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Intracellular Interactions Between Nucleos(t)ide Inhibitors of HIV Reverse Transcriptase

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Abstract

Current standard-of-care regimens recommended for the treatment of HIV infection include two or more nucleos(t)ide reverse transcriptase inhibitors (NRTI) in combination with a protease or non-nucleoside reverse transcriptase inhibitor. NRTIs are activated through interactions with the cellular machinery for regulating endogenous nucleoside triphosphate (NTP) pools. Once activated to their triphosphate form, NRTIs compete with natural 2'- deoxynucleoside triphosphates (dNTP) for incorporation by the virally encoded reverse transcriptase and host polymerases. Competitive inhibition, changes in enzyme expression, or allosteric modulation of cellular metabolizing enzymes may therefore alter NRTI activation or perturb cellular dNTP levels causing changes in NRTI antiviral activity and toxicity. This paper reviews the unique metabolic profiles of NRTIs and discusses methodologies for understanding the effects of combining them. Cell culture experiments assessing the antiviral synergy and intracellular metabolism of NRTI combinations have yielded valuable insights into the behavior of treatment regimens* in vivo*. The development of more reliable and convenient methods for detecting nucleotides, including those applying mass spectrometry, are helping to further elucidate the intracellular pharmacology of NRTIs. Studies assessing the potential for intracellular NRTI drug-drug interactions will facilitate a better understanding of the efficacy of current therapies, as well as the design of combination therapies with optimal activity and toxicity profiles.
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Key words

NRTI. HIV. HAART. Drug Interactions. In vitro. ***Metabolism.***

Introduction

Since the Food and Drug Administration (FDA) approved zidovudine (AZT) in 1987 for the treatment of HIV, nucleos(t)ide reverse transcriptase inhibitors (NRTI) have served as the cornerstones of successful HIV therapy. Following AZT, a number of NRTIs have been approved by the FDA including didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), tenofovir disoproxil fumarate (TDF, prodrug for oral delivery of the nucleotide analog tenofovir (TFV)) and emtricitabine (FTC) (structures shown in Fig. 1). Currently favored regimens for anti-HIV therapy contain two NRTIs and either a protease or nonnucleoside reverse transcriptase inhibitor (NNRTI) of HIV[1]. The use of more than one NRTI in current combination therapies and their dependence on metabolic activation makes an understanding of the intracellular interactions of NRTIs crucial. This review gives an overview of the pharmacology of NRTIs and discusses *in vitro* techniques for better understanding their interactions with each other. Select examples of clinically relevant interactions are given from literature citations, prescribing information, and recent conference proceedings.

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Metabolism of NRTIs

The intracellular pharmacology of NRTIs has been previously reviewed[2-7], and will only be discussed briefly here. All NRTIs are inactive in their parent forms

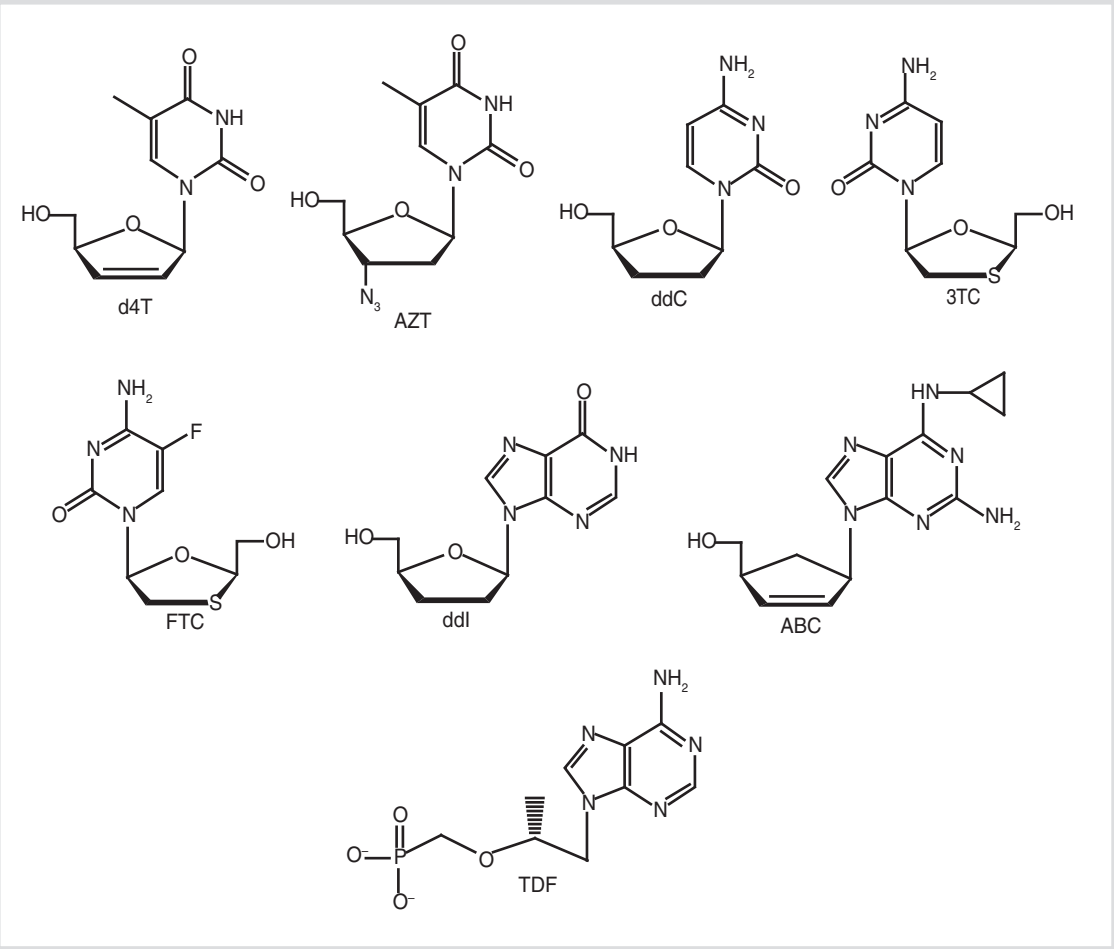

Figure 1. *Structures of the nucleoside/tide reverse transcriptase inhibitors currently approved by the United States Food and Drug Administration for the treatment of HIV.*

and must enter cells and be phosphorylated to nucleoside triphosphate analogs before being able to compete with natural 2'-deoxynucleoside triphosphates (dNTP) for incorporation by HIV reverse transcriptase. After incorporation, their lack of a 3'-hydroxyl group causes chain termination of viral reverse transcripts. While the plasma pharmacokinetics of NRTIs can be readily monitored, their dependence on intracellular activation makes intracellular concentration the most critical parameter in predicting antiviral activity and toxicity *in vivo*[8,9].

Permeation and transport

Figure 2 shows a general scheme for cellular factors important in the metabolism of NRTIs. NRTIs enter the cell by passive diffusion or carrier-mediated transport[10]. Carrier-mediated transport has been shown to contribute to the uptake of the cytidine analogs ddC[11,12], 3TC[13], and FTC[14]. Efflux transporters of the monophosphate forms of NRTIs have been upregulated in cell lines resistant to the cytotoxic effects of NRTIs[15,16]. Members of the adenosine triphosphate (ATP)-binding cassette transporter family capable of transporting nucleotide analogs, including the multidrug resistance-associated proteins (MRP) –4, –5 and –8, and their substrate specificity has recently been reviewed by Hoggard and Back[17] and Borst and colleagues[18].

Anabolism

Through interactions with the cellular machinery responsible for maintaining natural nucleotide pools, NRTIs are anabolized to their triphosphate analog forms.

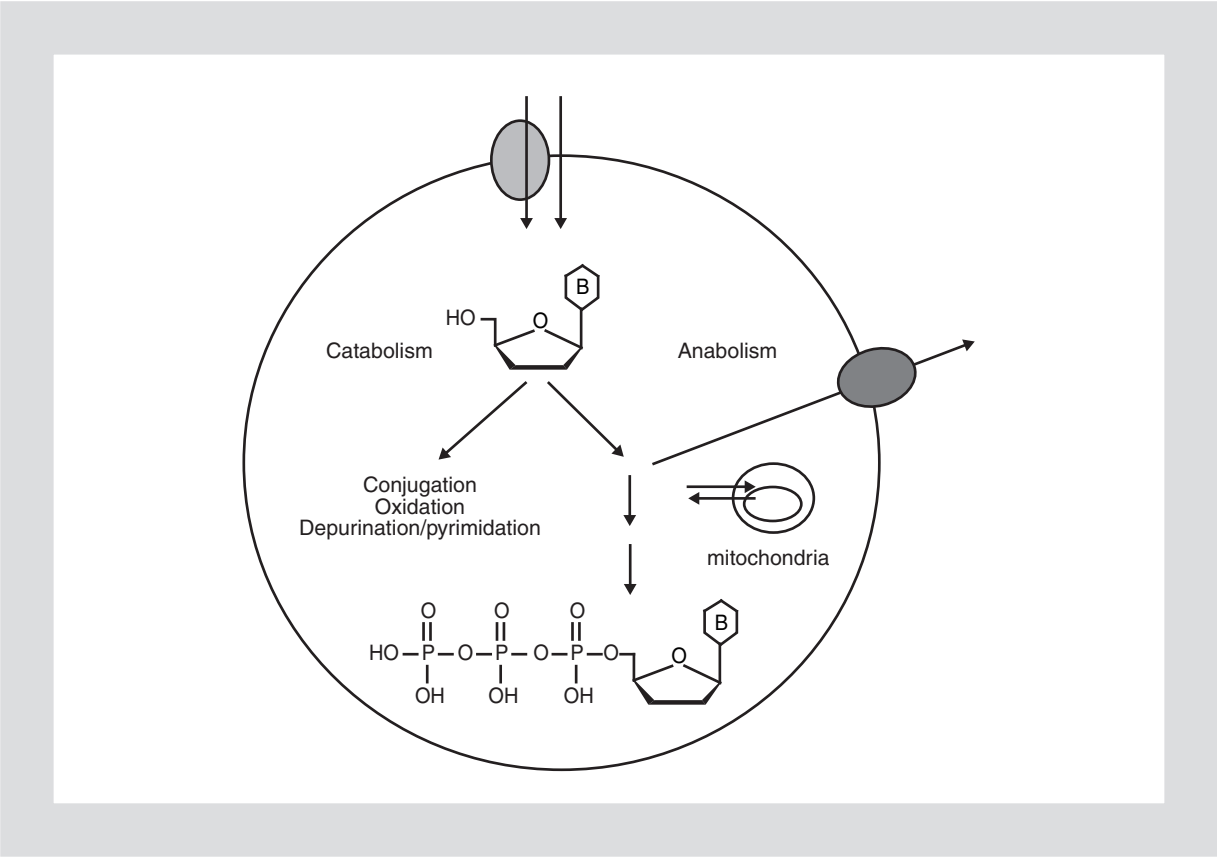

Figure 2. *Generic scheme for the metabolism of NRTIs. After entry into the cell by passive diffusion or carrier-mediated transport (transporter shown in green) NRTIs are subject to anabolic and catabolic pathways. Studies have shown that the monophosphate analog forms of NRTIs are subject to efflux out of the cell by transporters (transporter shown in red). Phosphorylation reactions may be catalyzed by cytosolic and mitochondrial enzymes. After forming their respective triphosphate analogs, NRTIs can be incorporated by viral or host polymerases, resulting in antiviral activity or toxicity, respectively.*

Table 1 summarizes what is known about the anabolic pathways for the FDA-approved NRTIs. Most research has been dedicated to nucleoside kinases and phosphotransferases responsible for the first phosphorylation step. Reduction in nucleoside kinase activity has been observed as a cellular resistance factor in experiments where cells were continuously passaged in the presence of increasing concentrations of AZT or ddC[19-21]. However, conflicting results have been observed *in vivo* and it is not known if the development of cellular resistance is a physiologically relevant resistance mechanism (reviewed by Sommadossi[3]). Both ddI and ABC have more complicated activation pathways including initial phosphorylation by phosphotransferases (using IMP or AMP as the phosphate donor, respectively) followed by base conversion to an adenosine or guanosine analog, respectively[22,23]. TFV is the only FDA-approved nucleotide analog, mimicking dAMP, and therefore its activity is not dependent on the action of nucleoside phosphorylating enzymes. Nucleoside monophosphate kinases, the enzymes responsible for addition of the second phosphate to NRTIs, have been reviewed in detail by Van Rompay, Johansson and Karlsson[24]. It was originally believed that the third and final phosphorylation step for NRTIs is predominantly catalyzed by nucleoside diphosphate kinase[25]. Recent studies have shown that creatine kinase and 3-phosphoglycerate kinase are more likely to catalyze this reaction for NRTIs in the D- or L-enantiomeric ribose ring conformation, respectively[26]. Since NRTIs are activated by cellular enzymes and compete with natural dNTPs, their antiviral activity is dependent on cell type[27], cell cycle, activation state, and dNTP pool size[28-31]. These dependencies are most apparent for AZT and d4T. The reason AZT is sensitive to the cell's activation state is its reliance on S-phase specific expression of cytoplasmic thymidine kinase 1 (TK1) for activation[32].

Mitochondria

Included in the generic anabolic pathway described in figure 2 are the mitochondria. The mitochondria have

Table 1. Summary of intracellular anabolism of FDA-approved NRTIs

NRTI	Nucleoside kinase	Active anabolite			Other enzymes involved in anabolism	Ref.
		Identity	Intracellular half-life (hr) (cell type)	*In vivo* patient PBMC concentration (fmol/million)		
AZT	TK1	AZTTP	7 (patient PBMC)	10 to 70; 68	TMP kinase	[8,32,66-68,138-141]
ddI	IMP phosphotransferase	ddATP	24 (patient PBMC)	6	Adenylosuccinate synthetase, Adenylosuccinate lyase, Adenylate Kinase	[22,142]
ddC	dCK	ddCTP			Creatine Kinase	[20,26]
d4T	TK1	D4TTP	7 (patient PBMC)	31	Creatine Kinase	[26,27,32,60,64, 140,142]
3TC	dCK	3TCTP	22 (patient PBMC)	2210 to 7290	3-Phosphoglycerate kinase	[8,26,138,141]
ABC	AMP phosphotransferase	CBVTP	20.64; 12 to 19 (patient PBMC) 3.3 (CEM)	29.6;141; 90	Cytosolic AMP deaminase	[23,117,143-146]
TDF	NA	TFVDP	≥ 60 (patient PBMC) 12 to 15 (activated PBMC) 33 to 50 (quiescent PBMC)	87.2	AK2	[117,147,148]
FTC	dCK	FTCTP	30 (patient PBMC)	200 to 2260	dCMP kinase	[14,149-151]

TK1: cytoplasmic thymidine kinase; dCK: deoxycytidine kinase; IMP: inosine-MP; AMP: adenosine-MP; NA: not applicable; CBVTP: carbovir-TP the active 2'-deoxyguanosine-TP analog metabolite of ABC; ddATP: 2',3'-dideoxyadenosine-TP the active 2'-deoxyadenosine-TP analog metabolite of ddI; TMP kinase: thymidylate kinase; AK2: adenylate kinase 2; dCMP kinase: 2'-deoxycytidine-MP kinase; -MP, DP and TP are added to reflect the mono-, di- and triphosphate forms of NRTIs, respectively.

their own genetic material and unique compartmentalized deoxynucleoside phosphorylating enzymes responsible for maintaining dNTP pools for mitochondrial DNA (mtDNA) replication[33,34]. The toxicity of some nucleoside analogs has been attributed to the unique substrate specificity of mitochondrial nucleoside kinases including the mitochondrial deoxyguanosine kinase[35,36]. However, some NRTIs capable of depleting mtDNA by chain termination in cellular experiments are not anabolized to their triphosphate form in isolated mitochondria[37], and evidence for the transport of nucleotide analogs into the mitochondria has been observed[38-40]. The exchange of nucleotide analogs between the cytoplasm and mitochondria has important implications for the activation of NRTIs and their resulting antiviral activity and toxicity[41].

Catabolism

NRTIs are catabolized and excreted by diverse mechanisms including oxidation, conjugation, and transport (also commonly referred to as phase I, II, and III metabolism, respectively). While ddC, 3TC, FTC and TFV show minimal biotransformation and a majority of the dose is recovered unchanged in the urine, AZT, ABC, D4T and ddI show extensive metabolism and excretion as catabolites (Table 2). When conducting drug interaction studies with extensively catabolized NRTIs, monitoring the effects of the coadministration of other agents on the formation of NRTI breakdown products should be considered. For example, ABC is metabolized by alcohol dehydrogenase and a drug interaction has been observed where coadministration of alcohol increases exposure to ABC[42]. Another catabolic drug-drug interaction between TFV and ddI will be described in the section on metabolic drug interactions.

Pathways for the anabolism and catabolism of NRTIs are diverse and complex. Often overlapping and poorly understood pathways for NRTI metabolism exist. This review focuses on the use of cellular experiments to understand the interactions between NRTIs because whole cells are the only *in vitro* system which can properly represent enzyme expression, co-substrate concentrations, natural nucleotide pool sizes and compartmentalization. While enzymatic studies can be informative in the determination of the molecular mecha-

Table 2. Summary of the catabolism and elimination of FDA-approved NRTIs

NRTI	Plasma half-life (hr)	% Urinary recovery of parent	Urinary metabolites (% recovered)	Other known catabolites	Enzymes involved in catabolism	Ref.
AZT	0.5 to 3	14	3'-azido-3'-deoxy-5'-*O*-β-*D*-glucopyranuronosylthymidine (74%)	3'-amino-3'-deoxythymidine	UGT, CYP3A4	[152]
ddI	1.5 ± 0.4	18 ± 8		Hypoxanthine, Uric acid, ddR-1-OH, ddR-1-phosphate	PNP	[22,71,153]
ddC	1 to 3	80	ddU (<15%)	ddCDP-choline and -ethanolamine	Cytidine deaminase, Choline-phosphotransferase	[11,12,20,154]
D4T	1.2 ± 0.4	39 ± 23	Unidentified	Thymidine, β-aminoisobutyric acid, unidentified sugar		[155-157]
3TC	5 to 7	71 ± 16	trans-sulfoxide (5.2% ± 1.4%)		FMO	[158]
ABC	1.45	1.2	5'-carboxylic acid (30%), 5'-glucuronide (36%), unidentified (15%)		ADH, aldehyde dehydrogenase, UGT	[42]
TFV	~17	70-80				[83, 159]
FTC	~10	86	3'sulfoxide diastereomers (~9%), 2'-O-glucoronide (~4%)		FMO, UGT	[160-162]

ddU: 2',3'-dideoxyuridine; ddR: 2',3'-dideoxyribose; UGT: UDP-glucuronosyltransferase; CYP3A4: cytochrome P450 3A4; PNP: purine nucleoside phosphorylase; FMO: flavin-containing monooxygenase; ADH: alcohol dehydrogenase.

nism for observed interactions, in the absence of the proper cellular data they can often be misleading.

Antiviral synergy

The most commonly used method for assessing interactions between NRTIs is the *in vitro* determination of their cell culture anti-HIV activity in combination. The most frequently used analytical method for synergy experiments is the Loewe additivity model, also known as the isobologram (Fig. 3)[43]. The data for the construction of an isobologram is generated by titrating the activity of one NRTI by twofold to fourfold serial dilution in the presence of fixed concentrations of the second NRTI at or below its effective concentration to cause 50% viral inhibition (EC_{50}) in 96-well plates. Isobolograms offer a qualitative assessment of drug interactions, but in their original form did not yield quantitative values relating to the magnitude of the drug interaction or the significance of the results. Methods have been introduced to quantitate and statistically assess the data generated from isobologram analyses[44-46].

Isobolograms only reflect one measure of activity (for example, antiviral EC_{50}) as apposed to a continuous range. If the continuous effect level is plotted against the concentration of the two compounds being tested, a three-dimensional surface (response surface) is obtained[47]. A cross-section of the dose plane of this surface reveals the familiar isobologram plot described in the previous paragraph[48]. Response surfaces offer the advantage over isobolograms of assessing synergy over a wider concentration range. For these analyses, activity is often measured for fixed ratios of two compounds and then plotted to determine whether the obtained points lie above or below a calculated additivity surface. Methods for analyzing drug synergy data, including isobolograms and response surfaces, are described in detail in a book by Tallarida[49].

There are many factors one should consider when designing and interpreting data from synergy experiments. Since NRTIs are activated by cellular enzymes by potentially overlapping metabolic pathways and compete with natural dNTP pools, studies in different cell types and activation states may yield different conclusions. While synergy with respect to antiviral activity is typically reported, synergy should also be assessed for markers of toxicity (for instance cytotoxicity, mtDNA content and lactic acid production). The therapeutic window for a combination that is synergistic with respect to both antiviral activity and toxicity may not allow for any therapeutic advantage. Interestingly, along with showing synergism with respect to antiviral activity, some L-analogs

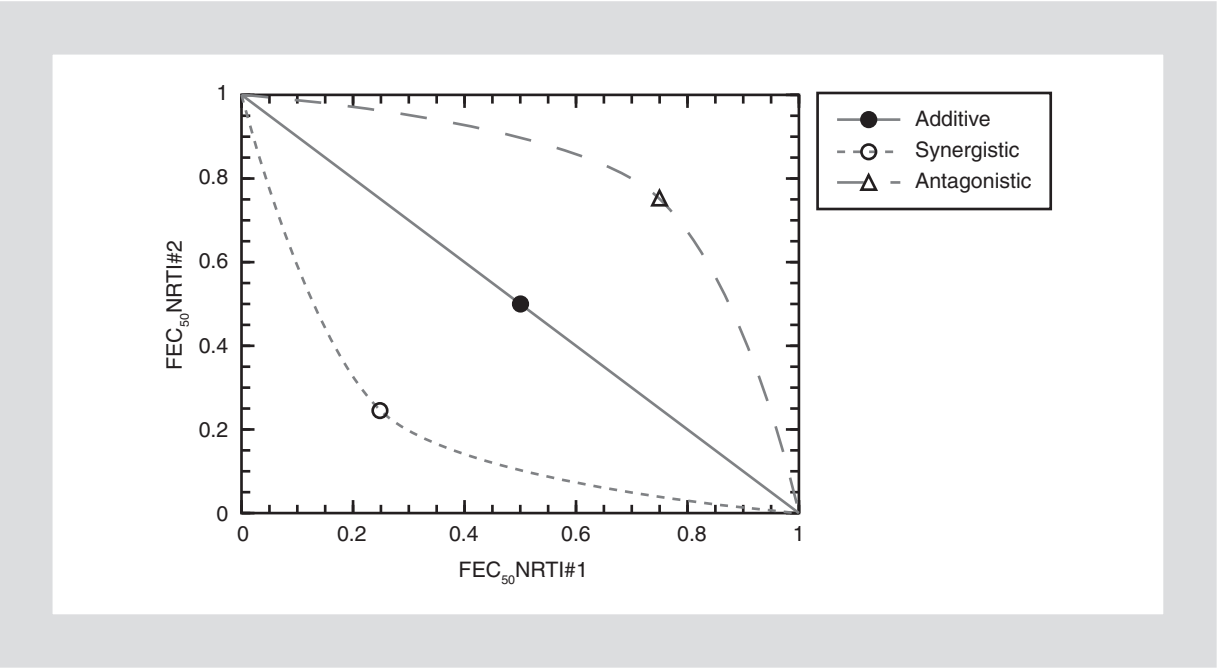

Figure 3. *The isobologram depicts the fraction effective concentration to inhibit HIV replication by 50% (FEC_{50}) for the first NRTI plotted against the FEC_{50} of the second. If the points fall on a line connecting the activity of each molecule on its own (coordinates 1,0 and 0,1, respectively) the combination is considered additive. If points deviate significantly below or above the line of additivity, the combination is considered to be synergistic or antagonistic, respectively.*

have been noted to reduce the mtDNA depletion caused by some D-analogs[50,51]. While experimentally NRTI combinations have shown synergistic activity, the underlying mechanism(s) is poorly understood[52,53] and may not be physiologically relevant. Antiviral synergy assays also appear to be somewhat insensitive to antagonistic drug interactions. The combinations of AZT/d4T and ddC/3TC both are additive in antiviral synergy experiments, despite negative metabolic interaction with respect to phosphorylation (further discussed in the next section).

Metabolic interaction studies

Antiviral synergy studies have many limitations: (i) Concentrations are limited to the linear range of HIV inhibition; (ii) No information is given explaining the potential underlying mechanism(s) for the changes in antiviral activity; (iii) Studies are confined to cells that can be efficiently infected with HIV *in vitro*. Direct measurements of metabolites after co-incubations in tissue culture can give important information for understanding the metabolism of NRTIs. Of most importance, incubations can be done at pharmacologically relevant concentrations. When deciding on an incubation concentration, it should be noted that intracellular accumulation is expected due to the long intracellular half-life of many phosphorylated NRTIs. The fold accumulation at steady-state of intracellular nucleotide reached after multiple dosing can be estimated from the equation: relative accumulation = $1/(1-e^{-kl})$, where k is the elimination rate ($k = \ln 2/\text{half-life}$) and l is the dosing interval. Calculations based on values given in table 1 would suggest that a twofold to fourfold accumulation should occur for NRTIs. As a result, incubation concentration should be adjusted accordingly to achieve appropriate pharmacologically relevant intracellular levels. Suprapharmacologic concentrations can also be used to determine if there is any evidence for overlapping anabolic pathways. When a drug-drug interaction is observed, determining concentrations of intermediate phosphorylation products and the kinetics of their appearance allows for an understanding of the step(s) inhibited and potentially the enzyme(s) affected.

When 3TC and ddC are coadministered at pharmacologically relevant concentrations in tissue culture, 3TC decreases the formation of ddCTP while ddC does not affect the formation of phosphorylated 3TC metabolites[54,55]. Evidence suggests that the source of this drug-drug interaction is ddC and 3TC's overlapping metabolic profiles including dependence on cytoplasmic deoxycytidine kinase (dCK). While ddC is a poor substrate for dCK[56,57], its reliance on the enzyme for activation has been established by its lack of activity in dCK-deficient cells[12]. Unlike ddC, 3TC is an excellent

Table 3: Results of antiviral and metabolic drug interaction studies for select NRTI combinations

NRTI	Co-incubated	Intracellular metabolism	Antiviral synergy	Ref.
3TC	AZT	↓3TCTP	Synergistic	[50,84,163,164]
d4T	AZT	↓d4TTP	Additive	[55,61,62]
ddC	3TC	↓ddCTP	Additive	[54,55,165]
ddI	TFV	↑ddI, ↓ddI catabolites, ↔ ddATP	Minor Synergy	[69-71]

substrate for dCK[58,59] and its tight binding interaction may effectively compete with the weaker interaction of ddC with the enzyme, thus limiting ddC activation.

Similar to the combination of ddC and 3TC, when d4T and AZT are incubated in combination, the formation of d4TTP is reduced while AZT phosphorylation is unaffected[55,60-62]. Based on both AZT and d4T being thymidine analogs, one might surmise that, similar to the two cytidine NRTIs, the inhibition of thymidine phosphorylating enzymes by AZT may be the cause of the decrease in d4T anabolism. In contrast to AZT, in enzymatic assays d4T has been shown to be poorly phosphorylated[60,63] or not phosphorylated at all[32] by TK1. Somewhat conflicting results have been found for the dependence of d4T on TK1 for phosphorylation in cells deficient in TK activity (reviewed by Hitchcock[64]). While only a slight decrease in d4T phosphorylation was observed in cells with reduced TK activity caused by continuous passage in the presence of increasing levels of AZT[65] or a TK-mutant cell line[66], in TK-minus mouse cells it was found that neither d4T nor AZT were phosphorylated[64]. Inefficient phosphorylation by TK1 [60,63] is consistent with the poor intracellular phosphorylation observed for d4T[27,60]. Therefore, these data may not necessarily indicate the contribution of another enzyme as has been suggested[32,66]. Taken together, phosphorylation of d4T seems to be at least in part dependent on TK1, and inhibition of this enzyme by AZT is a likely reason for the observed decrease in d4T phosphorylation. Another candidate for a molecular site of the interaction is thymidylate kinase (TMP kinase). Furman and colleagues have shown that AZTMP binds to TMP kinase more tightly than the natural substrate (TMP), but is only very slowly phosphorylated[67]. AZTMP is also found to accumulate to high intracellular levels[60,66,68]. The tight binding interaction of AZTMP with TMP kinase and its high intracellular levels make it a likely competitive inhibitor of d4TMP phosphorylation. These data suggest that both TK1 and TMP kinase may be responsible for the observed drug-drug interaction.

TFV and ddI are both activated to dATP analogs. Initial studies on the phosphorylation of ddI and TFV showed that neither affects the other's phosphorylation at pharmacologically relevant concentrations in quiescent or stimulated peripheral blood mononuclear cells (PBMC)[69]. This result is consistent with the reported slightly synergistic antiviral activity of the combination of ddI and TFV in cell culture[70]. Later studies have shown that TFV can inhibit the intracellular degradation of ddI[71]. Enzymatic experiments illustrate that acyclic nucleotides, including the anabolites of TFV, can inhibit purine nucleoside phosphorylase (PNP)[71-76], an enzyme associated with the catabolism of ddI (see further discussion below)[22,77].

As discussed below, the anabolic drug-drug interaction between AZT and d4T and the catabolic drug-drug interaction between TFV and ddI have manifested themselves clinically. While not discussed in this manuscript, combination studies of HIV NRTIs with nucleos(t)ide therapies used for other indications are also important. For example, a number of drug-drug interactions between ribavirin, a nucleoside analog used in the treatment of hepatitis C virus, and HIV NRTIs have been reported[62,78-81].

Clinical Relevance

Table 3 summarizes the results of studies on the metabolic and antiviral synergy interactions between selected NRTIs. The *in vitro* observed antagonism of AZT to d4T phosphorylation is likely the cause of the poor efficacy of AZT/d4T combination therapy in patients[82]. The inhibition of ddI catabolism by the enzyme PNP caused by the phosphorylated metabolites of TFV is likely the mechanism for the 44% to 60% increase in the plasma exposure to ddI during ddI/TDF coadministration in patients[71,83]. While *in vitro* results for AZT/d4T and ddI/TFV yielded data consistent with clinical findings, one should be cautious when interpreting the pharmacologic relevance of *in vitro* drug-interaction studies. For example, incubations of AZT and 3TC have shown that the presence of AZT can cause a slight but significant decrease in the formation of 3TCTP[84]. This interaction likely has little relevance as the combination of AZT and 3TC has shown clinical efficacy[85,86].

Proactive determination of the potential for pharmacologically relevant interactions between preclinical NRTI candidates and the FDA-approved NRTIs that they may be coadministered with is an important part of defining the most efficacious combinations for clinical assessment. A study illustrating the utility of preclinical *in vitro* experiments included the deoxycytidine analog SPD754. Similar to ddC, SPD754 was found to be additive with 3TC in anti-HIV synergy assays, while the intracellular formation of SPD754TP was inhibited by co-incubation with 3TC in metabolic studies. In HIV-infected patients there was no observation of a plasma drug interaction. However, similar to results from *in vitro* metabolic studies, PBMC levels of SPD754TP were reduced when SPD754 was coadministered with 3TC[87].

Nucleotide pools

The antiviral activity of NRTIs is dependent on both the levels of their 5'-triphosphates formed and the intracellular concentrations of endogenous dNTPs that they compete with for incorporation into proviral DNA[30]. It is therefore critical to determine the amount of active nucleotide analog formed relative to its corresponding natural dNTP. A complete metabolic interaction study should not only include the impact of coadministration on nucleoside-triphosphate analog concentrations, but also their respective competing dNTPs. The importance of nucleotide pools is clear from the effects of antimetabolite agents that modulate nucleotide pool sizes on the antiviral activity of NRTIs[56,88,89].

An understanding of the effects of single NRTIs and their combinations on dNTP pools could be an important factor in understanding their interactions. It is not implausible that NRTIs could affect dNTP pools: (i) NRTIs may directly inhibit nucleos(t)ide metabolizing enzymes; (ii) NRTIs may perturb allosteric interactions responsible for regulating cellular dNTP pools by enzymes including ribonucleotide diphosphate reductase [90,91]; (iii) It may also be possible for NRTIs to alter the expression of nucleos(t)ide metabolizing enzymes[21,65,92-97]. Although dNTP pool sizes could be affected by NRTIs, it has not been firmly established if, at pharmacologically relevant levels, currently approved NRTIs can alter dNTP levels in patients. Recent studies have lead McKee and colleagues to suggest that the association of AZT with mitochondrial toxicity is due to depletion of mitochondrial TTP pools due to the inhibition of mitochondrial thymidine kinase (TK2) and TMP kinase by AZT and AZTMP, respectively[98]. Similarly, whole cell TTP levels have previously been reported to be decreased by AZT[67,99].While requiring further study, these results may help to explain the clinical observation of symptoms often associated with mitochondrial damage during AZT therapy[100-103] despite AZTTP being a weak inhibitor of mtDNA polymerase gamma[104,105].

The finding that phosphorylated metabolites of TFV inhibit PNP[71], an enzyme important for the regulation of nucleotide pools, led Kukuda, Anderson and Becker to hypothesize that inhibition of this enzyme might be responsible for CD4+ cell decreases noted when non-dose adjusted ddI and TDF are combined[106,107] and for the observation of high rates of treatment failure, NRTI-resistance mutations, and virologic nonresponse in patients treated with triple-NRTI regimens including TDF and ABC[108-111]. This hypothesis is based on data showing that potent inhibitors of PNP can inhibit T-cell division[112] by increasing intracellular levels of guanine nucleotides[113], potentially explaining the decrease in CD4+ cells observed with TDF/ddI and the reduced antiviral effect of ABC (metabolized to an analog of dGTP) when administered with TDF in triple-NRTI combinations. However, in order to cause physiologically relevant effects with a PNP inhibitor requires nearly complete enzyme inhibition[114], and molecules far more potent than the metabolites of TFV with higher circulating levels have failed to show any immunosuppression *in vivo*[115]. If PNP inhibition by TFV metabolites was responsible for decreased CD4+ cells in TDF/ddI-treated patients, it would be expected that CD4+ declines would happen with therapies not including ddI. However, a wealth of clinical experience with TDF shows an increase in CD4+ cells, including the well-controlled studies 903 and 934 looking at the combination of TDF/efavirenz with 3TC and FTC, respectively. The presence of a drug-drug interaction between TFV and ABC is also not supported by the current data: (i) There is no systemic drug interaction in plasma[116]; (ii) No evidence for an intracellular antagonism of phosphorylation in patients treated with TFV and ABC[117]; and (iii) No intracellular antagonism of phosphorylation detected at concentrations up to 100 μM nor any effect of TFV, ABC or their combination on dATP or dGTP pools in cultured cells[118].

Analytical methods

The understanding of the intracellular pharmacology of NRTIs has been limited to some extent by the difficulties in detecting nucleotides. Nucleotides are readily separated by anion exchange or reversed phase ion-pairing liquid chromatography (LC) methods. While

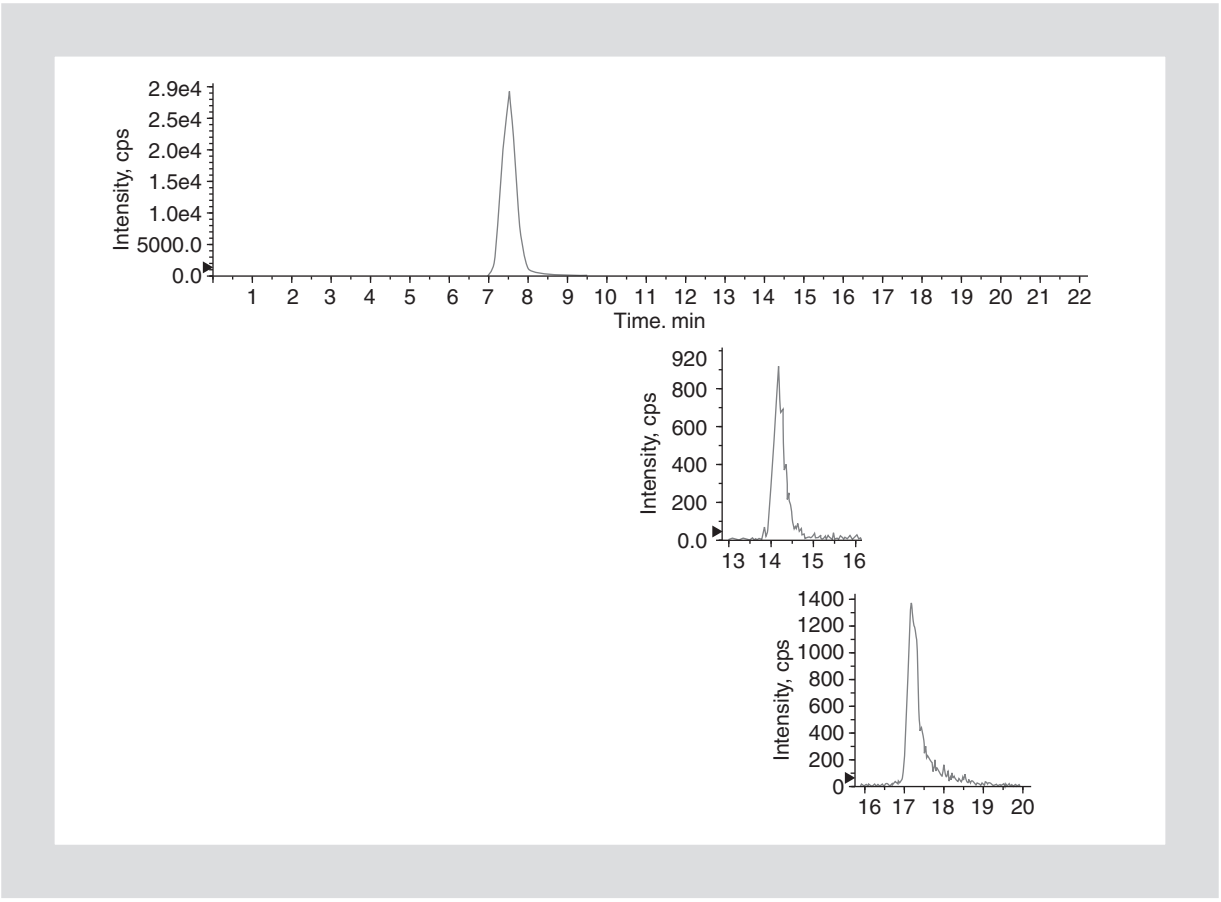

Figure 4. *Separation of tenofovir metabolites found after a 24 hour incubation at 10 µM in stimulated peripheral blood mononuclear cells by LC/MS/MS. One-million cells were isolated from media and extracellular drug by spinning through oil*[71] *and lysed by incubating in 70% methanol at -20 ºC overnight. Cellular debris was removed by centrifugation and the supernatant dried under vacuum. Dried cell extract was then re-suspended in 20 µL 10 mM tetrabutylammonium hydroxide (TBAH) and 10 mM ammonium phosphate (pH 7.0) per million cells. Extract from one-million cells was then injected on to a 1.0 x 100 mm 3.5 µm microbore reverse phase column. Separation of TFV metabolites was achieved by applying a step gradient from 6% to 20% acetonitrile in 0.25 mM TBAH, 4 mM phosphate (pH 6.5) at a flow rate of 40 µl/min. Analytes were detected by an Applied Biosystems/MDS Sciex API-4000 triple quadrupole mass spectrometer with an electrospray source running in positive mode. The retention times for TFV, TFV-MP, and TFV-DP were 7.6, 14.2, and 17.2 minutes respectively. The determined concentration from a standard curve showed approximately 7, 3, and 7 µM intracellular concentrations of TFV, TFV-MP, and TFV-DP, respectively.*

the heterocyclic bases absorb ultraviolet (UV) light, in most cases interference from endogenous nucleotides makes UV quantitation inaccurate. The limitations of UV analysis have made radiolabeled NRTIs the most commonly used method for quantitation of *in vitro* studies, despite the expense and difficulty of synthesizing labeled material. Depending on the type and position of the labeling, the radiolabel may also be unstable, causing contaminant peaks. Reincorporation of label into natural nucleotide pools can be especially problematic because of the similarities in analytical behavior of phosphorylated NRTIs and natural nucleotides[22,71,119].

Mass spectrometry (MS) allows for the sensitivity and specificity needed for performing intracellular measurements; however, it is not compatible with LC methods normally used for the separation of nucleotides. Both ion pairing and strong anion exchange chromatography typically use buffer systems with high ionic strength and containing nonvolatile components known to suppress the MS signal. One way to avoid the incompatibility between nucleotide analytical methods and MS detection is to pre-fractionate the sample, take the fractions containing the nucleotide of interest (for example the triphosphate) and dephosphorylate it to the nucleoside level using phosphatase. Detection of nucleosides by LC/MS is not problematic and this methodology has been used for clinical samples containing AZTTP, 3TCTP, and d4TTP[120,121].

In order to avoid radiolabeled synthesis or cumbersome sample preparation, a direct detection method for nucleotides using LC/MS is desirable. There is one report of the use of weak anion exchange LC coupled

to triple quadrupole mass spectrometry (MS/MS). Using a pH gradient, the authors were able to avoid the presence of high concentrations of salt normally necessary for anion exchange chromatography to detect the triphosphate of a clinical NRTI candidate[122]. The majority of methods use ion-pairing reversed phase LC to facilitate analytical separation using tetra-alkyl ammonium salts[123,124] or N, N-dimethyl-hexylamine[125-130]. One major advantage of ion-pairing techniques is the potential to develop single analytical methods capable of detecting the parent NRTI and all of its phosphorylated metabolites. Ion-pairing methods capable of detecting ABC and TFV in all their phosphorylated forms have been described[118,126] (Fig. 4). The most common way for accurately determining intracellular nucleotide levels in patients has been the time-consuming method of dephosphorylation followed by radioimmunoassay[131-137]. The development of effective and convenient LC/MS/MS methods promises to facilitate studies leading to the understanding of the intracellular pharmacology of NRTIs both *in vitro* and *in vivo*.

Conclusions

The use of *in vitro* antiviral synergy and metabolic drug interaction studies have helped in predicting pharmacologically relevant drug-drug interactions between NRTIs. The development of improved detection methods for NRTIs and their metabolites, including LC/MS/MS, should facilitate studies to further understand the intracellular pharmacology of NRTIs. The elucidation of the interactions between NRTIs is critical in understanding current HIV therapies and optimizing combinations for more efficacious future regimens.

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