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3′-Azido-3′-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria

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Abbreviations:
AIC, Akaike's information criterion
AIDS, acquired immunodeficiency syndrome
AZT, 3′-azido-3′-deoxythymidine
AZTMP, 3′-azido-3′-deoxythymidine-5′-monophosphate
AZTTP, 3′-azido-3′-deoxythymidine-5′-triphosphate
IC_{50}, 50% inhibitory concentration
HAART, highly active anti-retroviral therapy
HIV, human immunodeficiency virus
NRTI, nucleoside analog reverse transcriptase inhibitor

ABSTRACT

Long-term use of 3′-azido-3′-deoxythymidine (AZT) is associated with various tissue toxicities, including hepatotoxicity and cardiomyopathy, and with mitochondrial DNA depletion. AZT-5′-triphosphate (AZTTP) is a known inhibitor of the mitochondrial DNA polymerase γ and has been targeted as the source of the mitochondrial DNA depletion. However, in previous work from this laboratory with isolated rat heart and liver mitochondria, AZT itself was shown to be a more potent inhibitor of thymidine phosphorylation (IC_{50} of 7.0 ± 1.0 μM AZT in heart mitochondria and of 14.4 ± 2.6 μM AZT in liver mitochondria) than AZTTP is of polymerase γ (IC_{50} of >100 μM AZTTP), suggesting that depletion of mitochondrial stores of TTP may limit replication and could be the cause of the mitochondrial DNA depletion observed in tissues affected by AZT toxicity. The purpose of this work is to characterize the nature of AZT inhibition of thymidine phosphorylation in isolated rat heart and rat liver mitochondria. In both of these tissues, AZT was found to be a competitive inhibitor of the phosphorylation of thymidine to TMP, catalyzed by thymidine kinase 2. The inhibition constant (K_i) for heart mitochondria is 10.6 ± 4.5 μM AZT, and for liver mitochondria K_i is 14.0 ± 2.5 μM AZT. Since AZT is functioning as a competitive inhibitor, increasing thymidine concentrations may be one mechanism to overcome the inhibition and decrease AZT-related toxicity in these tissues.

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1. Introduction

AZT is an analog of the thymidine deoxynucleoside and is a member of the class called the nucleoside-analog reverse transcriptase inhibitors. AZT and other members of this class function by inhibiting the HIV reverse transcriptase. This halts the life cycle of the virus and slows the progression of AIDS. Originally, AZT was given at a high dosage in monotherapy, and long-term treatment resulted in many tissue toxicities, including myopathies, dilated cardiomyopathy, and hepatotoxicity [1–11]. When AZT was withdrawn from the patient's therapy regimen, these toxicities would resolve, suggesting that they are due to AZT and are not symptoms of AIDS [6,8]. The modern HAART regimen uses lower dosage AZT in combination with other drugs. This has caused these toxicities to become rare today but has lead to new problems related to AZT therapy, including lipodystrophy and hematological toxicities [12,13].

Mitochondrial damage due to mitochondrial DNA depletion is believed to be the cause of these toxicities [14–16]. The current prevailing hypothesis for NRTI toxicity is that the NRTI-triphosphate is inhibiting the mitochondrial DNA polymerase γ [17]. However, this mechanism seems less likely for AZT which is not readily phosphorylated beyond AZTMP since it is a poor substrate for thymidylate kinase [18,19]. As a result of this, AZTTP has never been detected at a concentration high enough to inhibit polymerase γ (IC_{50} of >100 μM) [16,18,20].

Previous work from this laboratory has suggested an alternative mechanism in which AZT inhibits the phosphorylation of thymidine. Trials in isolated rat heart and liver mitochondria carried out at 1 μM thymidine, the approximate physiological concentration [21], revealed an IC_{50} of 7.0 ± 1.0 μM AZT for heart mitochondria and 14.4 ± 2.6 μM AZT for liver mitochondria [22,23]. These IC_{50} values reflect inhibitory concentrations of AZT, not AZTTP, and are considerably lower than the concentration of AZTTP needed to inhibit polymerase γ. The steady-state 1.5 h post-dose peak serum concentration seen with chronic oral administration of 250 mg AZT every 4 h is 2.32 μM with a range of 0.19–5.46 μM [24]. This dosage is slightly higher than the recommended dosage of oral AZT of 200 mg every 4 h used in monotherapy; however, both of these dosages are in the range of dose-independent kinetics. The observed IC_{50} data from heart and liver mitochondria is close to these serum concentrations and suggests that AZT inhibition of thymidine phosphorylation could play a role in the clinical toxicity observed in AZT monotherapy. In the present day HAART regimen, AZT is dosed at 600 mg total per day (either 200 mg three times per day or 300 mg twice per day), resulting in a steady-state serum AZT concentration of ~0.8 μM [25]. This lower serum concentration may explain why the toxicities associated with the higher dosage monotherapy have become rare in current regimens.

Non-mitotic tissues, like heart and liver, may depend solely on thymidine kinase 2 to salvage thymidine and maintain the intracellular TTP pool. Given this inhibition, it is possible that in these tissues AZT inhibition of thymidine phosphorylation is depleting the TTP pool, which slows mitochondrial DNA replication and could lead to the observed mitochondrial DNA depletion associated with AZT toxicity. Since AZT and thymidine both are phosphorylated by the same enzyme, thymidine kinase 2, in isolated heart and liver mitochondria, it is likely that the observed inhibition is due to AZT directly competing with thymidine for phosphorylation by thymidine kinase 2. In this work, we hypothesize that AZT inhibition of thymidine phosphorylation best fits the model of competitive inhibition.

2. Materials and methods

2.1. Isolation and incubation of rat heart and liver mitochondria

Mitochondria were isolated from Harlan–Sprague–Dawley rat heart and liver using the methods described previously [22,23]. The isolated mitochondria were then incubated at 30 °C in a medium defined previously [22,23]. Concentrations and specific radioactivities of [methyl-^{3}H]-thymidine used in these incubations is noted in the figure legends.

2.2. Detection of mitochondrial phosphorylation of thymidine and AZT by direct precipitation

A 0.2 mL aliquot of the incubation medium was removed at various time points during incubation and was mixed with an equal volume of 10% trichloroacetic acid. This mixture was kept on ice for at least 10 min and then centrifuged. 0.35 mL of the acid-soluble supernatant were removed and neutralized with 350 mg of resin (AG-11A8) and 0.21 mL of water. The neutralized extract was filtered and analyzed by HPLC as described below. This method yields the total of the nucleoside and nucleotide components found in the medium and in the acid-soluble portion of the mitochondrial matrix and does not differentiate between phosphorylation within the matrix and phosphorylation outside of the matrix. However, previous work has demonstrated that phosphorylation is a matrix event [22].

2.3. HPLC analysis

[Methyl-^{3}H]-thymidine and its phosphorylated intermediates from the neutralized acid-soluble extract described in the previous section were identified and quantitated using reverse-phase HPLC with an Alltech absorbosphere nucleoside/nucleotide column connected to an in-line UV monitor (254 nm) and a Radiomatic flow-through scintillation counter using methods described previously [22].

2.4. Data treatment

Data was analyzed with Enzyme Kinetics Pro v2.36 to obtain values for AIC and K_i ± standard deviation. Graphs were generated with Sigma Plot 9.01.

3. Results

3.1. Time course of thymidine phosphorylation

Prior work from this laboratory has shown that thymidine phosphorylation in isolated rat heart and liver mitochondria is

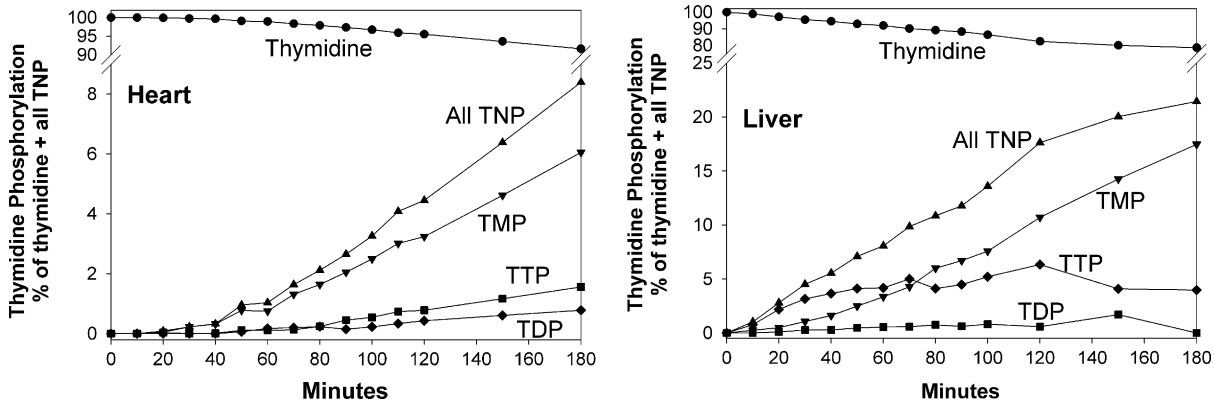

Fig. 1 – Time courses of thymidine phosphorylation in isolated rat heart and liver mitochondria. Mitochondria were incubated with 1 μM [methyl-^{3}H]-thymidine (8000 dpm pmol^{-1}), and the acid-soluble extracts were separated by HPLC as described in Section 2. In each tissue, data represents the mean of two trials and is expressed as the percentage of the sum of thymidine, TMP, TDP, and TTP (1.04 ± 0.08 pmol μl^{-1} for heart and 1.04 ± 0.14 pmol μl^{-1} for liver). The "All TNP" line represents the sum of TMP, TDP, and TTP.

nearly linear over time. This time course was extended to 3 h with more frequent sampling over the course of the incubation (Fig. 1). These trials were conducted with the addition of 1 μM [methyl-^{3}H]-thymidine and no AZT to the media. In this time course, TMP is the dominant phosphorylated form of thymidine in both heart and liver mitochondria, with the exception of the early time points in the liver mitochondria incubation where TTP was slightly higher than TMP. The total of all phosphorylated forms of thymidine increases linearly throughout the time course in liver mitochondria, and it increases linearly in heart mitochondria after an approximately 30 min delay.

3.2. *AZT inhibition of thymidine phosphorylation*

Isolated rat heart and liver mitochondria were incubated separately with the addition of [methyl-^{3}H]-thymidine (1–64 μM) and unlabeled AZT (0, 2, 8, and 20 μM) to the media. All samples were incubated for 2 h and the amount of thymidine phosphorylation observed was converted to a per hour rate. Graphical analysis of the rate data, using Lineweaver–Burk plots (Fig. 2), shows that the *y*-intercepts, representing the inverse of the V_{max}, are the same regardless of AZT concentration. The *x*-intercepts, representing the negative inverse of K_m, increase with increasing AZT concentrations.

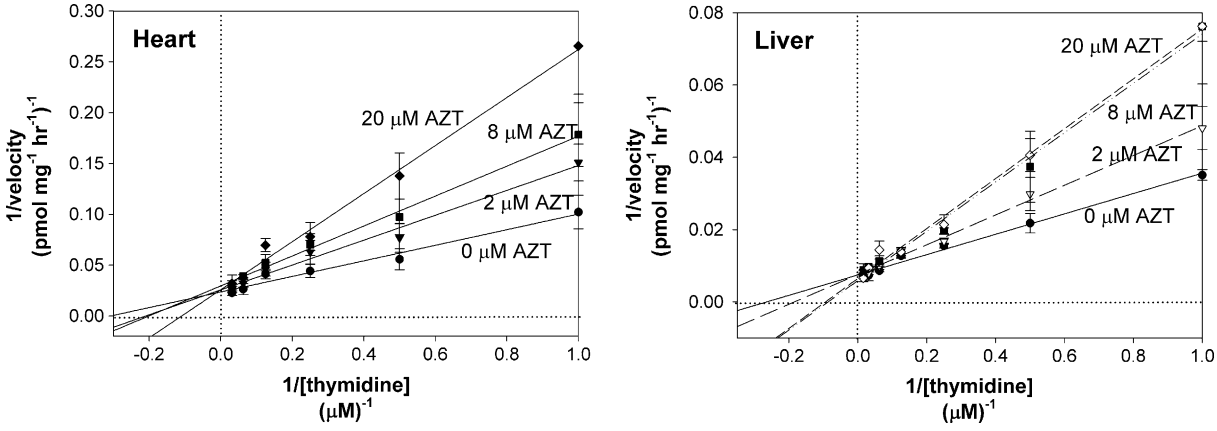

Fig. 2 – Inhibition of thymidine phosphorylation by AZT in isolated rat heart and liver mitochondria. Isolated rat heart and liver mitochondria were incubated separately for 120 min with [methyl-^{3}H]-thymidine and unlabeled AZT. AZT concentrations were 0, 2, 8, and 20 μM, and thymidine concentrations were 1, 2, 4, 8, 16, 32, and 64 μM. The specific radioactivities were 8000 dpm pmol^{-1} for samples containing 1, 2, 4, and 8 μM [methyl-^{3}H]-thymidine, and 5600, 2800, and 1400 dpm pmol^{-1} for samples containing respectively 16, 32, and 64 μM [methyl-^{3}H]-thymidine. The rate of thymidine phosphorylation in the acid soluble extracts of the incubated mitochondria was determined as described in Section 2. Data from three independent trials in each tissue are represented as the mean ± standard error of the mean. Inverse of the *y*-intercept is the apparent V_{max}, and negative inverse of the *x*-intercept is the apparent K_m.

Table 1 – Best fit analysis for AZT inhibition of thymidine phosphorylation to the mathematical models of inhibition

Inhibition model	AIC—heart mitochondria	AIC—liver mitochondria
Competitive	196.4	249.4
Noncompetitive	291.3	∞ (no convergence)
Uncompetitive	302.3	352.1
Mixed	198.0	∞ (no convergence)

This pattern of increasing K_m with no change in V_{max} as the concentration of inhibitor is increased suggests that the data best fits the model of competitive inhibition.

This conclusion was further corroborated with additional analysis using Enzyme Kinetics Pro v2.36. The data for AZT inhibition of thymidine phosphorylation was fit to the mathematical models of four types of inhibition, competitive, noncompetitive, uncompetitive, and mixed (Table 1). This analysis revealed that the model for competitive inhibition provides the best fit of the data for both heart and liver mitochondria as demonstrated by having the lowest AIC value [26]. Using the competitive inhibition model, values of K_i for AZT inhibition were calculated to be 10.6 ± 4.5 μM AZT for heart mitochondria and 14.0 ± 2.5 μM AZT for liver mitochondria.

Using the same data, the rates for phosphorylation of TMP to TDP and of TDP to TTP were calculated at each thymidine concentration for 0, 2, 8, and 20 μM AZT (data not shown). A comparison of these rates suggests that AZT has no effect on either of these reactions. These data support the hypothesis that AZT is a competitive inhibitor specifically of thymidine kinase 2.

4. Discussion

This work provides additional support to the alternative mechanism of toxicity proposed in prior work from this laboratory [22,23]. In this mechanism, AZT competitively inhibits thymidine kinase 2 in non-mitotic tissues, such as heart and liver. Since thymidine kinase 1 is only expressed during S phase of the cell cycle [27], thymidine kinase 2 is the only route for non-mitotic tissue to salvage and phosphorylate thymidine. With insufficient thymidine phosphorylation, it is possible that the TTP pool would become depleted. Song et al. have shown that imbalances in any of the dNTP pools will cause mitochondrial DNA mutations, deletions, and depletion [28,29]. Therefore, an imbalance in the TTP pool created by AZT inhibition of thymidine phosphorylation could account for the mitochondrial DNA depletion observed with AZT toxicities.

Additional strong support for this mechanism of toxicity comes from the inherited partial deficiency of thymidine kinase 2. In the few cases of this rare human genetic disorder that have been characterized, the patients have presented with a severe mitochondrial myopathy with mitochondrial DNA depletion and have died in early childhood [30,31]. Saada et al. found that in fibroblasts from patients with this disorder, TTP pools were markedly decreased within the mitochondria [32].

Since AZT inhibition of thymidine phosphorylation is competitive, a possible route to overcome the toxicity induced by AZT is to raise the thymidine level in the patient. This increase in thymidine concentration would allow more thymidine to be phosphorylated and would increase the TTP pool. Some similar work in this area has been done with supplementing uridine. Using HepG2 cells, Walker et al. has demonstrated that the addition of uridine to cells previously exposed to several NRTIs, including AZT, helped to lessen the apparent toxicity in vitro [33]. While the mechanism of action for uridine is not described by Walker et al., it is possible that uridine is acting to supplement the intracellular TTP pool. Salvaged uridine can be phosphorylated to UMP and then to UDP. Deoxynucleoside diphosphate reductase acts to convert UDP to dUDP, which is phosphorylated to dUTP and then quickly dephosphorylated to dUMP. Thymidylate synthase converts dUMP to TMP, which is phosphorylated to TTP. This supplementation of uridine does not appear to affect the efficacy of the NRTIs in combating HIV [34].

Human studies are underway to test the ability of mitocnol, a sugar cane extract, to treat the toxicities of various NRTIs [35,36]. Mitocnol raises the serum uridine concentration when administered to patients and may be acting in a similar fashion to the uridine given to the HepG2 cells mentioned previously via supplementation of the intracellular TTP pools in the tissues experiencing NRTI-related toxicity. However, not all tissues express the necessary enzymes to convert uridine to TTP. These tissues may be salvaging deoxyuridine or thymidine released into the blood stream by other tissues that can salvage uridine. Serum concentrations of deoxyuridine and thymidine have not been documented in patients administered mitocnol.

Understanding the mechanism of AZT toxicity can aid in the development of treatments, like mitocnol, for the toxic effects observed clinically with AZT and possibly other NRTIs. This can be particularly useful in maintaining patients on an effective therapy regimen for a longer period of time by lessening the adverse effects they may have otherwise experienced from taking these drugs. In turn, more effective and more tolerable therapies could lead to a better quality of life for patients with AIDS.

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