; Krishnan et al., 2002). The requirement for multi-step intracellular activation processes and the generation of highly polar and impermeable triphosphate analog species, results in a large disconnect between the readily measured plasma concentrations of NRTIs and the concentrations of the active metabolites in lymphoid cells and tissues. Although correlations between the in vitro and in vivo intracellular levels of active NRTI metabolites have yet to be established, accurate determination of the intracellular pharmacokinetic profile of the active triphosphate is critical for understanding optimal dosing frequency, drug interactions, and toxicity potential of each individual NRTI (Anderson et al., 2003; Becher et al., 2004; Harris et al., 2002; Hawkins et al., 2005; Piliero, 2004; Pruvost et al., 2005; Sommadossi, 1998; Wang et al., 2004). In recent years, this type of analysis became a standard part of preclinical and clinical development of new NRTIs.

2.2.3. Catabolism

In addition to dephosphorylation, some NRTIs are subject to catabolism. While ddC, 3TC, FTC and TFV are not markedly metabolized and a majority of the oral dose is excreted unchanged in the urine, AZT, ddI, d4T and ABC are primarily excreted as metabolites (Table 1). Unlike most drugs, NRTIs do not significantly interact with cytochrome P450 enzymes. AZT is a slight exception due to the minor formation of its 3′-amine containing metabolite through the non-specific action of cytochrome P450 and their reductases (Cretton and Sommadossi, 1993). In addition to interactions with cellular nucleoside and nucleotide metabolizing enzymes that result in formation of their active triphosphates, ddI and d4T are also catabolized by cellular nucleoside degradation pathways. Both ddI and d4T have their heterocyclic bases rapidly removed by depurination and depyrimidation, respectively (Ahluwalia et al., 1987; Cretton et al., 1993). While the identity of the enzyme catalyzing a thymidine phosphorylase-like degradation of d4T has not been unambiguously determined, ddI has been shown to be degraded by purine nucleoside phosphorylase (Stoeckler et al., 1980). NRTIs can also interact with general catabolic pathways. A majority of the administered AZT dose (74%) is excreted in the urine as its 5′-O-glucuronide due to metabolism by UDP-glucuronosyltransferase (UGT) (Resetar and Spector, 1989). ABC is also metabolized by UGT forming a 5′-O-glucuronide. In addition, the 5′-OH attached to the carbocyclic ring of ABC is also recognized by alcohol dehydrogenase (ADH). Combined, the Phase I (oxidation by ADH) and Phase II (conjugation by UGT) metabolism of ABC result in only 1.2% of the intact ABC dose being recovered in urine (McDowell et al., 1999) (Table 1).

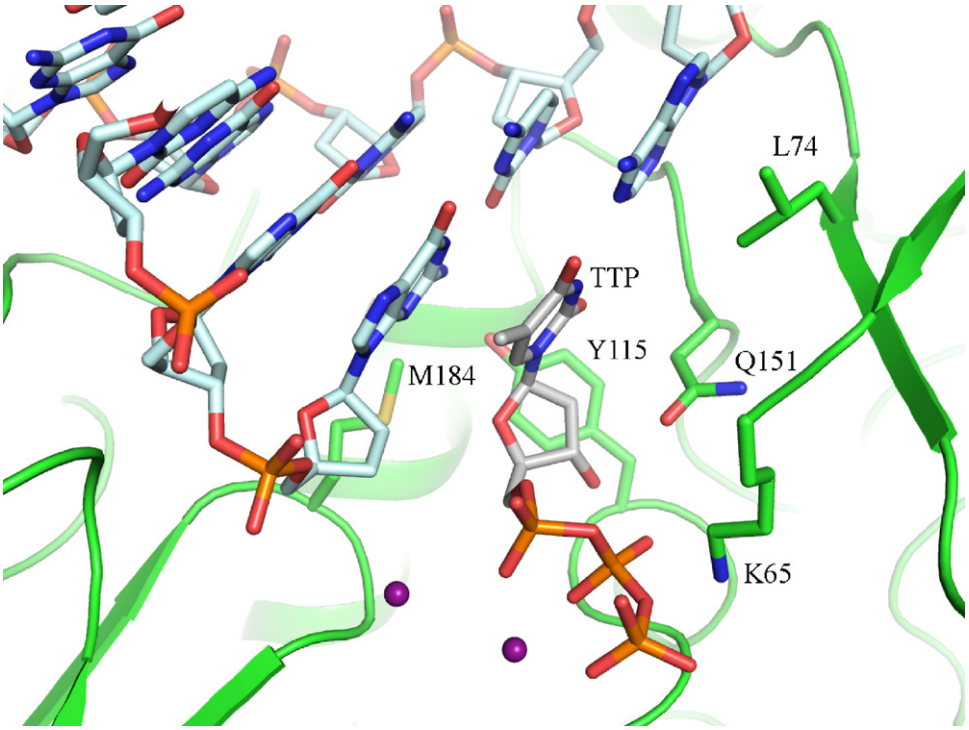

Fig. 3. Nucleotide binding in the active site of HIV RT. Residues that are in contact with the incoming dNTP and that often become mutated during drug resistance selection are labeled. Mg^{2+} ions are highlighted in purple. The figure was generated from the crystal structure of the ternary complex of RT bound to primer/templare and TTP.

2.3. Inhibition of reverse transcription

Following phosphorylation to their respective nucleoside triphosphate forms, NRTIs compete with endogenous dNTPs for incorporation by HIV RT. Active RT enzyme is a heterodimer with p51 and p66 subunits resulting from differential protease cleavage of the gag-pol polyprotein. The DNA- and RNA-dependent DNA polymerase activity is catalyzed by the p66 subunit and, coupled with RNAse H activity also residing in the p66 subunit, is responsible for the critical step of conversion of the minus stranded RNA viral genome into double stranded DNA for integration into the host cell genome. Like other polynucleotide polymerases, the overall structure of RT can best be described as a right hand with palm, fingers, and thumb subdomains (Steitz, 1999). The incoming dNTP binds between the palm and the finger subdomains and the ribose and base make important contacts with residues including L74, Y115, M184 and Q151 (Huang et al., 1998) (Fig. 3). A two-metal model has been proposed for the phosphoryl transfer reaction of polymerases including HIV RT (Steitz et al., 1994). In the RT active site, two Mg^{2+} ions are coordinated by the catalytic triad of D110, D185 and D186 that, along with residues including K65, interact with triphosphate and 3′-terminus of the primer (Huang et al., 1998). Structural studies support an induced fit model where proper base pairing by the incoming dNTP results in formation of a closed polymerase, primer/template, and dNTP complex with the incoming dNTP appropriately aligned to be attacked by the 3′-OH at the terminus of the elongating primer strand (Doublie et al., 1998; Sawaya et al., 1997). Kinetic studies showed a corresponding rate-limiting step that might reflect this conformation change (Kati et al., 1992; Reardon, 1992). The dependence of the rate-limiting step on correct dNTP binding and base-pairing forms the basis for the fidelity of polymerization (Joyce and Benkovic, 2004). Despite the structural diversity present in NRTIs used clinically, their active triphosphates are able to effectively mimic the structural contacts of natural dNTPs in the HIV RT active site, allowing for efficient incorporation (Feng and Anderson, 1999; Feng et al., 1999; Ray et al., 2002; Suo and Johnson, 1998). Perhaps best illustrating how effective NRTIs triphosphate can be at competing for incorporation by RT, d4T-TP has been found to be incorporated as efficiently as endogenous TTP in certain sequence contexts (Vaccaro et al., 2000). Once incorporated, the lack of a 3′-hydroxyl in all approved NRTIs results in obligate chain-termination of viral reverse transcripts. The structure and function of RT together with the mechanism of its inhibition by NRTIs was recently reviewed in detail (Sarafianos et al., 2009).

3. Current role of NRTIs in HIV therapy

NRTIs are the backbone of current combination antiretroviral therapy. The standard of care for HIV patients, referred to as highly active antiretroviral therapy (HAART), consists of three or more HIV drugs, most commonly two NRTIs in combination with a non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor or, most recently, integrase inhibitor. The common use of combinations of NRTIs and the potential for reduced pill burden and increased adherence has led to the clinical development of the fixed-dose combination pills AZT/3TC (Combivir), AZT/ABC/3TC (Trizivir), ABC/3TC (Epzicom), and TDF/FTC (Truvada). Recently, a one pill, once a day combination of TDF/FTC and the NNRTI efavirenz (Atripla) has been developed. A low cost generic fixed-dose combination including d4T/3TC and the NNRTI nevirapine has also been explored in the developing world.

The success of NRTIs in HIV therapy is due, at least in part, to the unique pharmacology described in the previous sections. One advantageous attribute of at least some NRTIs is the intracellular persistence. The intracellular retention of the active triphosphoraleted NRTI metabolites allows for more constant viral inhibition. For example, despite having only a 1.5 h plasma half-life, ddI is administered once a day because its active metabolite 2′,3′-dideoxyadenosine triphosphate (ddATP) has an intracellular half-life of 24 h (Table 1). The long intracellular half-lives of TFV-DP and FTC-TP have been suggested to contribute to the long-term clin-

ical efficacy of the combination of TDF, FTC, and efavirenz (Arribas et al., 2008). Since efavirenz has a very long plasma half-life that can potentially generate a "non-nucleoside tail" (Ribaudo et al., 2006; Stevens et al., 2004), its co-administration with TDF and FTC results in a combination of drugs with symmetrical pharmacokinetic profiles. Such a symmetry in combination therapy seems to be important since it should help avoid what could otherwise become essentially a monotherapy towards the end of the dosing interval, following a missed dose, or after discontinuation of treatment, potentially limiting the development of resistance, especially with drugs like efavirenz that have relatively low genetic barriers to resistance.

Based on the Adult and Adolescent Antiretroviral Treatment Guidelines updated by the Department of Health and Human Services (DHHS) in November 2008, the two preferred NRTIs for the first-line regimens are TDF and FTC (http://aidsinfo.nih.gov/contentfiles/AA_Tables.pdf). The third agent can be either efavirenz or one of the several protease inhibitors (atazanavir, darunavir, fosamprenavir, or lopinavir; all boosted with ritonavir). TDF/FTC was selected as the preferred NRTI combination primarily based on results of long-term clinical studies that have demonstrated potent and durable clinical efficacy and long-term safety and tolerability of the TDF/FTC combination when compared to other NRTI combinations (Arribas et al., 2008; Cassetti et al., 2007; Gallant et al., 2004, 2006; Smith et al., 2009). With the exception of NRTI combinations that should be avoided for various reasons, DHHS guidelines do not provide specific recommendations for the use of NRTIs in treatment-experienced patients, mainly because the status of each individual patient needs to be taken into consideration when selecting the best regimen. In these instances, the resistance profile of a particular NRTI or NRTI combination is usually weighted against potential side effects. Since M184V and TAMs are currently the most common mutations found in NRTI-experienced patients (McColl et al., 2008), drugs maintaining at least partial activity against these mutations such as ddI, TDF, and ABC are options for the second and third line treatment. FTC or 3TC are now more often retained in treatment regimens even in the presence of M184V/I mutation due to its effect on HIV fitness and pathogenicity, and the re-sensitization to some other NRTIs, e.g. TDF or AZT (Wainberg, 2004; White et al., 2002).

4. Limitations of approved NRTIs

While the unique pharmacology of NRTIs has helped them become the cornerstone of successful HAART, the effectiveness of NRTIs can be limited by drug–drug interactions, emergence of drug resistance, and adverse events. As there are more complete discussions of drug–drug interactions (Dickinson et al., 2010), antiretroviral resistance (Menendez-Arias, 2010), and adverse events of antiretroviral therapy (Hawkins, 2010) found elsewhere in this issue of Antiviral Research, we will focus on various concepts illustrating the unique molecular pharmacology of NRTIs and only discuss several specific examples.

4.1. Drug–drug interactions

4.1.1. Interactions between nucleoside analogs

NRTIs are metabolized by complex and often overlapping nucleotide metabolism pathways shared with the endogenous dNTPs they compete with for activity, resulting in the potential for intra-class pharmacokinetic and pharmacodynamic drug interactions (Ray, 2005). As thymidine analogs, AZT and d4T share common steps in their activation pathways including first and second phosphorylation steps catalyzed by TK1 and thymidylate kinase. The strong affinity of AZT and AZT-MP for these enzymes likely explains the reduced levels of d4T-TP observed when the two NRTIs are combined in vitro (Ho and Hitchcock, 1989) and their less than additive anti-HIV activity observed in a combination study in the clinic (Havlir et al., 2000). Similarly, an interaction observed in vitro between 3TC and ddC is likely related to their common dependence on dCK for activation (Veal et al., 1997). However, competition for phosphorylation, even by analogs containing the same base, does not always occur and most NRTIs are activated by lower affinity and higher capacity enzyme systems. In fact, not all nucleoside analog drug interactions are detrimental to levels of the active triphosphate. The plasma and intracellular levels of ddI in target cells can be increased by other nucleoside analogs via two very different mechanisms. A majority of the ddI dose is excreted as metabolites and the inhibition of its purine nucleoside phosphorylase-mediated catabolism has been proposed as the mechanism for increased ddI exposure when it is given with acyclic nucleoside and nucleotide analogs including tenofovir (Ray et al., 2004). In addition, as discussed in Section 2 above, the rate-limiting step in ddI activation is the initial phosphorylation catalyzed by a 5′-nucleotidase with IMP as a phosphate donor. The increase in IMP caused by inhibition of IMP dehydrogenase by ribavirin results in a marked increase in the amount of ddATP in cells (Hartman et al., 1991). While likely increasing antiviral potency by improving exposure to ddI and its active metabolites, these interactions also serve to increase ddI related adverse events (see Section 4.3 below). Therefore, the co-administration of ddI and ribavirin is contraindicated and ddI dose reduction is recommended when it is given with the acyclic nucleotide analog TDF (Videx prescribing information; http://packageinserts.bms.com/pi/pi_videx_ec.pdf). Treatment with NRTI-only therapy (triple-NRTI regimen) has been found to lead to high rates of virologic non-response, viral rebound, and resistance mutation selection (Gerstoft et al., 2003; Winston et al., 2004; Gulick et al., 2004; Gallant et al., 2005). This has led to the hypothesis that treatment failures observed with these regimens are due to negative NRTI drug–drug interactions and/or elevations in competing endogenous dNTP pools causing reduced NRTI potency. However, little evidence has been found for changes in dNTP pools in response to combinations of NRTI and lack of distribution to all sites of infection coupled with overlapping resistance profiles may be more likely explanation for these clinical findings (Vela et al., 2008).

4.1.2. Interactions with other classes of xenobiotics

The unique metabolism of NRTIs and, more specifically, the lack of a contribution of cytochrome P450 in their catabolism might lead to the belief that NRTIs would not have interactions with other classes of drugs. While drug–drug interactions have been less frequent and their magnitudes generally smaller, some surprising drug interactions related to the unique metabolism of individual NRTIs have been observed. The primary role of glucuronidation in the clearance of AZT has been shown to result in a measurable decrease in AZT exposure upon administration with the strong metabolizing enzyme inducer, including that of UGTs, rifampin (Burger et al., 1993). Given the role of glucuronidation in one of the main pathways for ABC catabolism, UGT induction may also explain the reduction in ABC levels observed when it was administered with some HIV PIs (Waters et al., 2007). The oxidative pathway for ABC catabolism mediated by alcohol dehydragenase also leads to an interesting interaction with ethanol. When ABC was administered with ethanol, a 41% increase in exposure and 26% increase in terminal half-life was observed for ABC with no change in ethanol exposure, likely reflecting saturation of alcohol dehydrogenase by ethanol (McDowell et al., 2000). Surprisingly, PIs have been observed to cause perturbations in TFV levels after co-administration with TDF. Changes in TFV exposure have ranged

between a 37% increase with atazanavir boosted with ritonavir to a 15% decrease when administered with fosamprenavir (Agarwala et al., 2005; Luber et al., 2006). These interactions have led to a great deal of scientific debate on the underlying molecular mechanism. It has been proposed that TFV increases are caused at the level of TFV renal clearance or TDF absorption resulting from HIV PI inhibition of a transporter in the kidney or small intestine, respectively (Kiser et al., 2008; Ray et al., 2008b; Tong et al., 2007). The elucidated low affinity, high capacity renal transport pathway for TFV (Ray et al., 2006) does not support a renal interaction due to the lack of overlap in transporters that TFV and PIs interact with (Cihlar et al., 2007). In contrast, TDF and HIV PIs have both been shown to interact with P-glycoprotein in the intestine, providing evidence that the intestine is likely the site of the interaction (Tong et al., 2007).

4.2. Resistance

The error-prone reverse transcription due to the lack of a proof-reading function of RT (Roberts et al., 1988) multiplied by the sheer number of replication cycles occurring in an infected individual facilitate the selection of drug resistant mutant strains of HIV (Coffin, 1995). In addition, it has been suggested that the genetic diversity of HIV including the emergence of resistance mutations may, at least under certain conditions, be promoted by the host DNA deaminase APOBEC3G that is known to induce G-to-A mutations in HIV genome (Mulder et al., 2008). Resistance selection can be further facilitated by sub-optimal drug levels in certain compartments that allow for sustained viral replication (Kepler and Perelson, 1998). Two general modes of resistance to NRTIs have been elucidated. The first mode of resistance affects the binding and rate of incorporation of the incoming nucleotide analog and primarily involves residues in direct contact with the incoming NRTI triphosphate (including K65R, L74V, Y115F, M184V/I and Q151M). The classic example is the mutation of M184V that causes steric hindrance to the proper binding of 3TC and FTC in the HIV RT active site (Sarafianos et al., 1999; Schinazi et al., 1993). The other set of mutations are in proximity to the triphosphate binding site and cause an increased rate of excision of an incorporated chain-terminating NRTI (including M41L, D67N, L210W, T215Y/F and K219Q/E/N/R) (Arion et al., 1998). While pyrophosphate is released during forward polymerization, data suggest that ATP may be the dominant species driving the reverse reaction, resulting in release of a dinucleoside tetraphosphate product (Meyer et al., 1999). Although the mutations enhancing the excision of NRTIs from terminated DNA were initially dubbed thymidine associated mutations (TAMs) due to their selection by combinations including AZT or d4T, they are truly multi-drug resistance mutations and their various combinations result in resistance to all approved NRTIs. Both the in vitro HIV resistance and the degree to which the clinical response to NRTIs is compromised usually increase with the number of TAMs present. Low number of TAMs does not appear to substantially affect susceptibility to ddI, TDF (Miller, 2004), and ABC (Lanier et al., 2003b).

The approval of potent agents coupled with factors improving adherence including reasonable tolerability, reduced pill burden, and convenient dosing schedules have allowed for the long-term suppression of HIV replication and reduced potential for the selection of drug resistance. However, drug resistance remains an important limitation considering the need for life-long antiviral therapy, especially when drugs with low genetic barrier for resistance development are part of a treatment regimen. For HIV patients initiating therapy in 1996 and viremic in 1998, it was found that greater than 70% had resistance to at least 1 NRTIs (Richman et al., 2004). While NRTIs resistance patterns changed between 1999 and 2002, these changes mostly reflected a shift in prescribing away from the use of thymidine analogs and a corresponding reduction in the prevalence of TAMs (Lanier et al., 2003a). An analysis of samples submitted for sequencing to commercial diagnostic laboratories between 2003 and 2006 found a relatively stable or, at most, slightly decreasing level of NRTI mutations (McColl et al., 2008). Reflecting the almost ubiquitous use of 3TC and FTC in combination therapy, approximately half of viremic patients harbor the M184V mutation.

Transmitted resistance can also compromise the efficacy of NRTI-containing combination therapy. The role of transmitted resistance in failure of a first-line therapy has become apparent with the growing number of patients on therapy, enhanced efficacy of current regimens, and improvement in viral sequencing techniques. Transmitted resistance to at least one antiviral drug has been established in 6–16% of patients (Ross et al., 2007) and patients with baseline resistance have been found to have a poorer response to therapy including further selection of drug resistance to other components in the regimen (Johnson et al., 2008; Little et al., 2002). In the light of these observations, treatment guidelines developed by the DHHS panel recommend genotypic analysis prior to selecting an initial treatment regimen in naïve patients (http://aidsinfo.nih.gov/contentfiles/AdultandAdolescentGL.pdf).

4.3. Adverse effects

For a class of obligate chain-terminating nucleoside and nucleotide analogs, there is a surprising diversity in the target organs and modes of toxicity for NRTIs. The mitochondria was initially implicated in NRTIs toxicity to explain general myopathy and cardiomyopathy associated with high dose AZT therapy (Dalakas et al., 1990; Lewis et al., 1991). The most dramatic illustration of the effects of nucleoside analog-induced mitochondrial dysfunction were observed in a clinical trial of the experimental anti-hepatitis B virus agent 2′-deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU). In a 15-person clinical trial, FIAU treatment was found to cause pancreatitis, neuropathy, myopathy, lactic acidosis, and hepatic failure. Even after discontinuation of the drug, of seven patients with severe hepatotoxicity, five died and two survived after liver transplants (McKenzie et al., 1995). Subsequently, inhibition of mitochondrial DNA polymerase gamma (mtDNA pol γ) has almost universally been suggested as the mechanism for all NRTI-related adverse effects (Brinkman et al., 1998). Factors contributing to the sensitivity of mitochondria to NRTIs include the presence of compartmentalized nucleoside/tide phosphorylating enzymes (see Section 2.2.2 above) and the inability fo mtDNA pol γ to discriminate between endogenous dNTPs and NRTI triphosphates (White, 2001). In addition, a low rate of removal of incorporated NRTIs from the 3′-end of terminated primer by 3′-to-5′ exonuclease activity associated with mtDNA pol γ has been suggested to play role in the mitochondrial toxicity of NRTIs (Hanes and Johnson, 2008). Transporter-mediated exchange of nucleotide analogs between the cytoplasm and mitochondria is also a factor in the mitochondrial toxicity of NRTIs (Chen and Cheng, 1992). As antiretroviral therapy has improved to reduce the morbidity and mortality associated with HIV infection, concerns about the long term effects of mitochondrial damage have arisen. Recognition of the role of the thymidine NRTIs d4T and AZT in lipoatrophy (Carr et al., 2000) has led to a shift in prescribing away from these analogs and towards NRTIs known to have less of an impact on mitochondria (primarily TDF, 3TC, FTC, and ABC). Non-cirrhotic portal hypertension has also been recently correlated with ddI use in patients on long term antiviral therapy (Maida et al., 2006).

While mtDNA pol γ inhibition is an important factor in the toxicity of some NRTIs, it is clearly not the only mediator of adverse events caused by NRTIs. While adverse events associated with AZT therapy, including myopathy, cardiomyopathy and anemia, have been attributed to mtDNA pol γ inhibition, kinetic studies have found that AZT-TP is a poor substrate for this polymerase (Johnson

et al., 2001). These findings have led to the proposal that AZT toxicity may actually be related to inhibition of thymidine phosphorylating enzymes and indirect inhibition of mtDNA replication through reduction in mitochondrial TTP pools (McKee et al., 2004). The active triphosphate analogs formed by ABC and TFV are poor substrates for mtDNA pol γ (Cihlar and Chen, 1997; Johnson et al., 2001) and do not deplete mtDNA in cell culture (Birkus et al., 2002), explaining their reduced contribution to lipodystrophy and, in part, the shift in prescribing to these NRTIs (Moyle et al., 2006).

However, adverse events have still been observed for these NRTIs that are likely caused by mechanisms other than the inhibition of mtDNA pol γ. ABC causes a hypersensitivity reaction reported in approximately 8% of patients during clinical trials (Ziagen prescribing information; http://us.gsk.com/products/assets/us_ziagen.pdf). The involvement of the major histocompatability complex class 1 human leukocyte antigen 57.1 haplotype (including HLA-B*5701) in this immune response has led to the establishment of a genetic test able to identify patients predisposed to ABC hypersensitivity (Mallal et al., 2002). A successful global clinical implementation of this test that is now being recommended by DHHS treatment guidelines has resulted in a dramatic reduction in the incidence of the hypersensitivity reaction in patients treated with ABC (Phillips and Mallal, 2009). The characteristic response to ABC in the cells from hypersensitive patients can be abrogated in situ with an alcohol dehydrogenase inhibitor (Martin et al., 2005) suggesting that reactive metabolites and resulting haptens, recognized by the immune system, play a role in initiating the hypersensitivity reaction (Martin et al., 2004). The finding in a large prospective observational cohort of HIV patients that recent (within the previous 6 months), but not cumulative, use of ABC increases the risk for myocardial infarction approximately 1.9-fold (Sabin et al., 2008) has sparked a great deal of recent debate (Brothers et al., 2009). Recent use of ddI, but not that of d4T, 3TC, or FTC, was also found to be associated with increased rates of myocardial infarction (relative risk of 1.49) (Sabin et al., 2008). Studies have reported an increase in inflammatory markers (SMART and DAD, 2008) and impaired endothelial function (Hsue et al., 2009) in patient receiving ABC, but a definitive agreement on the underlying molecular mechanism for this potential association has not yet been reached.

TDF therapy has been associated with the occurrence of renal adverse events characterized by changes in markers for the function of proximal tubules such as reduced creatinine clearance, proteinuria, glucosuria, phosphaturia, and others (Sax et al., 2007). Analysis of two randomized clinical trials in treatment naïve patients detected renal impairment in <2% patients with no discontinuation of TDF therapy after 144 weeks due to renal events (Gallant et al., 2008). In addition, analysis of a 4-year TDF expanded access program indicates that serious renal adverse events of any type was observed in 0.5% patients and elevation in serum creatinine occurred in 2.2% patients (Nelson et al., 2007). Baseline risk factors for the development of increased serum creatinine included elevated baseline serum creatinine, concomitant nephrotoxic medications, low body weight, advanced age, and lower CD4 cell count. While the molecular target for the renal toxicity of TDF is unknown, increased levels of TFV accumulation in renal proximal tubules due to influx transport via the human organic anion transporter 1 plays the major etiological role in this adverse effect (Cihlar et al., 2001).

5. NRTI development pipeline

As discussed in the above sections, most of the currently used NRTIs have some safety and/or pharmacological limitations affecting their successful long-term use for the treatment of HIV-infected patients, either in general or in certain specific populations such as individuals genetically or medically predisposed to NRTI-related adverse effects, or those with NRTI resistance. Currently, there are multiple NRTIs in various stages of clinical development (Fig. 4). As discussed in the section below, some of them exhibit various attractive pharmacological properties such as favorable resistance profile or a novel mechanism of action against RT that could make them suitable for the treatment of patient populations in need of new agents.

5.1. Apricitabine (ATC)

ATC [(−)-2′-deoxy-3′-oxa-4′-thiocytidine; AVX-754] is a deoxycytidine analog originally described in parallel with its (+)-enantiomer that is also active against HIV, but exhibits more pronounced cytotoxicity (de Muys et al., 1999). Although ATC is somewhat less potent in vitro compared to some other NRTIs, it maintains its activity against a broad spectrum of HIV-1 variants with NRTI resistance mutations (Bethell et al., 2005; Gu et al., 2006). The most significant loss of activity (>10-fold) was observed with viruses carrying the Q151M mutation complex (Gu et al., 2006; Ntemgwa et al., 2007). In contrast, viruses with combinations of TAMs with or without M184V showed only a minor, usually 2-fold or less, shift in the in vitro susceptibility to ATC (Bethell et al., 2005; de Muys et al., 1999). Minor resistance due to the K65R mutation has also been found; this is further increased in combination with M184V (Cox and Southby, 2009). In vitro, ATC has been shown to independently select for individual RT mutations K65R, V75I, or M184V (Cox and Southby, 2009). ATC exhibits a favorable in vitro toxicity profile with low-level general cytotoxicity, lack of effects on mtDNA (de Baar et al., 2007), and a low potential for myelotoxicity (Gu et al., 2006). The triphosphate of ATC is approximately 2-fold more potent inhibitor of RT than 3TC-triphosphate and does not inhibit mtDNA pol γ (de Muys et al., 1999).

ATC is currently in the final stage of clinical development for the treatment of NRTI-experienced patients. In Phase I, ATC monotherapy for 10 days once or twice daily at doses ranging from 400 to 1600 mg/day resulted in a viral load reduction of up to $-1.65\ \log_{10}$ (Cahn et al., 2006a). The drug was rapidly absorbed, generating significant levels of triphosphate in patients' PBMCs. The following Phase IIb study (AVX-201) demonstrated efficacy and safety of ATC in combination with other antiretrovirals in treatment-experienced patients with the M184V mutation. In the initial 21-day functional monotherapy phase of the study, both 600 and 800 mg twice daily resulted in a viral load reduction of approximately $-0.8\ \log_{10}$, while the comparative 3TC arm was ineffective. After 48 weeks following the addition of optimized background regimen (OBP), 85% patients treated with 800 mg ATC twice daily reached viral load of <50 c/mL (Cahn et al., 2008). Genotypic analysis at week 24 showed no emergence of new primary RT mutations; reversion of M184V back to wild-type was observed in several patients (Cox et al., 2008). An extension of the AVX-201 study to a total of 144 weeks is in progress (www.clinicaltrials.gov; NCT00367952). An ongoing Phase III study compares the efficacy of two different doses of ATC (800 and 1200 mg twice daily) with a standard dose of 3TC (150 mg twice daily) in a combination with OBR in treatment-experienced patients carrying M184V/I mutation (www.clinicaltrials.gov; NCT00612898). Based on an interim 16-week analysis, the commercial sponsor (Avexa, Ltd.) selected 800 mg twice daily as the final dose for approval of the drug (http://www.avexa.com.au).

If approved, ATC will likely be used primarily in NRTI-experienced patients including those failing prior 3TC or FTC therapy due to the M184V mutation. However, because of the current twice daily dosing, ATC may represent a less attractive option for the first-line therapy. In addition, because of negative drug–drug interactions, ATC can not be combined with other deoxycytidine analogs such as 3TC or FTC that are frequently used as a part of

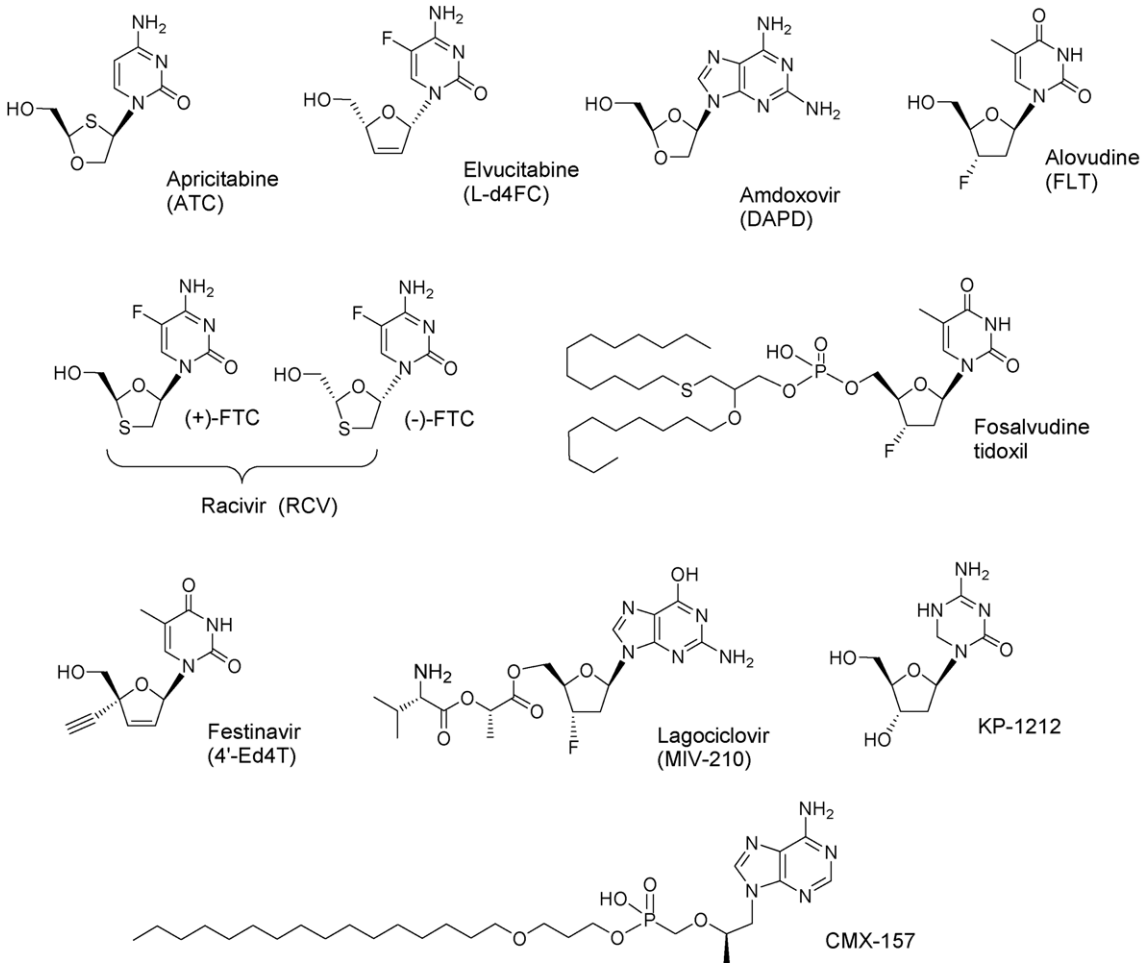

Fig. 4. Structures of NRTIs currently in clinical development.

first-line regimens (Bethell et al., 2007). A comprehensive review of pharmacology and early stage clinical development of ATC has been recently published (Cox and Southby, 2009).

5.2. Elvucitabine (L-d4FC)

L-d4FC (L-β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine; L-d4FC; ACH-126,443) is an L-nucleoside analog with more potent anti-HIV activity than 3TC, in part because of high intracellular levels of its triphosphate metabolite (Dutschman et al., 1998). Although L-d4FC shows in vitro cytotoxicity against some cell types, it does not negatively affect the replication of mtDNA (Lin et al., 1996) due to a reduced affinity of its triphosphate for mtDNA pol γ relative to the corresponding D-form (Murakami et al., 2004). L-d4FC is also claimed to have a protective effect against mitochondrial toxicity of other NRTIs (Dutschman et al., 1998). In vitro, L-d4FC selects for HIV-1 variants with M184I/V mutation in RT and with >20-fold reduced susceptibility (Hammond et al., 2005). In addition, K65R mutation has been shown to reduce the susceptibility to L-d4FC (Parikh et al., 2005).

Achillion Pharmaceuticals, Inc. initiated the development of L-d4FC in 2000. A Phase I study in patients with M184V mutation demonstrated that the replacement of 3TC with L-d4FC at 50 or 100 mg once-daily resulted in a $-0.7\log_{10}$ reduction in plasma viral load after 4 weeks of treatment (Dunkle et al., 2003). However, because of significant hematological toxicity, manifested as reversible leucopenia and neutropenia, the development of L-d4FC continued at lower doses. Subsequent 7-day monotherapy at 10 mg once-daily demonstrated the efficacy of L-d4FC in treatment naïve patients, producing the viral load reduction of $-0.85\log_{10}$ (Colucci et al., 2006). A larger Phase II trial (ACH-015) in treatment-naïve subjects compared 10 mg L-d4FC to 300 mg 3TC, both once-daily, in combination with TDF and efavirenz. After 48 weeks, similar efficacy was observed in both treatment arms, but there was a trend towards a lower recovery of CD4+ cells observed in patients treated with L-d4FC (DeJesus et al., 2008). Despite recently published data from a multidose pharmacokinetic analysis demonstrating a prolonged terminal half-life of L-d4FC in plasma ($T_{1/2}$ > 100 h) (Colucci et al., 2009), it is not yet clear how L-d4FC, if approved, would be used in the clinic since once-daily administration does not appear to show better efficacy than the currently well established regimens for treatment-naïve patients containing FTC or 3TC and the drug resistance profile combined together with the reduced dose of L-d4FC may limit its efficacy in NRTI-experienced patients.

5.3. Amdoxovir (DAPD)

DAPD (1-β-D-2,6-diaminopurine dioxolane) acts as a water-soluble prodrug that is converted to the antivirally active nucleoside 1-β-D-dioxolane guanosine (DXG) via deamination by adenosine deaminase (Furman et al., 2001). In cells, DXG is phosphorylated through the action of various kinases and phosphotransferases including 5′-nucleotidase, GMP kinase, creatine kinase, and NDP kinase (Feng et al., 2004b). DXG triphosphate is an efficient substrate for RT and, despite showing a similar K_i/K_m ratio for both RT and mtDNA pol γ (Furman et al., 2001), concen-

trations up to 50 μM DXG do not appear to affect mtDNA. On the other hand, DAPD at the same concentrations reduced the levels of mtDNA by >60% (Furman et al., 2001). However, this effect may not be clinically relevant because of the rapid deamination of DAPD to DXG in vivo (Furman et al., 2001). HIV variants with multiple TAMs or M184V show minimal resistance to DXG (Gu et al., 1999; Mewshaw et al., 2002). On the other hand, HIV strains expressing K65R, L74V, or Q151M are 4- to >10-fold resistant to DXG (Bazmi et al., 2000; Gu et al., 1999; Mewshaw et al., 2002; White et al., 2002). In vitro, DXG selected for approximately 10-fold resistance due to the emergence of K65R mutation in RT (Bazmi et al., 2000).

The clinical development of DAPD was initiated by Triangle Pharmaceuticals, Inc. in late 1990s after its licensing from Emory University and University of Georgia Foundation. Phase I study evaluated 14-day DAPD monotherapy once or twice daily in treatment-naive patients (Thompson et al., 2005). A parallel study was conducted in treatment-experienced patients with DAPD twice daily added to existing therapy (Thompson et al., 2005). DAPD at 500 mg twice daily dose reduced the plasma viral load by −1.3 and −0.66 $\log_{10}$ among the treatment-naïve and experienced patients, respectively. Following the merger of Triangle Pharmaceuticals with Gilead Sciences in 2002, the clinical development of DAPD continued under the sponsorship by RFS Pharma, LLC. The drug was evaluated in various combinations including those with mycofenolate mofetil (Margolis et al., 2007) and enfuvirtide (Gripshover et al., 2006) in highly treatment-experienced patients, and showed a measurable clinical effect in the former, but not the latter study. More recently, RFS Pharma conducted a 10-day study with a DAPD + AZT combination in subjects not receiving therapy (Murphy et al., 2008). The combination was significantly more potent than either of the two drugs alone. The highest viral load reduction (−1.97 $\log_{10}$) was observed in patients treated with 500 mg DAPD + 200 mg AZT. Nausea and headache were the most frequent effects in the two-drug arms occurring in 50% subjects. No serious adverse events were identified. Based on this data, authors of the study proposed further development of the fixed-dose combination of DAPD and AZT as a second-line NRTI therapy (Murphy et al., 2008).

5.4. Racivir (RCV)

RCV [(±)-β-2′,3′-dideoxy-3′-thia-5-fluorocytosine; (±)-FTC] is a 1:1 racemic mixture of (−)-FTC and (+)-FTC. Despite observations from pre-steady state enzyme kinetics that the triphosphates of both FTC enantiomers are equally efficient substrates for RT (Feng et al., 1999), (+)-FTC is a substantially less potent inhibitor of HIV replication than (−)-FTC in cell culture (Schinazi et al., 1992), in part due to the less efficient phosphorylation (Shewach et al., 1993). Unlike (−)-FTC, that selects for the M184V resistance mutation in RT, exposure to the (+)-enantiomer leads to emergence of the T215Y mutation (Schinazi et al., 1997). Due to the orthogonal resistance profile of the two enantiomers, the emergence of in vitro resistance against the racemic mixture might be slower compared to the resistance selection with each individual isomer (Herzmann et al., 2005) and references therein). This feature together with the lower cost of manufacturing the racemate are the major rationales justifying the development of RCV. The drug is currently being developed by Pharmasset, Inc. for the treatment of HIV and HBV infection.

In Phase Ib/IIa study in treatment-naïve patients, a once-daily monotherapy of RCV was administered for 14 days at doses of 200–600 mg in combination with d4T and efavirenz. Viral load suppression was observed in all dosage groups, with mean reductions reaching up to −2.43 $\log_{10}$ (Herzmann et al., 2005). The drug appeared well tolerated with dose proportional pharmacokinetic parameters and the maximum plasma exposure exceeding the EC_{90} values for in vitro activity against the wild-type HIV-1 (with the caveat that the ratio of the two enantiomers in plasma has not been determined). Subsequent Phase IIb study assessed RCV at 600 mg once-daily in treatment-experiences patients on 3TC therapy with M184V mutation who either switched to RCV or continued with 3TC. After 28 days, the mean viral load change was −0.4 and +0.13 $\log_{10}$ in the RCV and 3TC arms, respectively. In a subset of RCV-treated patients with less than 3 TAMs, the mean viral load reduction was −0.7 $\log_{10}$ (Cahn et al., 2007b).

Advantages of RCV include once-daily dosing, the orthogonal resistance profile of the two enantiomers, and lower cost of production compared to individual enantiomers, which would be an important factor for its use namely in resource-limiting settings. RCV can be considered a fixed-dose combination of two separate NRTIs, one of them being emtricitabine ((−)-FTC). This raises a question whether RCV could provide any benefit compared to the fixed-dose combination of TDF and emtricitabine (Truvada). Similar to (+)-FTC, TDF also exhibits an orthogonal resistance profile to (−)-FTC. However, the long-term safety profile of RCV still remains to be fully explored especially since (+)-FTC is a D-nucleoside and although no effects on mtDNA have been observed at low concentrations (Cui et al., 1996), the discrimination of natural substrate over (+)-FTC-triphosphate by mtDNA pol γ is approximately 36-fold (Feng et al., 2004a), whereas that of tenofovir-diphosphate is >11,000-fold (Johnson et al., 2001).

5.5. Alovudine (FLT) and fosalvudine tidoxil (HDP 99-0003)

FLT (3′-deoxy-3′-fluorothymidine; formerly MIV-310) inhibits HIV with a potency similar to that of AZT (Kim et al., 2001; Kong et al., 1992). However, unlike AZT and some other NRTIs, FLT retains its activity against multiple NRTI-resistant strains of HIV including those with TAMs (Kim et al., 2001). FLT triphosphate is an efficient substrate and inhibitor of RT, but at the same time interferes with the activity of mtDNA pol γ (Martin et al., 1994), resulting in potent depletion of mtDNA in treated cells (de Baar et al., 2007; Faraj et al., 1996). In addition, FLT showed in vitro hematopoietic toxicity (Dornsife and Averett, 1996; Faraj et al., 1996) and unacceptable levels of hematologic toxicity occurred in a concentration-controlled clinical trial (Flexner et al., 1994) that led to a decision by the commercial sponsor, American Cyanamid, to terminate further development. Subsequent clinical activities were conducted by Medivir AB at reduced doses to avoid adverse effects. In Phase II pilot study, 7.5 mg once daily was given as add-on therapy to patients with multiple TAMs for 4 weeks, resulting in a median viral load reduction of −1.13 $\log_{10}$. Notably, d4T in background therapy substantially reduced the clinical response (−0.57 $\log_{10}$ and −1.88 $\log_{10}$ for patients with and without d4T, respectively) (Katlama et al., 2004). Subsequent study with doses of FLT further reduced to 2 mg once daily for 4 weeks produced only modest antiviral responses in treatment-experienced patients when added to the existing therapy (Ghosn et al., 2007). Currently, FLT is being developed by Beijing Mefuvir Medicinal Technology that licensed the compound from Medivir AB.

Because of the dose-limiting side effects of FLT, Heidelberg Pharma AG designed a phospholipid-based prodrug of FLT (fosalvudine tidoxil) with a goal to reduce toxicity by altering the drug pharmacokinetics and tissue distribution. Rapid generation of phosphorylated FLT metabolites and potent antiretroviral activity in cells treated with fosalvudine tidoxil have been demonstrated in vitro (Reuss et al., 2006). In Phase I study, a single oral administration of fosalvudine tidoxil at doses ranging from 5 to 40 mg was well tolerated (Cahn et al., 2006b). A short systemic half-life was observed for the prodrug, presumably due to its rapid distribution into tissues, but the levels of parent nucleoside, FLT, have not been determined in the study. In a subsequent Phase II dose-finding study, treatment-naïve patients were administered

fosalvudine tidoxil for 14 days (Cahn et al., 2007a). Doses ranging from 5 to 40 mg resulted in the plasma viral load reduction of −0.43 to −1.00 $\log_{10}$. No dose dependent adverse events were observed.

Despite an attractive resistance profile and considerable clinical potency of FLT in short-term trials, including those conducted in treatment experienced patients with multiple NRTI mutations, the long-term safety including the potential for mitochondrial toxicity remains a concern both for FLT and its lipid prodrug (Venhoff et al., 2009).

5.6. Festinavir (4′-Ed4T)

4′-Ed4T (2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine; OBP-601) is a d4T analog with a 4′-ethynyl substitution (Haraguchi et al., 2003) that shows 5–10-fold fold improved potency (Nitanda et al., 2005) and a reduced in vitro toxicity, including less effects on mtDNA compared to d4T (Dutschman et al., 2004). The active metabolite 4′-Ed4T triphosphate has longer in vitro intracellular retention than the triphosphates of AZT and d4T (Paintsil et al., 2009a; Wang et al., 2009) and consequently, 4′-Ed4T showed more persistent anti-HIV activity after drug removal than the other thymidine analogs (Paintsil et al., 2007, 2009a). The prolonged intracellular half-life of 4′-Ed4T metabolites appears to be in part due to reduced catabolism by thymidine phosphorylase (Dutschman et al., 2004) and in part due to limited cellular efflux (Wang et al., 2009). Computational analysis suggests that the 4′-ethynyl group provides additional binding energy through its interaction with a hydrophobic pocket in the active site of RT (amino acids A114, Y115, M184, F160). This interaction increases the affinity of 4′-Ed4T-triphosphate to RT approximately 5-fold compared to d4T-triphosphate (Yang et al., 2007) and at the same time reduces the interactions with mtDNA pol γ (Yang et al., 2007).

4′-Ed4T shows approximately 10-fold reduced activity in the presence of multiple TAMs or M184V (Nitanda et al., 2005). In contrast, K65R and Q151M multidrug complex did not affect the potency of 4′-Ed4T against HIV (Nitanda et al., 2005). In fact, hypersensitivity of Q151M-containing HIV strains to 4′-Ed4T has been demonstrated (Weber et al., 2008). Resistance selection experiments with 4′-Ed4T resulted in the emergence of P119S, T165A, and M184V mutations in RT and >100-fold reduced susceptibility to the drug together with a cross-resistance to 3TC (Nitanda et al., 2005).

4′-Ed4T is currently being developed by a Japanese sponsor Oncolys BioPharma under a license from Yale University. In April 2008, Investigational New Drug (IND) application for 4′-Ed4T was approved by FDA. Phase Ia study assessed the safety and pharmacokinetics of a single oral dose ranging from 10 to 900 mg. Results indicated a linear dose-proportionality in the pharmacokinetics of 4′-Ed4T with no food effects on plasma levels (Paintsil et al., 2009b). A 10-day efficacy and safety study in HIV infected patients is being currently conducted (www.oncolys.com/en).

5.7. Lagociclovir (MIV-210)

MIV-210 is a valyloxy-propionyl ester prodrug of 3′-deoxy-3′-fluoroguanosine (FLG). In comparison with FLT, another 3′-fluoro-dideoxynucleoside, FLG shows somewhat less potent anti-HIV activity with EC_{50} values in low μM range (Herdewijn et al., 1988; Zhang et al., 2002). However, FLG also shows a favorable in vitro resistance profile including maintained activity against HIV strains with multiple TAMs, Q151M, or T69S insertion in RT (Zhang et al., 2002). Selection of resistance in the presence of FLG resulted in the emergence of the M184V mutation (Harmenberg et al., 1998). Unlike FLT, FLG is also active against HBV (Schroder et al., 1998). Recently, potent efficacy of MIV-210 against chronic hepatitis virus infection in a woodchuck model has been reported (Michalak et al., 2009). The availability of additional information on the pharmacology of FLG or MIV-210, including their toxicity profiles, is somewhat limited.

Medivir AB initiated the clinical development of MIV-210 in 2001 and presented data from the Phase I study in healthy volunteers in 2002 (Harmenberg et al., 2002). The study evaluated a single oral administration at doses of MIV-210 ranging from 25 to 1500 mg. Peak plasma level of FLG reached approximately 5000 ng/mL (18 μM) at the highest dose, with the oral bioavailability reaching 50%. Subsequent development progressed rather slowly with multiple partners being involved in the program including GlaxoSmithKline and Tibotec (Medivir AB Press Releases from 2004 to 2007; http://www.medivir.se). In 2007, Medivir outlicensed MIV-210 to a Chinese partner company Hainan Noken Pharmaceutical to develop the drug for the treatment of both HIV and HBV infection (Medivir Press Release, September 4, 2007; http://www.medivir.se).

5.8. KP-1212 and KP-1461

KP-1212 (5-aza-5,6-dihydro-2′-deoxycytidine) is a deoxycytidine analog with a modified base and a 3′-hydroxyl that allows for its non-chain terminating DNA incorporation by RT. Since the modified base of KP-1212 can efficiently base-pair with both G and A, its presence in DNA induces mutations in viral genes, resulting in the inhibition of HIV replication. This effect is, in principle, analogous to that of cellular cytidine deaminase APOBEC3G (Soros and Greene, 2007), except that it is chemically induced. Serial passaging of HIV in the presence of KP-1212 substantially increased mutational rate of the virus (Harris et al., 2005). While this mutagenic effect results in the clearance of virus in vitro (Harris et al., 2005), it is unlikely to lead to the elimination of latent HIV reservoirs in patients (Smith et al., 2005). Although in vitro data are encouraging in that high concentrations of KP-1212 did not exhibit short-term host genotoxicity (Harris et al., 2005), longer-term studies using more sensitive methods as well as rigorous assessment of potential incorporation by host replicative DNA polymerases should still be performed. In addition, mtDNA pol γ has been shown to efficiently interact with KP-1212 triphosphate, potentially leading to long-term mitochondrial toxicity (Murakami et al., 2005). Thus far, this concern has not been confirmed in initial in vitro mitochondrial toxicity studies in T-lymphoid cells (Harris et al., 2005).

KP-1461, an orally available prodrug of KP-1212 is currently being developed by Koronis Pharmaceuticals, Inc. According to a statement from the commercial sponsor, Phase Ib study in treatment-experienced patients who received escalating doses of KP-1461 demonstrated a statistically significant decrease in plasma viral load (http://www.koronispharma.com/KP1461forHIV). Subsequently, Phase IIa open-label trial has been initiated to examine 1600 mg of KP-1461 twice daily for up to 124 days, but the study was suspended by the sponsor to examine interim clinical results.

5.9. CMX-157

Nucleoside phosphonates are suitable for coupling with long aliphatic side chain to generate prodrugs mimicking natural phospholipids (Hostetler, 2009). CMX-157 is a lysolecithin-derived prodrug of TFV (hexadecyloxypropyl-TFV) with potent in vitro activity against both HIV and HBV (Painter et al., 2007). The potency of CMX-157 against HIV is substantially enhanced compared to parental TFV, reaching EC_{50} values of <1 nM (Painter et al., 2007). This potency may in part be due to a proposed binding of CMX-157 to cell-free virions through a direct insertion into viral envelope, resulting in subsequent facilitated delivery of TFV into infected cells (Lanier et al., 2009a). CMX157 has shown good oral bioavailability (Painter et al., 2007) and doses up to 200 mg/kg were well toler-

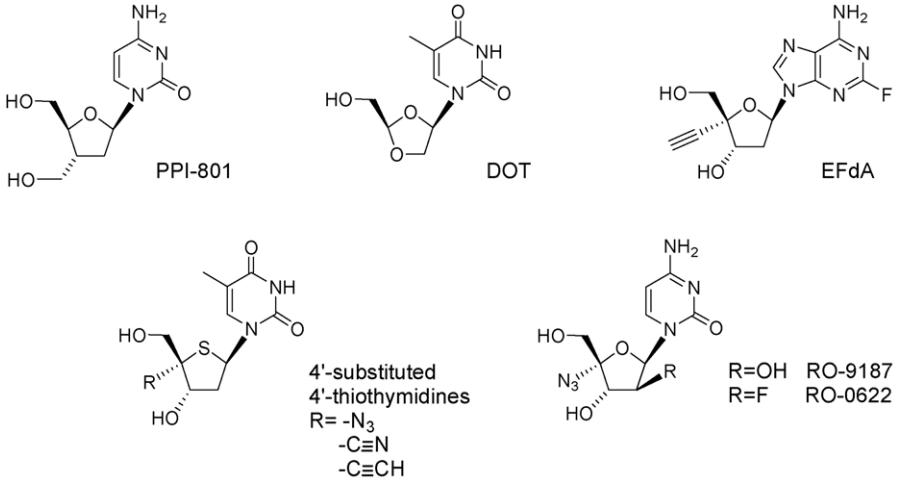

Fig. 5. Structures of novel nucleoside analogs.

ated in 28-day toxicology studies in rats and monkeys with the dose-limiting toxicity being of gastric nature (Lanier et al., 2009b). Chimerix, Inc., the commercial sponsor developing CMX-157, has filed an IND application for CMX-157 development in April 2009 (Lanier et al., 2009b).

6. Novel NRTIs and their profiles

Although the total number of approved NRTI-based drug products together with compounds currently in clinical development exceeds twenty, the design and profiling of new NRTIs remains an active area of research, yielding a wide variety of novel HIV inhibitors with interesting profiles. In this section, three examples of structurally diverse classes of nucleosides will be reviewed (Fig. 5) followed by an update on the design of novel nucleoside phosphonates and their prodrugs (Fig. 6), all with distinct features that make at least some of them attractive development candidates.

6.1. PPI-801 (MIV-410) and PPI-802

PPI-802 is a prodrug of 2′,3′-dideoxy-3′C-hydroxymethyl cytidine (PPI-801; MIV-410). This deoxycytidine analog contains a 3′-hydroxymethyl substitution that allows for a unique mechanism of action against RT. The triphosphate of PPI-801 is a substrate for RT, but unlike other NRTIs does not act as an immediate chain terminator. Instead, the 3′-hydroxymethyl substitution allows for the incorporation of one additional dNTP and only after this step a delayed DNA termination at +1 position occurs (Zhang et al., 2007). Importantly, this penultimate DNA termination reduces the accessibility of incorporated PPI-801 for excision by RT. Since the enhanced efficiency of 3′-end primer excision is the primary mechanism of resistance mediated by TAMs, the penultimate termination may favorably affect the activity of PPI-801 against viruses harboring these mutations. PPI-801 has shown potent in vivo activity in SIV-infected macaques (Bottiger and Oberg, 2000; Zhang et al., 2007). Recently, Medivir AB out-licensed PPI-801 to Presidio Pharmaceuticals, Inc. that plans to develop its oral prodrug PPI-802 (http://www.presidiopharma.com/hiv).

6.2. Dioxolane NRTIs

After obtaining the clinical proof-of-concept for dioxolane purine NRTIs in studies with DAPD, additional nucleosides from this class were explored including pyrimidine-containing dioxolanes such as β-D-dioxolane thymidine (DOT) and β-D-dioxolane 5-fluoro-cytidine (FDOC), both potent inhibitors of HIV-1 with in vitro

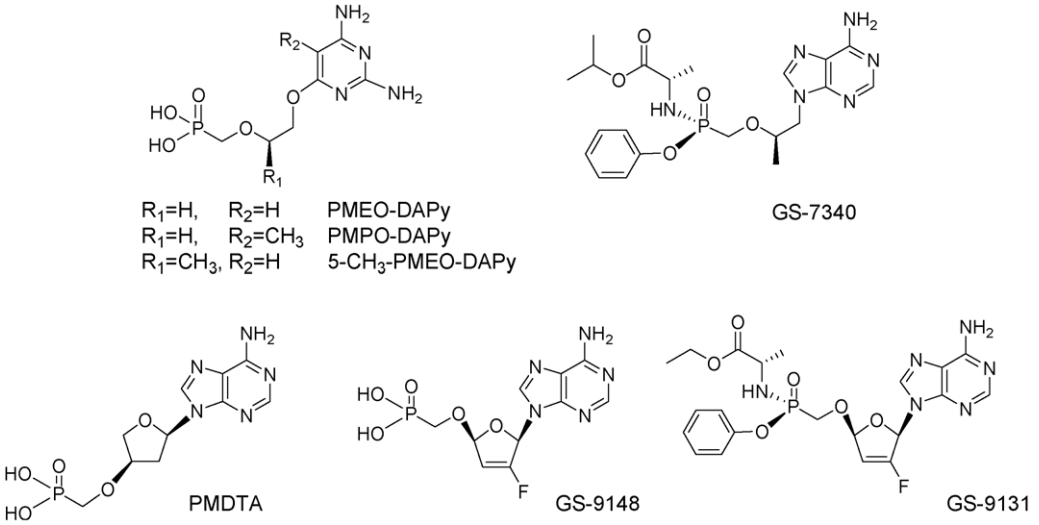

Fig. 6. Structures of novel nucleoside phosphonates and their prodrugs.

activity at sub-μM concentrations (Chu et al., 2005). In the initial profiling, FDOC induced profound effects on mtDNA levels in Hep2 cells and therefore was not considered for further development (Cui et al., 1996). While the data on the potential of DOT to cause mitochondrial toxicity has yet to be published, DOT represents the first thymidine analog significantly active against viruses with multiple TAMs (Chu et al., 2005). In addition, DOT retains its full potency against viruses containing other NRTI resistance mutations such as K65R, M184V, or L74V (Chu et al., 2005). These data were in part confirmed in the inhibition studies with DOT-triphosphate and various RT mutants (Lennerstrand et al., 2007). The compound is currently in preclinical development by RFS Pharma and the sponsor has recently conducted pharmacokinetic studies in rhesus monkeys that demonstrated good CNS permeability and identified a glucuronidation-dependent metabolism of the compound (Asif et al., 2007). Over the recent years, several types of DOT prodrugs have been explored including phosphoramidates (Liang et al., 2006) and 5′-esters (Liang et al., 2009). Some of these prodrugs exhibited up to 10-fold improved in vitro antiviral activity compared to the parental nucleoside (Liang et al., 2006).

6.3. 4′-Substituted NRTIs

Maag et al. (1992) described 4′-azido-thymidine, the first 4′-substituted deoxynucleoside with potent anti-HIV activity. The compound was shown to have better antiviral potency than AZT, but also higher cytotoxicity. Notably, unlike in the case of clinically used NRTIs, the presence of 3′-hydroxyl turned out to be critical for maintaining the activity of 4′-substituted deoxynucleosides. Among a wide range of subsequently synthesized 4′-substituted NRTIs (reviewed in Hayakawa et al., 2004), 2′-deoxy-4′-C-ethynyl-2-fluoroadenosine (EFdA; Fig. 5) stands out as one of the most potent NRTIs ever identified (Nakata et al., 2007). EFdA is up to 100-fold more potent than AZT, reaching even better in vitro activity than some HIV protease inhibitors (Nakata et al., 2007). Despite the presence of 3′-hydroxyl, EFdA triphosphate acts as a chain terminator upon its incorporation into DNA by RT. This chain termination arises from a difficulty of DNA to translocate in the RT active site following the compounds incorporation at the 3′-end of primer (Marchand et al., 2008). EFdA triphosphate has longer persistence in cells than AZT triphosphate, translating into a prolonged protection of pretreated cells against HIV infection (Nakata et al., 2007). One potential limitation may be the reported inhibitory effect of EFdA triphosphate towards the mtDNA pol γ (Nakata et al., 2007). EFdA retains its potency against a wide range of NRTI-resistant mutant HIV strains including those with TAMs or the Q151M complex (Kawamoto et al., 2008). Viruses with the M184V mutation show moderate resistance to EFdA (Kawamoto et al., 2008). In vitro, EFdA selects for an HIV variant with I142V, T165R, and M184V mutations (Kawamoto et al., 2008). Recently, EFdA has shown in vivo activity against HIV-1 infection in a NOD/SCID mouse model (Hattori et al., 2009).

Exploration of novel 4′-substituted 4′-thiothymidines containing sulfur atom in the 4′-substituted sugar ring yielded potent inhibitors of HIV with EC_{50} values ranging from 20 to 300 nM (Haraguchi et al., 2008). In this series, 4′-azido- and 4′-cyano-thionucleosides were approximately 10-fold more potent than the 4′-ethynyl-containing compounds. The authors noted that unlike most of the other 4′-substituted NRTIs, the thiothymidine series appeared to maintain potent activity against viruses with the M184V mutation (Haraguchi et al., 2008).

Recent reports by Klumpp et al. (2008, 2009) described novel 4′-azido-2′-deoxycytidine analogs with a dual antiviral activity against both HIV and HCV. In this class, 2′-deoxy-2′-β-fluoro-4′-azidocytidine (RO-0622) and 2′-deoxy-2′-β-hydroxy-4′-azidocytidine (RO-9187) that have been originally explored for their potent inhibitory effects in replicons derived from multiple HCV genotypes also exhibited excellent potency against HIV. In particular, RO-0622 inhibited the replication of HIV with EC_{50} value of <1 nM, an activity approximately 6000-fold better than that of 3TC (Klumpp et al., 2009). Triphosphates of RO-9187 and RO-0622 inhibited both HIV RT and HCV RNA polymerase, and exhibited high selectivity against host DNA polymerases (Klumpp et al., 2009).

6.4. Nucleoside phosphonates and their prodrugs

Following the successful introduction of TDF into clinical practice, the focused search for novel types of nucleoside phosphonates has continued, yielding a number of new molecules including a series containing 6-substituted 2,4-diaminopyrimidine (DAPy) base that mimics the structure of the purine bases (Balzarini et al., 2002). Similar to tenofovir and adefovir, their respective DAPy analogs PMEO-DAPy and PMPO-DAPy are both active against HIV and HBV (Balzarini et al., 2002; De Clercq et al., 2005). Among other PMEO derivatives, 5-CH_3-PMEO-DAPy in particular has shown promising activity against NRTI-resistant strains of HIV (Hockova et al., 2003; Ying et al., 2005).

To enhance the in vivo delivery of nucleoside phosphonates into lymphoid cells and tissues, various mono- and bis-amidate prodrugs have been explored. Among these, the mono-alaninyl mono-phenyl ester of tenofovir (GS-7340) has shown approximately 1000-fold improved potency against HIV-1 in vitro relative to parent TFV (Lee et al., 2005). Importantly, GS-7340 is substantially more stable in blood and plasma than TDF, but undergoes rapid hydrolysis in lymphocytes, resulting in enhanced intracellular accumulation of TFV and TFV-diphosphate (Lee et al., 2005). When administered orally to dogs, GS-7340 distributes more favorably into peripheral lymphocytes and lymphatic tissues than TDF, increasing TFV levels in these compartments by 15–30-fold without changing the liver, kidney, and systemic exposures (Lee et al., 2005).

In contrast to acyclic nucleoside phosphonates, comparatively fewer active cyclic nucleoside phosphonates have been identified to date, mainly reflecting challenges in their chemical synthesis. Nucleotides containing a 2′-deoxythreose sugar are of note since the adenine derivative (PMDTA) exhibits similar in vitro anti-HIV activity and selectivity as TFV (Wu et al., 2005). Within the series of cyclic phosphonates containing 2′,3′-dideoxy-2′,3′-didehydro-ribose, the adenine derivative (d4AP) has been reported in the past as a potent inhibitor of HIV (Kim et al., 1991). However, d4AP diphosphate is efficiently utilized by mtDNA pol γ, indicating a potential for mitochondrial toxicity. GS-9148 is a 2′-fluoro analog of d4AP rationally designed to reduce the mitochondrial toxicity potential (Cihlar et al., 2008; Ray et al., 2008a). GS-9148 exhibits a favorable in vitro resistance profile, retaining its activity against viruses with a wide range of NRTI resistance mutations including those with multiple TAMs, K65R, L74V, M184V, and their various combinations (Cihlar et al., 2008). This property is rather unique among NRTIs and is likely due to the ability of the active metabolite, GS-9148 diphosphate, to closely mimic the interactions of dATP with the active site of RT, as demonstrated by a recently determined high-resolution X-ray crystal structures (Lansdon et al., 2009). GS-9131, the ethylalaninyl phenyl ester mono-amidate prodrug of GS-9148 enhances the antiviral potency of GS-9148 approximately 100-fold in vitro (Cihlar et al., 2008) and allows for a substantial accumulation and a prolonged retention of GS-9148 diphosphate in peripheral lymphocytes in vivo (Cihlar et al., 2008; Ray et al., 2008a). Low doses of GS-9131 administered orally to dogs yielded concentrations of GS-9148 diphosphate in PBMCs that are approximately 20-fold higher compared to those of TFV diphosphate detected in PBMCs of HIV patients treated with TDF (Cihlar et al., 2008; Ray et al., 2008a). Given that similar intracellular levels of

TFV diphosphate and GS-9148 diphosphate are required for effective inhibition of HIV-1, low doses of orally administered GS-9131 are expected to translate into a potent clinical antiviral activity (Ray et al., 2008a). Overall, GS-9131 exhibits favorable pharmacological profile including a lower potential for renal toxicity compared to acyclic nucleoside phosphonates (Cihlar et al., 2009) and remains an attractive candidate for clinical development.

7. Future roles of NRTIs in the management of HIV infection

The contribution of NRTIs to highly effective long-term HIV suppression is at least in part due to their synergistic effects in combination with other classes of antiretrovirals. In addition, intracellular accumulation and prolonged retention of active metabolites of some NRTIs allows for their once daily dosing, is more forgiving towards non-adherence, and can buffer the pharmacokinetic fluctuations in levels of other drugs in any given regimen. Because of these unique properties, NRTIs are likely to remain, at least in the near future, the backbone of most drug combinations for the treatment of both naïve and experienced patients. Although multiple two- and three-drug NRTI-sparing regimens have been studied over the past years, most have shown inferiority to the best NRTI-containing drug combinations. For example, the recently explored lopinavir/ritonavir + efavirenz regimen was almost as effective as 2 NRTIs + efavirenz in the virologic suppression, but showed higher frequency of resistance mutations and metabolic adverse effects (Haubrich et al., 2009; Riddler et al., 2008). Similar outcomes were observed in other studies assessing NRTI-sparing regimens (Calmy et al., 2007; Fischl et al., 2007). Multiple other NRTI-sparing two-drug combinations are currently being explored, but, with at least some of them, it might be difficult to achieve similar potency and durability when dosed once daily. Three-drug combinations of novel highly potent drugs such as raltegravir + etravirine + darunavir/ritonavir may represent an NRTI-sparing option for treatment-experienced patients (Fagard et al., 2009).

The demonstrated long-term safety and efficacy of Atripla, the first complete once-daily fixed-dose regimen (FDR), increased the interest in the development of additional NRTI-containing FDRs. The combination of TDF, FTC, integrase inhibitor elvitegravir, and pharmacoenhancer GS-9350 in a single pill given once-daily is already in advanced stages of clinical development (Mathias et al., 2009). The collaborative development of another once-daily FDR consisting of TDF, FTC, and NNRTI rilpivirine (TMC-278) has been recently announced by commercial sponsors and could provide potential advantages over Atripla (Gilead press release; http://www.gilead.com/pr_1308630). However, it should be noted that all FDRs have been thus far explored only in treatment naïve patients, underscoring the need of exploring FDR options also for patients with drug-resistant viruses.

Reducing latent reservoirs with the ultimate goal of a cure is currently emerging as one of the new high profile strategies for antiretroviral treatment (Richman et al., 2009). Irrespective of the approaches considered for the effective activation of latent virus reservoirs, they all would have to be applied in conjunction with antiretrovirals capable of blocking de novo infection in all physiologically relevant body compartments. The newly designed highly active NRTIs or NRTI prodrugs with potentially improved permeability into multiple virus reservoirs will likely have an indispensable place in this strategy.

Future control of HIV epidemics through reducing new infections globally with the long-term prospect for HIV eradication will most likely require approaches relying on an efficacious prophylactic vaccine. However, highly effective pre-exposure prophylaxis (PrEP) using antivirals could be a possible option in the absence of such a vaccine. Recent studies showed promising effects of NRTIs in preventing or substantially reducing infection rates in animal models for HIV transmission (Garcia-Lerma et al., 2008). In part due to encouraging results from animal testing, clinical trials are being conducted to assess the efficacy of PrEP with topical tenofovir gel, oral TDF, and oral TDF/FTC in several countries with high incidence of HIV infection (Karim and Baxter, 2009). Recent epidemiological modeling studies suggest that effective implementation of PrEP could substantially reduce the incidence of HIV transmission in populations at high risk of HIV infection in the United States (Paltiel et al., 2009). However, price reduction and increase in efficacy would be necessary to make PrEP cost-effective nationally. Nevertheless, in the face of uncertainty about the development of effective vaccine, these data encourage further systematic exploration of various PrEP strategies including combinations of NRTIs with other antiretroviral classes.

Finally, the future role of NRTIs in controlling the HIV epidemics in resource-limited settings should be the most obvious one. Although several NRTIs are already being provided to developing countries under reduced costs or as generic products, all currently approved single NRTIs will become generic within the next several years. This should further reduce the cost and expand the accessibility of a broader repertoire of NRTI-containing regimens to larger populations of infected patients. Cost-effectiveness, simplified regimens of the best NRTIs, and stability under various storage conditions will be among the most important factors for successful use of NRTIs in combating the HIV epidemics in resource-limited regions. However, utilizing NRTIs the their full potential in these settings will still require unconditional support and coordinated action of the developed nations.

8. Conclusion after 25 years of NRTIs: solid answers, yet many questions

There is no doubt that over the quarter of a century following the discovery of AZT as the first potent inhibitor of HIV, NRTIs have evolved into the cornerstone of effective antiretroviral therapy. When Mitsuya et al. (1985) were writing their first communication on the activity of AZT, they could hardly have envisioned the expansion and utilization of this class of drugs as we know it today. Since 1985, countless numbers of structurally diverse nucleoside and nucleotide analogs, as well as their prodrugs have been designed, synthesized, and tested for antiretroviral activity. Perhaps surprisingly, a large proportion have been found to be active and to possess attractive pharmacological properties that have permitted their progression into clinical development. Although many have not become drugs, either for the lack of clinical efficacy or, in most cases, unacceptable adverse effects, those that did, immediately became part of life-saving therapies. The NRTI class now includes eight individual drugs, four NRTI fixed-dose combinations, and one complete single pill antiretroviral regimen. Most of these drugs were approved by the FDA under the fast track status, each representing a milestone on the journey towards better controlling the HIV infection. They all were much needed, improving patient care and redefining treatment paradigms. However, it still remains to be seen how much current NRTIs can be improved upon. How much further can the potency be increased while maintaining the safety and tolerability required for current antiretroviral therapies? Would it be ultimately possible to use just one highly active NRTI instead of the usual two? Can we design NRTIs more immune to resistance? NRTI prodrugs offer the promise of improving pharmacology but will this promise be realized to lower doses and increase selective distribution to sanctuary sites? Continuing efforts in the discovery and development of novel NRTIs should bring answers to at least some of these questions.

Acknowledgement

We would like to thank Eric Lansdon of Gilead Sciences for the preparation of Fig. 3.

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