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A Transient Kinetic Approach to Investigate Nucleoside Inhibitors of Mitochondrial DNA polymerase γ

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Abstract

Nucleoside analogs play an essential role in treating human immunodeficiency virus (HIV) infection since the beginning of the AIDS epidemic and work by inhibition of HIV-1 reverse transcriptase (RT), a viral polymerase essential for DNA replication. Today, over 90% of all regimens for HIV treatment contain at least one nucleoside. Long-term use of nucleoside analogs has been associated with adverse effects including mitochondrial toxicity due to inhibition of the mitochondrial polymerase, DNA polymerase gamma (mtDNA pol γ). In this review, we describe our efforts to delineate the molecular mechanism of nucleoside inhibition of HIV-1 RT and mtDNA pol γ based upon a transient kinetic approach using rapid chemical quench methodology. Using transient kinetic methods, the maximum rate of polymerization (k_{pol}), the dissociation constant for the ground state binding (K_d), and the incorporation efficiency (k_{pol}/K_d) can be determined for the nucleoside analogs and their natural substrates. This analysis allowed us to develop an understanding of the structure activity relationships that allow correlation between the structural and stereochemical features of the nucleoside analog drugs with their mechanistic behavior toward the viral polymerase, RT, and the host cell polymerase, mtDNA pol γ . An in-depth understanding of the mechanisms of inhibition of these enzymes is imperative in overcoming problems associated with toxicity.

1. Introduction

As mentioned in several other chapters in this volume, a cornerstone of antiviral therapy, especially for treatment of AIDS, centers on the use of nucleoside analogs as drugs that inhibit the viral polymerase, HIV-1 reverse transcriptase (RT), by halting DNA replication. Nucleoside analogs have a long history of use as therapeutics for treatment of cancer and viral infections. Nucleoside RT inhibitors (NRTIs) are substrate analogs that act as prodrugs that must be transported into the cell and phosphorylated by cellular kinases to the metabolically activate triphosphate form to wield their therapeutic effect [1,2].

The *in vivo* and *in vitro* mechanisms of cytotoxicity by the nucleoside analogs are not well understood and are dependent on the individual nucleoside. It has been suggested that among the many factors which are certain to be crucial in cellular toxicity, one may be the inhibition of the host mitochondrial DNA polymerase gamma (mtDNA Pol γ) [3-7].

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Accordingly, the triphosphate form of the nucleoside analog may serve as a substrate, resulting in inhibition of cellular DNA synthesis [8]. Two lines of evidence support this hypothesis: (1) ddCTP is a potent inhibitor of the mtDNA Pol γ and prolonged exposure of the cell to ddC results in decreased mitochondrial DNA synthesis [9-11] and (2) nucleoside analogs such as AZT, in general, have a much higher affinity for the mtDNA Pol γ compared with other host cell polymerases (α , β , δ and ϵ) [8,12]. This evidence suggests that there are similarities between the mtDNA Pol γ and HIV-1 RT. A tragic case of serious drug toxicity resulting in the death of five people and was thought to be related, in part, to mitochondrial toxicity induced by the anti-hepatitis B virus (HBV) drug, Fialuridine (FIAU), which was being evaluated in clinical trials [13,14]. Long term oral administration of the FIAU nucleoside analog was accompanied by severe, delayed onset toxicity characterized by multi-organ involvement, microvesicular fat infiltration of the liver, and severe and refractory lactic acidosis [13].

An in-depth understanding of the mechanisms of inhibition of the mtDNA polymerase γ is very important in overcoming problems associated with cytotoxicity [7]. Understanding structural similarities and differences will identify features important in the selectivity of interacting with HIV-1 RT over the mtDNA polymerase γ .

In this chapter we describe transient kinetic methodology as an approach for delineating molecular mechanism and inhibition of mtDNA Pol γ and RT and provide examples of how this approach has yielded important information for understanding selectivity and toxicity for host and viral polymerases.

2. Transient Kinetics as A Methodology to Dissect Molecular Mechanism

Transient kinetic methodology offers unique contributions toward dissecting the molecular mechanisms of enzyme catalysis and inhibition. This information aids in the design of novel inhibitors as well as to identify key features of current therapies associated with drug resistance and/or toxicity.

Standard enzymological assays employ steady-state kinetic analyses to determine K_m , k_{cat} , and K_i for nucleotide substrates and NRTI inhibitors. A comparison of steady state and transient kinetic methodologies is shown in Figure 1.

Steady-state kinetic analysis offers a useful starting point in examining enzymatic reactions by allowing an assessment of substrate binding order and specification of molecules at the active site; however, the kinetic parameters are complex. A transient kinetic approach offers the ability to examine the mechanism in detail by allowing the pathway to be established directly, determination of kinetic parameters for individual steps in the reaction, and most importantly the ability to monitor reactions at the active site of the enzyme. This includes providing information on enzyme intermediates or discrete conformational states (purple box, Fig. 1).

As illustrated in Fig. 1, steady-state assays provide an initial evaluation for assessing mechanism and compound inhibition of the viral (HIV-1 RT) and cellular (mtDNA pol γ) polymerases. However, a steady-state kinetic approach only examines the rate-limiting step in the overall kinetic pathway, which for most polymerases, including RT and mtDNA pol γ , has been shown to be the release of the elongated DNA product from enzyme (Scheme 1) [15,16]. There are examples where sole reliance on steady-state kinetic assays has lead to incorrect assumptions concerning the enzyme mechanism and inhibition [17-19].

2.1 Rapid Chemical Quench Methodology

As exemplified in Fig. 1, a transient kinetic approach, as a complementary strategy, provides *unique* information regarding kinetic events occurring at the *active site* of the enzyme. This approach encompasses a variety of technologies including rapid chemical quench and stopped-flow fluorescence methodologies that provide detailed mechanistic information regarding substrate and inhibitor binding, conformational changes, and individual steps involved in enzyme chemical catalysis.

Rapid chemical quench techniques involve the use of enzyme in quantities stoichiometric with radiolabeled substrate. A chemical quenching agent is used to stop the enzyme reaction at a fixed time point allowing one to examine the extent to which chemical catalysis has occurred. A number of excellent comprehensive reviews have been published [19-24].

A schematic illustrating a rapid chemical quench experiment is shown in Figure 2 [25-28]. In this type of experiment, the enzyme is loaded into one sample loop and the radiolabeled substrate is loaded into the other sample loop. A precisely controlled motor drives the buffer syringes pushing the substrate and enzyme through the reaction loop where enzyme catalysis occurs and then the reaction is terminated by denaturing the enzyme with a chemical quenching agent such as strong base or acid, a metal chelating agent, or neat organic solvent. In the selection of the most appropriate quenching agent, one must make sure that substrate, product, (and any putative intermediates) are stable under the quenching conditions employed and concurrently that the enzymatic reaction is very rapidly terminated. The apparatus shown below is designed to allow the use of very small sample volumes (<15 μ L) and allows one to examine reaction times in the millisecond time range.

The sample is then collected and analyzed to quantify the conversion of substrate to product by an appropriate analytical method based upon the enzyme chemistry. These analytical methods may include HPLC, protein or DNA gel analysis in combination with radiolabeled detection or spectroscopic methods such as NMR and mass spectrometry. In the case of DNA polymerases, one uses a 5'-radiolabeled primer, and monitor the incorporation of the elongated DNA primer using DNA sequencing gel analysis and EDTA as a quenching agent as previously described [28].

2.2 Stopped-flow fluorescence methodology

A companion technique is stopped-flow fluorescence methodology. As illustrated in Figure 3, this approach involves monitoring an optical signal associated with alterations elicited by substrate binding, catalysis, or conformational changes [28]. This strategy has been utilized for a number of polymerases to derive mechanistic information on the nucleotide incorporation and exonuclease proofreading by using intrinsic protein fluorescence or a fluorescently labeled probe such as an oligonucleotide substrate containing 2-aminopurine [28]. This approach and application to a variety of polymerases has recently been summarized therefore will not be discussed in detail here [29].

2.3 Aspects of experimental design for transient kinetics

Using rapid chemical quench or stopped-flow fluorescence methodology, in a transient kinetic approach, one of the most definitive experiments to examine reactions at the enzyme active site is termed a single turnover experiment. This type of experiment involves following the conversion of substrate to product in a single enzyme turnover with enzyme in excess over the limiting substrate that contains the radiolabel. These experiments permit one to directly examine events occurring at the active site of the enzyme since the reaction proceeds with 100 % conversion of substrate to product. The following issues may limit the usefulness of a single turnover experiment. In general, in setting up a single turnover

experiment, it is preferable to use substrates that have tight binding constants ($K_d < 50 \mu\text{M}$) rather than weak binding ($\sim 1 \text{ mM}$) due to the requirement that substrate binding is not the rate-limiting step. This obstacle can be overcome by conducting the experiment at very high enzyme concentrations ($>1 \text{ mM}$) however, potential technical problems can arise if the protein precipitates or aggregates under these conditions. The k_{cat} or enzyme turnover rate sets a limit for the slowest step in the pathway and allows an assessment of whether the reaction would be within the time domain of the rapid mixing methods currently available. With the rapid mixing apparatus described in Figure 2, reliable time points can be collected for as short as 3 milliseconds. It should be noted that enzyme systems having K_m values in the mM range and $k_{\text{cat}} > 500 \text{ s}^{-1}$ are not readily amenable to rapid chemical quench methodology. The limits of sensitivity for detection are also dependent upon the nature of the radioisotope in the substrate as well as the analytical method employed to separate and detect substrate, intermediate, and products. In our experience, radiolabels are particularly effective with a detection limit of 4-5% of the total radiolabeled species comprising intermediate for less energetic isotopes like ^{14}C or ^3H , and less than 1% detection limit for more energetic ^{32}P isotopes.

3. Transient kinetics to investigate the mechanism of RT and mtDNA pol γ

3.1 Mechanism of HIV-1 RT

Using a transient kinetic approach, our studies have allowed us to develop the kinetic model shown in Scheme 1 describing the mechanism of polymerization for HIV-1 RT. This is an ordered mechanism with the DNA binding first followed by the nucleotide. The DNA, containing a specified number of bases (DNA_n), binds tightly to the RT forming an E^*DNA complex that is slow to dissociate. The binding of the dNTP follows. A two step binding process of the dNTP controls the selectivity involved in incorporating the correct nucleotide. The dNTP first binds to the E^*DNA complex in a loose conformational state that is largely controlled by the base-pairing free energy upon recognition of the correct dNTP over the incorrect dNTP [17]. The binding of the *correct* dNTP induces a rate-limiting protein conformational change designated by $\text{E}^*\text{DNA}_n\text{dNTP}$. The formation of this tight ternary complex allows the critical transition state to be reached leading to very rapid chemistry to produce the elongated DNA_{n+1} . After nucleotide incorporation, the enzyme must return to the loose state in order to release the PPi and a translocation step to allow the next round of reaction [15,30,31]. Additional evidence for the two conformational states of the ternary RT enzyme, DNA, dNTP complex are provided by the kinetic studies with non-nucleoside inhibitors [18,32]. Several crystal structures of RT corroborate this kinetic model [33-36]. The rate-limiting conformational state of the protein induced by the binding of the correct dNTP was visualized in the structure of the catalytic complex by the fingers domain closing in toward the palm domain to form a very tight enzyme*-DNA-dNTP complex [34].

3.2 Mechanism of mtDNA pol γ

Similar transient kinetic studies revealed an analogous mechanism for mtDNA pol γ with the exception that the host cell polymerase contains a 3'-5' proofreading exonuclease activity to remove incorrect dNTPs [16,37,38]. The structure of mtDNA pol γ has recently become available to correlate with functional studies [39,40]. Mechanistic aspects of mtDNA pol γ and its importance in mitochondrial disease, have recently been reviewed [41].

The replication fidelity of a polymerase is defined as the catalytic ability of the polymerase active site to discriminate between correct and incorrect base pairs for incorporation. In contrast to RT, mtDNA pol γ has exonuclease proofreading activity that allows correction of errors if an incorrect dNTP is incorporated. As illustrated in Scheme 2, if the mtDNA pol γ stalls, then the incorrect dNTPs are incorporated much more slowly. If a misincorporation

does occur, incorporation of a correct base pair after a mismatch is unfavorable, as determined by a decrease in both the rate and binding affinity for the dNTP [38]. This allows for transfer of the mispaired primer-template to the exonuclease site where the mispair is removed. Dissociation of the mispaired primer-template may also take place and the k_{off} is increased ~10-fold relative to the properly paired primer-template. In addition, the rate of exonuclease removal is enhanced in the presence of a buried mismatch, illustrating the ability to repair buried mispairs.

As illustrated by the kinetic pathway in Scheme 1, a determination of the affinity, K_d , and maximum rate of nucleotide incorporation, k_{pol} , defines the efficiency of incorporation (k_{pol}/K_d) using a transient kinetic analysis. These key parameters allow one to quantitatively dissect how various nucleotide analogs are recognized and utilized by wt and mutant forms the viral and mtDNA polymerases relative to the corresponding natural dNTP substrates.

4. Nucleoside analogs as inhibitors of DNA polymerization: molecular target versus host specificity

The structures of a number of clinically relevant nucleoside analogs including the currently approved NRTIs are illustrated in Figure 4. The NRTI analogs lack a 3'-hydroxyl group required for primer extension thereby halting the extension of the primer. While the goal of designing an antiviral nucleoside is to achieve maximal efficiency of incorporation by the viral polymerase, in order to avoid mitochondrial toxicity, the incorporation efficiency of the nucleoside analog by mtDNA Pol γ should be minimal.

4.1 Kinetic parameters governing nucleoside analog discrimination by mtDNA pol γ

Steady-state [42] and transient kinetic studies have been used to examine the incorporation efficiency of the triphosphate form of these nucleoside analogs by RT and mtDNA Pol γ [41,43,44]. Knowledge of the incorporation efficiency coupled with an assessment of the proofreading exonuclease activity for the mtDNA polymerases for removal of NRTIs has been used to develop a toxicity index that allows us to predict NRTI toxicity at the level of the triphosphate.

We used recombinant exonuclease-deficient (E200A), reconstituted human mtDNA pol γ holoenzyme in single turnover kinetic studies to determine the maximum rate of incorporation (k_{pol}), binding affinity (K_d), and incorporation efficiency or specificity constant (k_{pol}/K_d) for the 8 clinically relevant NRTIs (Fig. 4) approved by the FDA to treat AIDS. Table 1 summarizes this comprehensive study which determined the ability of mtDNA pol γ to distinguish the nucleoside analog (AnalogTP) relative to the natural dNTP substrate as defined by the discrimination that reflects the ratio of k_{pol}/K_d (natural dNTP) / k_{pol}/K_d (AnalogTP). The smaller the discrimination number for the AnalogTP, the higher the likelihood the analog will serve as a substrate for mtDNA pol γ . The discrimination varies more than 500,000-fold for the series ddC > ddA (active metabolite of ddI) > d4T > (-)3TC > PMPA > AZT > (-)FTC > Carbovir (CBV the prodrug of Abacavir) [43-46]. More recent studies with AZTTP and other 3'-azido substituted nucleoside analogs suggest the reaction kinetics are more complex due to a slow rate of pyrophosphate product release [47]. The hierarchy of discrimination mirrors the mitochondrial toxicity observed in the clinic, with ddC being the most toxic and abacavir among the least toxic. Other studies examining the rates of excision have similar findings [42].

The nucleoside analog, FIAU, targeting the hepatitis B polymerase rather than HIV-1 RT, was also examined and found to have a discrimination of 4.8. In contrast to the anti-HIV nucleoside analogs in Table 1, that result in DNA chain termination; FIAUTP is

incorporated and readily extended at a rate of 0.16 s^{-1} . The presence of FIAUMP in mtDNA suggests that it is stably incorporated and highly mutagenic [43,44,48,49].

The exonuclease removal of each analog after incorporation was also examined using the exonuclease proficient human mtDNA pol γ holoenzyme. The rates of excision ranged from 0.06 to 0.0004 s^{-1} with the fastest being FIAU and the slowest rate of removal being ddC. The rates of removal for ddA and d4T were also quite slow [43]. Additional studies indicate that these analogs bind more tightly to the polymerase active site during incorporation than the natural dNTPs and the higher affinity may contribute to slower excision rate by the proofreading exonuclease [50].

A similar approach has recently been utilized to evaluate the inhibition of mtDNA pol γ by the anticancer drug, gemcitabine [51] and the antiviral drug for herpes simplex, acyclovir [52].

4.2 The role of stereochemistry and fluorine substitution in nucleoside analog discrimination

Among all of the NRTIs used as antiretroviral therapy, two of the analogs, (–)3TC and (–)FTC are oxathiolane nucleosides with the unnatural L-configuration. (–)FTC closely resembles (–)3TC except the former contains a fluorine substituent in the 5 position of the cytosine ring. Earlier cell culture studies with L-isomer (–)3TC and the D-isomer (+)3TC, suggested that (–)3TC was more potent and less toxic but the mechanistic basis for the stereoselectivity and differential toxicity were not completely understood [7,53-58]. Transient kinetic studies were used to investigate the interactions of the (+) and (–) 3TCTP and FTCTP with mtDNA pol γ . Molecular studies comparing these nucleoside analogs along with ddCTP and the natural substrate, dCTP are summarized in Table 2 [46].

Several interesting structure activity relationships (SAR) are noted from this study. The structural modification of ddCTP by introduction of a sulfur atom at the 3' position of the sugar moiety to produce 3TCTP decreases the incorporation efficiency by mtDNA pol γ . The absolute stereochemistry has a profound effect on selectivity. There is a 16-fold difference in selectivity for the (+) versus the (–) enantiomers of 3TC. The substitution of a fluorine in the 5-position of the cytosine ring resulted in a further amplification of selectivity as there was a > 8000-fold difference in (+)FTCTP versus (–)FTCTP. The exonuclease rates of removal for each of these oxathiolane analogs was in a range similar the dCTP natural substrate. Previous studies in our lab have demonstrated a clear difference in SAR, for HIV-1 RT versus mtDNA pol γ , in that incorporation efficiencies are slightly *higher* for unnatural L-isomers, (–)3TCTP and (–)FTCTP [46,59].

Collectively, these studies have shown that several design aspects of newer generation NRTIs have dramatically decreased mtDNA pol γ inhibition, including the use of unnatural L-nucleosides ((–)3TC and (–)FTC), acyclic nucleosides (tenofovir) and carbocyclic nucleosides (abacavir). The use of L-nucleosides such as (–)3TC and (–)FTC allows for potent inhibition of RT while serving as poor substrates for the mitochondrial polymerase imparting low mitochondrial toxicity [45,46,59,60]. Fluorination of the C-5 position of cytosine base in (–)FTC results in further decreased incorporation by mtDNA pol γ compared to (–)3TC [46]. Acyclic nucleoside analog PMPA and carbocyclic analog carbovir, the active metabolites of tenofovir and abacavir respectively, present among the highest therapeutic indices by also minimizing incorporation by mtDNA pol γ [43]. Utilization of transient kinetic approach in the analysis of nucleoside analog therapeutics represents a powerful tool in understanding and predicting mitochondrial toxicity.

While the use of L-nucleosides often provides an improved toxicity profile compared to their D-enantiomers, there are noteworthy exceptions. For instance, a notable example is the highly toxic L-nucleoside analog troxacitabine (L-dioxolane cytosine, Fig. 4) [61,62], which is in phase II clinical trials as an anti-cancer agent. Moreover, phase III clinical trials of clevudine (L-FMAU) for the treatment of HBV infection were recently halted due to unexpected mitochondrial toxicity [63-65], which was undocumented in preclinical studies (Fig. 4) [66,67]. Biochemical analysis showed L-FMAUTP was not a substrate of mtDNA pol γ or other host polymerases [68] suggesting the possibility of an alternative mechanism of mitochondrial toxicity. Further study is required to understand the mechanism of clevudine toxicity.

5. A Predisposition to NRTI Toxicity

5.1 Single nucleoside polymorphisms in mtDNA pol γ that may be associated with drug-induced toxicity

Long term treatment with NRTIs can lead to mitochondrial toxicity of varying degrees dependent upon the efficiency of incorporation and exonuclease removal by mtDNA pol γ [69]. Neurological syndromes attributable to mtDNA mutations have been linked to NRTI therapy [70]. Among the most toxic is ddC and the least toxic is abacavir spanning a toxicity index range of over 150,000-fold [43]. Although significant strides have been made in characterizing the toxicity of NRTIs, one question that remains unclear was why certain individuals tolerate treatment with NRTIs, whereas others encounter issues with mitochondrial toxicity. Clearly, this is a multi-factorial problem, but one possible answer could be found in a genetically inherited predisposition to NRTI toxicity caused by a mutation or polymorphism in the Pol γ coding sequence. This suggestion has recently been examined in a clinical setting using a pharmacogenomic/toxicogenomic approach to antiretroviral treatment with an emphasis on NRTI therapy and toxicity in HIV/AIDS [69].

In 2007, Yamanaka, et al. described the identification and initial characterization of the first such proposed mutation of Pol γ linked to stavudine (d4T) mediated mitochondrial toxicity [71]. In this report, an HIV infected individual undergoing stavudine treatment, presenting to the clinic with lactic acidosis was found to harbor a homozygous R964C mutation in Pol γ . Steady-state inhibition assays using the exonuclease proficient catalytic subunit alone suggested little change in K_i values for inhibition by ddTTP, AZTTP, or d4TTP between mutant and wild type Pol γ . However, a decrease in specific enzyme activity, as well as steady-state dTTP incorporation rate, was noted [71]. This preliminary kinetic data, coupled with data from experiments demonstrating a significant decrease in mtDNA levels in hetero- and homozygous R964C cultured lymphoblast cell lines in response to d4T treatment, lead to the speculation that the mutation impaired the normal enzymatic activity of the polymerase. Further challenge with d4T treatment then was hypothesized to overcome a threshold for mitochondrial toxicity, leading to lactic acidosis [71].

To address this question at a molecular level, we recently performed a pre-steady-state kinetic analysis to assess the functional impact of this mutation and to determine its effect on natural dNTP and d4TTP binding and incorporation [2]. Our experiments determined that the R964C mutation imparted a 25% decrease in overall dTTP incorporation efficiency relative to wild type. This was caused by a decrease in the maximum rate of polymerization, with no significant change in nucleotide binding affinity. The mutation also imparted a 2-fold *increased* binding affinity for d4TTP by the R964C mutant versus wild type with similar incorporation rates, resulting in a 2-fold increased incorporation efficiency. Therefore, the mutant enzyme was 3-fold less able to discriminate between the natural substrate and d4TTP, implying a mechanism of increased stavudine-induced mitochondrial toxicity [2]. Identification of a Pol γ mutant that demonstrates greater susceptibility to NRTI

inhibition represents an important step in utilizing pharmacogenomics to prevent antiviral drug toxicity.

5.2 Future Possibilities in Pharmacogenomics for NRTI therapy

This goal of pharmacogenomics in NRTI therapy is limited by the identification of additional mutations attributable to the side effects and the prevalence of such mutations. A recent report predicted a link between the E1143G polymorphism, one of seventeen documented polymorphisms of mtDNA pol γ , and an increased prevalence of lipodystrophy in response to d4T treatment [72]. E1143G is found combination with the W748S mutation in patients with ataxia-neuropathy syndrome and progressive external ophthalmoplegia (PEO) patients [73,74]. Biochemical analyses of the single mutants and the double mutant were performed illustrating the effect of each mutation on Pol γ activity. The E1143G polymorphism demonstrated increased steady-state catalytic activity, DNA binding affinity, and thermostability relative to the wild-type enzyme, with no apparent change in fidelity [75]. This improvement in polymerase activity served to counteract the decrease in DNA binding and catalytic activity of the W748S mutant, as the double mutant exhibited increased DNA binding affinity, polymerase activity, compared to the W748 mutant alone, albeit with decreased thermostability [75]. Therefore, further experiments are required for determining how the E1143G mutation alone may be affecting d4T-mediated toxicity. Future experiments will focus on understanding this phenomenon, as well as serve as a call for more genetic analysis of patients demonstrating lactic acidosis and mitochondrial toxicity in response to NRTI treatment.

5.3 Mutation of the mitochondrial DNA polymerase γ and genetic disorders

Mutation of mtDNA pol γ has been linked to several different genetic mitochondrial disorders. These include PEO, Alpers syndrome, parkinsonism, male infertility, SANDO (sensory ataxic neuropathy, dysathria, and ophthalmoparesis) among others. Several excellent reviews are available on this topic (see [41,76] and other chapters in this issue).

6. Conclusions

Our studies using a transient kinetic approach have defined the interaction of a number of clinically relevant nucleoside analogs with the viral target enzyme and the host enzyme that have provided important molecular insights. This analysis has allowed us to identify key structural and kinetic features that may begin to allow us to predict which analogs would be the most active with RT while being the least toxic to mitochondria based upon interactions with the mtDNA Pol γ . This analysis has defined a toxicity index based upon the increased time required to replicate the mitochondrial genome in the presence of NRTIs [43-45]. The toxicity index reflects the toxicity observed when these NRTIs are assayed at a cellular level and clinically. These studies provided an explanation for the toxicity of FIAU as well as the very wide range of toxicities that are observed for those nucleoside analog currently used clinically. Moreover, the toxicity index can be used as a tool to predict potential toxicity for new NRTIs and other compounds at very early stages of development as well as clinical observations at a molecular level.

The profile for an ideal NRTI would have high incorporation efficiency for the viral polymerase and a low efficiency and toxicity index for the mitochondrial polymerase. This unique assessment of NRTIs in the manner allows one to rationalize, predict, and prioritize potential novel therapies.

Moreover, comprehensive structure-function studies are essential for delineating mechanisms of mitochondrial disease, as well as the design of safer, less toxic nucleoside

analogs. Many mutations of Pol γ associated with mitochondrial disease have yet to be characterized biochemically. We believe that using a pre-steady-state kinetic approach to examine mutant forms of Pol γ will allow more precise assessment of nucleotide incorporation efficiency and may yield important mechanistic data that would contribute to understanding mitochondrial disease.

7. Appendix: Resources for Performing Transient Kinetic Analysis

1. A more complete, detailed description of equipment, experimental protocols, and kinetic data analysis is provided in, "Kinetic Analysis of Macromolecules: A Practical Approach, Oxford Press, Johnson, K.A. Ed. [28].
2. Equipment to conduct rapid chemical quench and stopped-flow absorbance and fluorescence studies can be found at: <http://www.kintek-corp.com>
3. A comprehensive hands on training class for using rapid kinetic equipment and collecting and processing data is also available. See the 8th Annual "Dawn of the New Enzymology Kinetics Workshop" in Summer 2010 at <http://kintek-corp.com/workshop/index.shtml>

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Figure 1.
Comparison of Steady-state and Transient Kinetics

Figure 2.
Schematic of a pulsed-quench-flow apparatus [21,23].

Stopped-flow Fluorescence

Figure 3.
Schematic of stopped-flow fluorescence apparatus.

Scheme 1.

Scheme 2.

Kinetic pathway for Pol γ polymerization. Schematic demonstrating rate constants for dNTP incorporation and removal by mtDNA Pol γ as determined by pre-steady state kinetic analysis. Figure adapted from [38].

Figure 4.
Structures of clinically relevant antiviral and anticancer nucleoside analogs

Table 1Discrimination of Nucleoside Analogs by mtDNA pol γ

AnalogTP	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\text{s}^{-1} \mu\text{M}^{-1}$)	Discrimination ^a
ddCTP	0.66 ± 0.02	0.041 ± 0.007	16	2.9
ddATP	0.31 ± 0.02	0.022 ± 0.009	14	4
d4TTP	0.24 ± 0.01	0.045 ± 0.007	5.3	7.4
(-)-3TCTP	0.125 ± 0.005	9.2 ± 0.9	0.014	2,857
PMPApp	0.21 ± 0.01	40.3 ± 5.7	0.005	11,400
AZTTP	0.20 ± 0.04	187 ± 54	0.001	37,103
(-)-FTCTP	0.0086 ± 0.001	62.9 ± 8.4	0.00014	288,800
CBVTP	0.0018 ± 0.0001	13 ± 1.5	0.00014	902,777

^a defined as ratio of k_{pol}/K_d (natural dNTP) / k_{pol}/K_d (AnalogTP)

Table 2Differential dNTP selectivity for mtDNA pol γ : Stereochemistry & Fluorine Substitution

dNTP	k_{pol} (s^{-1})	K_{d} (μM)	$k_{\text{pol}}/K_{\text{d}}$ ($\text{s}^{-1} \mu\text{M}^{-1}$)	Discrimination ^a
dCTP	44 ± 2	1.1 ± 0.1	40	-
ddCTP	0.66 ± 0.02	0.041 ± 0.007	16	2.9
(+)-3TCTP	0.35 ± 0.01	1.5 ± 0.1	0.23	173
(-)-3TCTP	0.125 ± 0.005	9.2 ± 0.9	0.014	2,857
(+)-FTCTP	0.84 ± 0.03	0.79 ± 0.07	1.1	36
(-)-FTCTP	0.0086 ± 0.001	62.9 ± 8.4	0.00014	288,800

^a defined as ratio of $k_{\text{pol}}/K_{\text{d}}$ (natural dNTP) / $k_{\text{pol}}/K_{\text{d}}$ (AnalogTP)