A computational model of mitochondrial AZT metabolism

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The mechanisms of the mitochondrial toxicity of AZT (azidothymidine; zidovudine) are not clear. The two main contenders are the incorporation of phosphorylated AZT into the mtDNA (mitochondrial DNA) and the competitive inhibition of natural deoxynucleotide metabolism. We have built a computational model of AZT metabolism in mitochondria in order to better understand these toxicity mechanisms. The model includes the transport of non-phosphorylated and phosphorylated forms of AZT into mitochondria, phosphorylation, and incorporation into mtDNA. The model also includes the mitochondrial metabolism of the natural deoxynucleotides. We define three simulated cell types, i.e. rapidly dividing, slowly dividing and postmitotic cells. Our standard simulation indicates that incorporation of AZT into mtDNA is highest in rapidly dividing cells because of the higher mitochondrial AZTTP (3'-azidothymidine-5'-triphosphate)/dTTP ratio in this cell type. However, under these standard conditions the rate of incorporation into mtDNA is too low to be a major

cause of toxicity. These simulations relied on the assumption that phosphorylated AZT is transported with the same kinetics as phosphorylated thymidine. In simulations with mitochondria set to have a limited ability to transport phosphorylated AZT, AZTTP accumulates to toxic levels in the mitochondria of postmitotic cells, while low levels are maintained in mitochondria from rapidly dividing cells. This result is more consistent with the tissue toxicities observed in patients. Our model also predicts that inhibition by AZT of mitochondrial deoxycytidine phosphorylation by thymidine kinase 2 may contribute to the mitochondrial toxicity, since in simulations using a typical peak plasma AZT level the mtDNA replication rate is decreased by 30 % in postmitotic cell simulations.

Key words: antiviral toxicity, AZT, deoxynucleotide metabolism, mitochondrial DNA, polymerase γ , zidovudine.

INTRODUCTION

The current treatment for HIV infection is HAART (highly active anti-retroviral therapy). This therapy typically consists of two or more nucleoside reverse transcriptase inhibitors along with a protease inhibitor [1]. HAART has proven to be very effective in reducing viral load in patients [1], but it is not a cure and must be considered a life-long therapy. The continuation of HAART for many years is complicated by the high incidence of toxic side effects in this therapy. In a study of 862 patients undergoing HAART, 25.5 % of the patients had discontinued the therapy due to toxicity side effects by the end of only 1 year of treatment, whereas only 7.6 % discontinued due to failure of the therapy to control the virus [2].

Anti-retroviral therapies are known to have a wide range of adverse side effects [3]. Arguably the most severe of these is lactic acidosis, which occurs with an incidence of 1.3–3.9 out of 1000 [4]. A milder form of this condition, hyperlactataemia, occurs in an estimated 10–20 % of patients receiving nucleoside analogue drugs [3]. Other severe adverse side effects are cardiomyopathy, peripheral neuropathies, pancreatitis and hepatotoxicity. This is by no means a complete list of the known adverse side effects of HAART, but are those believed to be due to mitochondrial toxicity [5].

Nucleoside analogues are phosphorylated within cells through the same metabolic pathways used by the four natural deoxynucleosides [deoxyadenosine (dA), deoxycytidine (dC), deoxyguanosine (dG), and deoxythymidine (dT)]. Each analogue is a slightly modified form of one of these metabolites and typically shows altered activity with the enzymes that affect the natural

deoxynucleoside [6,7]. Most analogues can be phosphorylated and incorporated into replicating DNA, but lack the chemical structure (3'-OH) necessary for the continuation of DNA polymerization, causing the termination of DNA replication (referred to as 'chain termination'). The analogues that are effective in antiviral treatments are substrates for the viral DNA polymerase in the cytoplasm, but are not good substrates for nuclear DNA polymerases.

Nucleoside analogues that can be phosphorylated to the triphosphate form, in either the cytoplasm or the mitochondrion, can enter the mitochondrial matrix and potentially disrupt the replication of mtDNA (mitochondrial DNA) [5]. This is the most widely accepted explanation for the mitochondrial toxicity of these chemicals. Another possibility is that they inhibit natural deoxynucleotide metabolism through competitive inhibition of enzymes or transporters involved in this metabolism [8–10]. This might alter the relative levels of the dNTP pools within the mitochondria and cause mutations to occur during mtDNA replication [10,11] or mtDNA depletion. Since different sets of deoxynucleotides share enzymes at different points in metabolism (see Figure 2), the extensive coupling between the metabolism of the four natural deoxynucleotides and any nucleoside analogues makes it difficult to predict the effects of competitive inhibitions without a simulation of the coupled metabolism.

AZT (azidothymidine; zidovudine) was the first nucleoside analogue approved to combat HIV, and continues to be a major component of HAART. Like other nucleoside analogues, specific tissue toxicities are associated with the prolonged use of AZT. Symptoms of AZT toxicity often include mitochondrial myopathy and cardiomyopathy with mtDNA depletion in patients, but other

Abbreviations used: AZT, azidothymidine (zidovudine); AZTDP, 3'-azidothymidine-5'-diphosphate; AZTMP, 3'-azidothymidine-5'-monophosphate; AZTTP, 3'-azidothymidine-5'-triphosphate; DNC, deoxynucleotide carrier; HAART, highly active anti-retroviral therapy; mtDNA, mitochondrial DNA; NDPK, nucleoside diphosphate kinase; PBMC, peripheral blood mononuclear cells; pol- γ , mtDNA polymerase- γ ; TK2, thymidine kinase 2; TMPK, thymidine monophosphate kinase; the superscript 'mt' is used to denote concentrations within the mitochondrion.

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Scheme 1 General deoxynucleoside metabolism in mitochondria

Nucleoside reverse transcriptase inhibitors follow the same metabolic pathways as their natural analogue forms, and can interact with them through competitive inhibition. They can also interact with pol- γ to disrupt mtDNA replication.

symptoms may occur. AZT also causes increased oxidative stress that may lead to mtDNA mutations [12]. Furthermore, AZT may be converted into D4T (stavudine), a slightly more toxic nucleoside analogue in patients [13]. The exact mechanism by which AZT causes toxicity is not known.

Initial studies with purified pol- γ (mtDNA polymerase- γ) indicated that this enzyme is the target of inhibition by AZTTP (3'-azidothymidine-5'-triphosphate) [14]. Additional studies, in which AZT inhibited mtDNA replication in isolated rat liver mitochondria [15], were viewed as support for this mechanism. However, more recent studies indicated that AZTTP is not formed in detectable amounts in isolated rat liver [9] or rat heart mito-

chondria, or even in the entire perfused rat heart [10]. AZTTP was demonstrated to have similar pol- γ kinetics as other nucleoside analogue triphosphates that are much less toxic [16]. AZT was also shown to cause an immediate decrease in the cellular ATP level prior to mtDNA depletion [17]. These studies, along with cell culture studies implicating AZTMP (3'-azidothymidine-5'-monophosphate) as the toxic metabolite [8,9], cast doubt on the assumption that AZTTP is the primary toxic metabolite in the mitochondrion.

We have previously published a model of natural mitochondrial deoxynucleotide metabolism and mtDNA replication [18]. We refer the reader to that paper for details of the modelling of natural deoxynucleotide metabolism. Here we have added to that model a network of biochemical reactions describing the transport of phosphorylated and non-phosphorylated AZT between the cytoplasm and the mitochondrial matrix, the phosphorylation of AZT, and the incorporation of AZT into mtDNA. Scheme 1 describes the general metabolism of the system, emphasizing the relationship between cytoplasmic AZT and deoxynucleotide metabolism. Scheme 2 shows the complete set of transport and phosphorylation reactions within the mitochondrion. The deoxynucleotides that act as competitive inhibitors of AZT metabolism are different for different phosphorylation reactions, leading to a complicated net of parallel reactions that directly or indirectly affect AZT metabolism. Using these simulations, we sought to clarify the cell type-dependent variation in AZT toxicity.

MATERIALS AND METHODS

Mitochondrial deoxynucleoside and deoxynucleotide transport and phosphorylation were modelled using Michaelis–Menten and Hill enzyme kinetics [18], except for the polymerization reaction, for which we have developed our own model. Differential equations using these kinetic equations were constructed to define the

Scheme 2 Mitochondrial deoxynucleotide metabolism with inhibitions

The reactions inside the boxes represent those occurring in the mitochondrial matrix space. The 'T-intersection' symbol is used here to represent inhibition, and the inhibiting substrates are listed beside each inhibition symbol. All of these inhibitions are modelled as competitive inhibitions of substrate binding. For emphasis, inhibitions due to the various forms of AZT are shown in italics. Enzymes that are believed to exist, but for which a specific gene has not been identified, are denoted by question marks.

Table 1 Cytoplasmic AZT, AZTDP and AZTTP concentrations used

Cytoplasmic AZTMP levels are not needed, since this metabolite is not transported into the mitochondrion. AZT levels were taken from [45] and are assumed to be constant in all cell types. AZTDP levels were set equal to AZTTP levels [45]. AZTDP and AZTTP values were set 10-fold lower than dTTP levels, using data from patient PBMC [20]. The range of values is listed in parentheses next to the mean value.

Metabolite	Concentration (μM)		
	Rapidly dividing cells	Slowly dividing cells	Postmitotic cells
AZT	0.15	0.15	0.15
AZTDP and AZTTP	2 (0.8–3.8)	0.25 (0.01–0.56)	0.025 (0.001–0.056)

rate of change of the mitochondrial deoxynucleotide metabolite pools over time. These equations are described in the Appendix.

Numerical solution of the differential equations

To solve the system of differential and algebraic equations in our model, the simulation was implemented in Mathematica (version 5). The Mathematica numerical differential equation solver uses an adaptively selected step size, and switches automatically between the Adams Predictor–Corrector Method for non-stiff and Backward Difference Formulas (Gear Method) for stiff differential equations. A human readable version of the Mathematica code is available (see Supplementary Material at <http://www.BiochemJ.org/bj/392/bj3920363add.htm>).

Defining simulated cell types

We define three broad categories of cells, i.e. rapidly dividing cells, slowly dividing cells and postmitotic cells, with mitochondrial protein levels constant across these three categories, as described in [18]. We distinguish each cell category by the cytoplasmic dNDP and dNTP concentrations, as published previously [18]. Cytoplasmic deoxynucleotide levels are lowest in postmitotic cells and highest in cells that are rapidly dividing, due to the cell cycle regulation of deoxynucleotide synthesis [19]. Cytoplasmic deoxynucleoside and AZT nucleoside concentrations were kept at a constant level in all three cell categories. In all cell categories cytoplasmic AZTDP (3'-azidothymidine-5'-diphosphate) and AZTTP concentrations (Table 1) were set 10-fold lower than the dTTP concentration, as measured in patient PBMC (peripheral blood mononuclear cells) [20].

Unknowns in the model

Kinetic data are lacking for some of the enzymes and transporters of mitochondrial AZT metabolism, including TMPK (thymidine monophosphate kinase), NDPK (nucleoside diphosphate kinase, and the DNC (deoxynucleotide carrier). For TMPK and NDPK, kinetic parameters from homologous cytoplasmic enzymes were used [21,22]. For the DNC, kinetic values for the analogous thymidine deoxynucleotide were used [6] as our standard values, and the effects of varying the K_m for the DNC acting on AZT were explored using the simulation.

RESULTS

Mitochondrial AZT and thymidine deoxynucleotide levels

In order to avoid confusion between cytoplasmic and mitochondrial concentrations, in the following we use the superscript 'mt' to denote concentrations within the mitochondrion. In Figures 1(A), 1(C) and 1(E) we show simulation results for dT^{mt}, dTMP^{mt}, dTDP^{mt} and dTTP^{mt} levels through the replication of one mtDNA molecule in a simulated rapidly dividing, slowly dividing and

Figure 1 Mitochondrial concentrations of the different phosphorylated thymidine and AZT forms at steady state and during one mtDNA replication event

(A, B) Rapidly dividing cells; (C, D) slowly dividing cells; (E, F) postmitotic cells.

postmitotic cell respectively. The simulation begins in a steady state. At time 100 min in rapidly dividing cells, and at times 200 and 600 min in slowly dividing and postmitotic cells respectively, we begin the replication of the mtDNA molecule. The dTTP pools in the mitochondrion are drained significantly during the mtDNA replication event. The deoxynucleotide concentrations equilibrate to levels where the net influx rate matches the loss due to the mtDNA polymerization. The deoxynucleotides reach different steady-state levels during the beginning, middle and end of polymerization due to the asynchronous strand replication of mtDNA [23] and the biased deoxynucleotide compositions on each strand. For further details of the mtDNA replication model, see [18].

Mitochondrial AZT metabolite levels during an mtDNA replication event are shown in Figures 1(B), 1(D) and 1(F). In the simulated rapidly dividing cells, these levels quickly reach a steady state, with AZTDP^{mt} and AZTTP^{mt} levels being higher than AZT^{mt} or AZTMP^{mt} levels. This is in contrast with cytoplasmic levels, where experiments have shown AZTMP levels to be the highest. These differences in the mitochondrial and cytoplasmic concentrations of AZTMP occur because AZTMP is not transported across the mitochondrial inner membrane [10]. The AZTMP^{mt} concentration is highest (compared with the other phosphorylated states) in slowly dividing and postmitotic cells. When mtDNA replication begins, AZTMP^{mt} levels rise substantially. This is in contrast with the natural deoxynucleotides, levels of which generally fall during replication. A fall in the dTTP^{mt} and dCTP^{mt} levels is responsible for this rise in the AZTMP^{mt} level, as competitive inhibitions on TK2 (thymidine kinase 2), the enzyme that catalyses the phosphorylation of the AZT^{mt} nucleoside, are

Figure 2 The AZTTP and dTTP concentrations during mtDNA replication determine the amount of AZTTP incorporated into mtDNA

(A) Mean dTTP concentration during replication on varying the cytoplasmic dNDP and dNTP concentrations in the three different cell types (using definitions from [18]). (B) Incorporation of AZT into mtDNA. Cytoplasmic deoxynucleotides were increased over the physiological range as in (A). Cytoplasmic AZTDP and AZTTP concentrations were maintained 10-fold lower than that of dTTP.

released. Note that the rise in AZTMP^{mt} does not carry through to the AZTDP^{mt} and AZTTP^{mt} pools. The action of the DNC buffers these concentrations in the mitochondrion.

AZT incorporation into mtDNA

Since the incorporation of AZT into mtDNA is thought to be controlled by the AZTTP/dTTP ratio in the mitochondrion [24], we examined the average levels of dTTP^{mt} (Figure 2A) and AZTTP^{mt} during replication in the three simulated cell categories, i.e. rapidly dividing cells, slowly dividing cells and postmitotic cells. We varied the cytoplasmic deoxynucleotide concentrations to cover the range observed experimentally and defined by the three cell categories [18]. In the model we set the cytoplasmic AZTDP and AZTTP concentrations to 10-fold lower than the dTTP concentration, a ratio that has been measured in patient PBMC [20,25]. During the simulations, AZTTP^{mt} equilibrates with the cytoplasmic AZTTP concentration in all cell types. Mitochondrial dTTP^{mt} levels are higher than the cytoplasmic levels in postmitotic cells, whereas they fall below cytoplasmic levels in dividing cells (Figure 2A). Therefore the average AZTTP/dTTP ratio in the mitochondrion during replication increases with the mitotic status of the cell (Supplementary Figure S1; <http://www.BiochemJ.org/bj/392/bj3920363add.htm>).

We modelled the incorporation of AZT into the mtDNA as a competition between dTTP^{mt} and AZTTP^{mt} (for the mathematical details of the model, see the Appendix). We plotted the amount of AZT incorporation during a single replication event in our model throughout the range of cytoplasmic deoxynucleotide concentrations defining the three different simulated cell categories (Figure 2B). The highest rate of incorporation of AZT in these simulations in any cell type is 0.2 molecules per replication event, or one AZT incorporation for every five times an mtDNA molecule replicates. Since values for AZT incorporation per mtDNA replication event are <1, they must be interpreted as probabilities. The highest incorporation rate occurs in rapidly dividing cells, with slowly dividing cells having lower rates of incorporation. These different incorporation rates occur even though the cytoplasmic AZTTP/dTTP ratios were set to the same value in all cell types. The mitochondrial AZTTP/dTTP ratio is increased in rapidly dividing cells for two reasons. First, the steady-state dTTP^{mt} level in rapidly dividing cells is lower than the cytoplasmic level, while it is higher than the cytoplasmic level in postmitotic cells. Secondly, dTTP^{mt} pools are drained to a greater degree

Figure 3 AZT incorporation into mtDNA

The cytoplasmic AZTTP/dTTP ratio was varied using the standard conditions for cytoplasmic dNDP and dNTP levels and varying AZTTP. AZTDP was set equal to AZTTP in all cell types. The horizontal line denotes the range of values reported from patient PBMC [20,25]. The vertical arrow denotes the ratio used in our standard simulations.

during replication in rapidly dividing cells than in postmitotic cells.

The effect of varying the cytoplasmic AZTTP/dTTP ratio on the amount of AZT incorporated into mtDNA for the three simulated cell categories is shown in Figure 3. Ratios of 0.02, 0.10 and 0.20 have been measured in PBMC from different patients [20,25]. In our simulations, the cytoplasmic AZTTP/dTTP ratio must be nearly 0.25 in rapidly dividing and 0.4 in slowly dividing cells to yield an average of one AZTTP incorporation per mtDNA replication event. AZTTP/dTTP ratios have not yet been measured in tissues such as heart or muscle that seem to be the most adversely affected by AZT.

Flux of AZT through the mitochondrion

Mitochondrial deoxynucleotide metabolism consists of a reversible phosphorylation chain and transporters for the non-phosphorylated and most phosphorylated metabolites. Therefore mitochondria can be in a state of net AZT phosphorylation, where AZT enters the mitochondrion through the nucleoside transporter and exits the mitochondrion into the cytoplasm through the DNC in a phosphorylated state (Scheme 1). Alternatively, a reverse flow of AZT through the mitochondrion would result in a state of net AZT dephosphorylation. The simulated net mitochondrial AZT transport fluxes for the three cell types outside mtDNA replication events are shown in Supplementary Figure S2 (<http://www.BiochemJ.org/bj/392/bj3920363add.htm>). In the mitochondria of postmitotic and slowly dividing cells a net phosphorylation of AZT occurs, while in rapidly dividing cell mitochondria a net dephosphorylation occurs.

In order to understand the relative importance of AZT phosphorylation in the mitochondrial and cytoplasmic compartments, we performed simulated labelling experiments to determine whether the AZT incorporated into mtDNA in postmitotic cells is phosphorylated in the cytoplasm before transport into the mitochondrion (DNC transport) or whether it is transported as AZT into the mitochondrion first and then phosphorylated (nucleoside transport). Results indicated that 97% of the AZT incorporated in mtDNA is transported by the DNC. Even when the cytoplasmic AZT concentration is increased to 5 μM, 91% of the incorporated AZT is transported by the DNC. The percentage is even higher in the dividing cell types. Therefore it appears that phosphorylation of AZT by TK2 and TMPK within the mitochondrion

Figure 4 Effects of varying the K_m of the DNC for AZTDP and AZTTP on the rate of incorporation into mtDNA

A higher K_m indicates a lower transport rate. The K_m of AZTTP was varied from the K_m of dTTP, and the K_m of AZTDP was varied from the K_m of dTDP. Standard cell type definitions were used.

plays no significant role in the subsequent generation of mitochondrial AZTTP under our standard simulation conditions. Almost all mitochondrial AZTTP incorporated into mtDNA is transported directly from the cytoplasm.

Varying the kinetics of AZTDP and AZTTP transport

The kinetic parameters for the transport of AZTDP and AZTTP either by isolated mitochondria or directly by the mitochondrial DNC have not been measured, to the best of our knowledge. The kinetic parameters for dideoxy analogues with the DNC have been measured and are of the same magnitude as for the corresponding natural deoxynucleotides [6]. Based on this, we have applied the kinetic parameters for dTDP and dTTP transport to AZTDP and AZTTP transport by the DNC as our standard simulation condition. Given the lack of information on the transport kinetics, we have used the simulation to explore how varying the K_m for AZTDP and AZTTP for the DNC affects the incorporation of AZT into the mtDNA (Figure 4). From these simulations, we see that the K_m values for AZTDP and AZTTP with the DNC must be at least 80 times larger in postmitotic cells and 450 times larger in slowly dividing cells than the K_m s for the corresponding thymidine deoxynucleotides in order for the probability of incorporation of AZT to exceed one molecule per mtDNA replication. Changing the kinetics of the DNC does not greatly affect AZT incorporation in rapidly dividing cells. The reason for this is that higher dCTP and dTTP levels in these simulated cells inhibit TK2-mediated phosphorylation of AZT.

How varying AZT levels affects incorporation into mtDNA

Plasma levels of AZT can vary greatly from the time of ingestion of the drug until the next dose. AZT equilibrates across the plasma membrane into the cytoplasm in some cell types [26]. Therefore we performed simulations varying the cytoplasmic AZT concentration in postmitotic and rapidly dividing cells (Supplementary Figure S3; <http://www.BiochemJ.org/bj/392/bj3920363add.htm>). We simulated cytoplasmic AZT concentrations of 0.15, 1 and 5 μ M, the peak concentration found in plasma after a 500 mg oral dose [27]. We also increased the cytoplasmic AZTDP and AZTTP levels in rapidly dividing cells 2-fold for 1 μ M AZT and 3-fold for 5 μ M AZT to approximate the corresponding increase in the phosphorylated levels caused by phosphorylation in the cytoplasm [28]. Increasing the cytoplasmic AZT concentration

from 0.15 to 5 μ M increases AZT incorporation into mtDNA 3–4-fold. In postmitotic cells, this amount of AZT incorporation into mtDNA is still much too low to be toxic. However, in rapidly dividing cells, the increased AZT in the cytoplasm following drug ingestion may have a substantial effect on toxicity.

Inhibition by AZT of mitochondrial deoxynucleotide metabolism

It has been suggested that inhibition of natural deoxynucleotide metabolism by the nucleotide analogues may be responsible for mitochondrial toxicity [10]. Therefore we analysed all of the inhibitions in the model, shown in Scheme 2, for rapidly dividing and postmitotic cells during replication of mtDNA. In the standard simulation the maximum inhibition, defined as $[C_1]/K_i$, due to any form of AZT was 0.1. This occurred in postmitotic cells for the TMPK-catalysed reaction of dTMP^{mt} to dTDP^{mt}, and the inhibition was due to AZTMP^{mt}, which reaches a high concentration during mtDNA replication (see Figure 1F).

Another potentially important inhibition in our model is that of TK2 by AZT^{mt}. The Michaelis–Menten inhibition term $[C_1]/K_i$ for this reaction in our standard model is only 0.075 (0.15/2). When the intracellular AZT concentration equilibrates with peak plasma levels (5 μ M) [26,27,29], this inhibition term increases to 2.5 and would be significant. We have performed metabolic control analysis on simulations of all three cell categories [18]. Results from postmitotic cells indicated that TK2 has the second highest flux response coefficient on the polymerization rate of any enzyme in our model (NDPK had the highest). Therefore inhibition of TK2 by AZT^{mt} is likely to have a significant effect on natural mitochondrial deoxynucleotide metabolism in postmitotic cells.

To explore this point, we performed simulations of rapidly dividing (Supplementary Figure S4; <http://www.BiochemJ.org/bj/392/bj3920363add.htm>) and postmitotic (Figure 5) cell mitochondria, setting the cytoplasmic AZT concentration to 0.15, 1 and 5 μ M. AZT^{mt} and AZTTP^{mt} levels nearly equilibrate with cytoplasmic levels in both rapidly dividing and postmitotic cells. The AZTMP^{mt} concentration only increases 3–4-fold when cytoplasmic AZT is increased over 30-fold. In mitochondria from rapidly dividing cells, AZT does not decrease mitochondrial dCTP and dTTP levels. However, in postmitotic cell mitochondria, an AZT concentration of 1 μ M decreases most deoxypyrimidine reaction rates and average concentrations during replication by approx. 20% (Figures 5A–5D). Raising the cytoplasmic AZT concentration to 5 μ M causes a 55% decrease in phosphorylation by TK2 during replication. This inhibition lowers the rate of the other reactions of mitochondrial deoxypyrimidine metabolism and the concentrations of the pyrimidine deoxynucleotides in the model, leading to a 30% decrease in the polymerization rate.

AZTMP levels

In many cell types in culture, the toxicity of AZT has been correlated with increasing concentrations of AZTMP [9] in the cytoplasm. Increasing AZT concentrations in cell culture (0–10 μ M) gave near linear increases in cellular AZTMP levels and toxicity, while AZTTP levels increased only slightly [30]. In these experiments AZTMP levels in cells can rise to extremely high levels. We note here that AZTMP is the only form of AZT without a direct mechanism of transport between the cytoplasm and the mitochondrion (Scheme 1). In Figure 6(A) we show AZTMP^{mt} levels during and outside mtDNA replication in postmitotic, slowly dividing and rapidly dividing cells. In postmitotic and slowly dividing cells, the AZTMP^{mt} levels decrease as the cytoplasmic AZTDP and AZTTP concentrations increase. In rapidly dividing cells, AZTMP^{mt} levels increase back to near postmitotic levels. AZTMP^{mt} levels do not exceed 1 μ M in any simulated cell type.

Figure 5 Inhibition by AZT of mitochondrial deoxypyrimidine metabolism in postmitotic cells

The values shown are for deoxynucleotide concentrations (A, C and E) and reaction rates (B, D and F). The graphs are organized by the reaction substrates deoxycytidine (A and B), thymidine (C and D) and AZT (E and F). Standard conditions for postmitotic cells were used while varying the cytoplasmic AZT nucleoside concentration.

To determine how AZTMP^{mt} levels may vary in different patients, we varied the ratio of cytoplasmic dTTP to AZTTP for the three cell categories (Figure 6B). The range chosen covers and extends the values measured in HIV patient PBMC [20]. The AZTMP^{mt} level increases moderately in rapidly dividing cells with a high cytoplasmic AZTTP/dTTP ratio, but increases only slightly in slowly dividing and postmitotic cells. Even the increase observed in rapidly dividing cells at a cytoplasmic AZTTP/dTTP ratio of 1 is only roughly equal to the AZTMP levels measured in the cytoplasm of PBMC from these patients (~1–2 μM) [27,29]. Therefore our standard simulations give no indication that the AZTMP^{mt} concentration rises to a potentially toxic level.

DISCUSSION

Mitochondrial transport of phosphorylated AZT

In our model, the transport of phosphorylated AZT metabolites by the DNC plays a very important role in toxicity. The DNC transports both AZT diphosphates and triphosphates. Because of the limited activity of matrix space NDPK for AZTDP^{mt} and AZTTP^{mt}, the flux through the DNC from each of these pools is quite distinct (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/392/bj3920363add.htm>). In postmitotic cells the activity of the DNC plays a partially protective role by transporting AZTDP^{mt} out of the matrix space. At the same time (in our

Figure 6 Mitochondrial AZTMP concentrations

(A) Cytoplasmic AZTDP and AZTTP concentrations were set 10-fold lower than the cytoplasmic dTTP concentration. All cytoplasmic deoxynucleotide concentrations were increased proportionally as in Figure 4. The steady-state AZTMP concentration outside replication or the average AZTMP concentration during replication was plotted. (B) The cytoplasmic AZTTP/dTTP ratio was varied by changing the cytoplasmic AZTDP and AZTTP concentrations for our standard cell type dNDP and dNTP concentration definitions as in Figure 5. The steady-state mitochondrial AZTMP concentration outside replication was plotted.

standard simulation) the small amount of AZT that is incorporated into mtDNA in this cell type is derived almost completely from AZTTP influx through the DNC from the cytoplasm. Therefore the transporter can have both a protective and a deleterious effect.

One hypothesis generated by the model is that AZT incorporation into mtDNA and chain termination of newly synthesized mtDNA strands would occur with a reasonably large probability in postmitotic cells if phosphorylated AZT nucleotides have slow kinetics for transport across the mitochondrial inner membrane, thus concentrating the mitochondrially phosphorylated AZTTP^{mt} inside the mitochondrial matrix space instead of releasing it into the cytoplasm. This is due to the net flow of non-phosphorylated AZT into the mitochondrion and of phosphorylated AZT out of the mitochondrion in postmitotic cells (see Supplementary Figure 3 at <http://www.BiochemJ.org/bj/392/bj3920363add.htm>). The simulations show little AZT incorporation into mtDNA in slowly dividing and postmitotic cell types if the transport kinetics of AZTDP and AZTTP are the same as those of dTDP and dTTP. This points out how experiments measuring AZTDP and AZTTP transport kinetics between the mitochondrion and the cytoplasm may be critical to understanding the mechanism of AZT toxicity. These experiments would test our (possibly) counterintuitive hypothesis that decreased activity of the DNC with AZTDP and AZTTP would lead to greater incorporation and toxicity. This hypothesis also relies on AZTMP^{mt} phosphorylation (within the mitochondrion) that may or may not occur in human cells.

Inhibition by AZT of natural deoxynucleotide metabolism

Another hypothesis generated by the model is that inhibition of natural deoxynucleotide metabolism by AZT may contribute to toxicity in postmitotic cells by decreasing dCTP^{mt} or dTTP^{mt} levels. This could decrease the mtDNA replication rate and lead to mtDNA depletion or an increased rate of mutation. High concentrations of non-phosphorylated AZT occur only transiently after a drug dose. It is possible that the long time scales generally required for the mitochondrial toxicity of AZT may be due to these short-duration (but constantly repeated) periods of increased vulnerability of mtDNA to damage.

Tissue and patient variability of AZT toxicity

The potency of nucleoside analogue antivirals, as well as their toxicity, correlates well with the ratio of the nucleoside analogue reverse transcriptase inhibitor triphosphate to the competing natural dNTP [24]. It is possible the tissue variability in this ratio (including variability in the natural dNTP levels) is the cause of the variability in toxicity. During polymerization in simulated slowly and rapidly dividing cells, the natural mitochondrial dNTPs are drained, and fall to lower concentrations than in the cytoplasm. However, in the postmitotic cell simulations, dTTP^{mt} levels remain higher than the cytoplasmic levels, whereas AZTTP^{mt} levels mirror cytoplasmic AZTTP levels in all cell types. For this reason, the higher mitochondrial AZTTP/dTTP ratio present in dividing cells may contribute to the toxicity of AZT in tissues such as bone marrow and gastrointestinal epithelia, where a high percentage of rapidly dividing cells are located. These tissues were greatly affected in patients undergoing AZT therapy before AZT dosage levels were lowered.

With the current AZT dosage levels, and with the possible exception of infants exposed trans-placentally [31], most toxicities of AZT are associated with postmitotic tissues. Since cytoplasmic dNTP levels are very low in these tissues, mitochondrial phosphorylation of deoxynucleosides is very important to provide dNTPs for mtDNA synthesis. TK2 levels are known to be quite low in muscle [32], and so the tissue is sensitive to perturbations that would lower dTTP synthesis further. The similar muscle myopathies observed in TK2-deficient patients and patients taking AZT suggest that mitochondrial dTTP^{mt} or dCTP^{mt} limitation may lead to both pathologies. However, more data are needed before it can be shown conclusively that AZT significantly inhibits natural deoxynucleotide metabolism *in vivo*.

AZT administration results in toxicity in only approx. 10–20% of patients. What could be responsible for the interpatient variability? The AZTTP/dTTP ratio may be the most important factor here too. This ratio (measured in the cytoplasm) can vary greatly between patients [20,25], and may have a profound effect on the amount of AZT incorporated into mtDNA.

AZT removal from mtDNA and mitochondrial toxicity

In addition to incorporation into mtDNA, removal of incorporated AZT is an important factor in determining the possible toxicity of

AZT. Measurements have indicated that it takes pol- γ 40 min on average to remove an incorporated AZT molecule from the end of a strand of DNA [16]. A toxicity index based upon the incorporation and removal kinetics of nucleotide analogues by pol- γ has been calculated [33]. AZT was calculated to have a toxicity index of 0.4, corresponding to a 40% increase in the time taken to replicate one copy of mtDNA when AZTTP^{mt} and dTTP^{mt} are present at equal concentrations. Another group confirmed the slow kinetics of the removal of incorporated AZT by pol- γ [34]. These data indicate that more than one AZT incorporation per mtDNA replication event is probably needed for toxicity. However, since each AZT incorporated is removed so slowly, only a limited number could be incorporated and removed before mtDNA levels become limiting for the function of the cell.

Model limitations

Two of the most severe limitations in the model are the unknown mitochondrial transport kinetics of AZTDP and AZTTP and the unknown kinetics of AZT metabolites with the mitochondrial isoforms of TMPK and NDPK. As shown in Figure 6, altered kinetics for the transport of phosphorylated AZT would have a large effect on AZT incorporation into mtDNA in postmitotic cells (assuming mitochondrial phosphorylation of AZTMP^{mt} and AZTDP^{mt}). Recent data also indicate that transporters other than the DNC might be responsible for the transport of deoxynucleotides and nucleoside analogue phosphates across the mitochondrial inner membrane [35]. Our simulations indicate that these kinetics are important for the mitochondrial toxicity mechanisms of AZT.

Conclusions

In the current project we have modelled the incorporation of AZT into mtDNA. AZT may also cause mtDNA mutagenesis, which we have not included in the model. AZT also induces other forms of cellular toxicity. Maternally administered AZT is incorporated into the nuclear DNA of the infant *in utero* [31,36]. AZT causes increased mutagenesis, cancer and telomere shortening in perinatal animal models of AZT toxicity [37,38]. In addition, cells in culture show AZT-induced chromosomal instability [39] and cell cycle gene expression changes [40]. All of these factors must be considered in order to gain an understanding into the complex mechanisms of cellular AZT toxicity.

APPENDIX

In this appendix we give the mathematical details of the simulation model, concentrating on AZT metabolism. For further details of the modelling of natural deoxynucleotide metabolism in the mitochondrion, see [18].

Nucleoside transporter and DNC models

An equilibrative nucleoside transporter, allowing deoxynucleosides and AZT [41] to move between the mitochondrial matrix and the cytoplasm, is present in the mitochondrial inner membrane. Michaelis–Menten kinetics with competitive inhibitions are used to model the flux of AZT into and out of the matrix space:

$$R_{AZT} = \frac{V_{\max}[AZT]_{\text{cyto}}}{K_m \left(1 + \sum_i \frac{[C_i]}{K_{i,i}} \right) + [AZT]_{\text{cyto}}} - \frac{V_{\max}[AZT]_{\text{matrix}}}{K_m \left(1 + \sum_i \frac{[C_i]}{K_{i,i}} \right) + [AZT]_{\text{matrix}}} \quad (\text{A1})$$

where C is the concentration of an inhibitor and i is summed over the three inhibitors dA, dC and dT.

The DNC exchanges nucleotide and deoxynucleotide diphosphates and triphosphates across the mitochondrial inner membrane. We modelled the DNC as being able to exchange deoxyribonucleotides for ribonucleotides [6,18]. At the time of publication the kinetics of AZTDP and AZTTP transport into and out of the mitochondrial matrix were unknown, so kinetic values for dTDP and dTTP were used. AZTDP and AZTTP compete with all eight natural deoxynucleotide di- and tri-phosphate substrates for the DNC in the model.

Deoxynucleoside and deoxynucleotide phosphorylation

Our simulation calculates the changing concentrations of the non-phosphorylated and different phosphorylated forms of AZT and natural deoxynucleotides within a mitochondrion. We calculate the reaction rates using the Michaelis–Menten equation including competitive inhibitions. The exceptions to this are the TK2-catalysed phosphorylation reactions of dT and AZT. The kinetic

Table A1 K_m , V_{max} and K_i values for the reactions

The TK2 reaction for AZT has negatively co-operative Hill kinetics, with h denoting the Hill coefficient. V_{max} values were multiplied by the enzyme molecular mass in kDa and the number of enzymes per mitochondrion [18] to yield the units of molecules/min per mitochondrion used in the simulation. Values were taken for nucleoside transport from [41], TK2 from [42], dNT-2 from [44], TMPK from [21], NDPK from [22], AZTDP and AZTTP transport from [6], and pol- γ from [16]. AZT nucleoside also has a K_i of 8 μ M on adenylate kinase [41] and of 10 μ M on NDPK. K_m (μ M) values were converted to units of molecules/mitochondrion for the simulation. dNT-2, deoxynucleotidase-2.

Parameter	Nucleoside transport	TK2	dNT-2	TMPK	NDPK	AZTDP transport	AZTTP transport	Pol- γ
V_{max} (μ mol/min per mg)	13.3	0.036	8.4	0.025	1.2	0.85	0.85	0.1 nucleotide/s
K_m (μ M)	10	90 ($h=0.4$)	2500	8.6	125	117	595	187
K_i (μ M)	10	2	2500	8.6	125	117	595	0.86

K_m , V_{max} and K_i values for natural deoxynucleotide metabolism are given in [18], and those for AZT are given in Table A1.

AZT phosphorylation by TK2 has negatively co-operative Hill kinetics (similar to dT). The kinetic model for this is:

$$R_{AZT} = \frac{V_{max}[AZT]^h}{K_m^h \left(1 + \sum_i \frac{[C_i]}{K_{i,i}} \right) + [AZT]^h} \quad (A2)$$

where h is the Hill cooperativity. We model the inhibitors as not having co-operative kinetics, based on experimental data [42].

mtDNA polymerization

We modelled mtDNA replication as in [18]. Each of the four deoxynucleotides and AZT are polymerized individually at different rates, denoted by rA , rC , rG , rT and $rAZT$, with each rate depending on the individual triphosphate concentration. We use Michaelis–Menten dynamics to model these rates, including competitive inhibition between dTTP and AZTTP. V_{max} and K_m values for AZTTP and for each dNTP were determined from the literature [16,43].

The total time t taken to polymerize a segment of mtDNA strand of length L is modelled as a linear function, since deoxynucleotides are added linearly to a replicating strand of mtDNA. In the following equation we define $rT_{sum} = rAZT + rT$. Then:

$$t = \left(\frac{fA}{rA} + \frac{fC}{rC} + \frac{fG}{rG} + \frac{fT}{rT_{sum}} \right) L \quad (A3)$$

where fA , fC , fG and fT are the fractions of each of the deoxynucleotides to be polymerized into the mtDNA segment. From this, after some algebra, we can define a total polymerization rate R_{poly} as:

$$R_{poly} = \frac{L}{t} = \frac{rA \times rC \times rG \times rT_{sum}}{fA \times rC \times rG \times rT_{sum} + fC \times rA \times rG \times rT_{sum} + fG \times rA \times rC \times rT_{sum} + fT \times rA \times rC \times rG} \quad (A4)$$

This is the rate at which pol- γ replicates a strand of mtDNA. Replication then proceeds as an asynchronous polymerization of both strands [23]. The nucleoside analogue (AZT) and its natural nucleotide equivalent (T) in our model are a special case. Each time the polymerase adds T to the growing DNA strand, there is some probability that AZT will be added instead. We define the rate of polymerization of AZT as:

$$R_{poly}^{AZT} = \left(\frac{rAZT}{rT_{sum}} \right) \times (fT \times R_{poly}) \quad (A5)$$

The first term in parentheses is the AZT polymerization rate as a fraction of the total rate of AZTTP and dTTP polymerization. The second term is the fraction of the time that either a dTTP or AZTTP will be incorporated into a strand of mtDNA multiplied by the polymerization rate. The total amount of AZT polymerized into the mtDNA is the integral of R_{poly}^{AZT} taken over the time of replication. When R_{poly}^{AZT} is small, it can be interpreted as a probability of AZT incorporation. A similar equation substituting rT for $rAZT$ describes the rate of dTTP polymerization. At equal concentrations, one AZT molecule is predicted to be incorporated for every 37000 T incorporations [16].

Differential equations

The individual kinetic equations described above are used in differential equations that alter the size of the four AZT (AZT, AZTMP, AZTDP and AZTTP) and 16 natural (dN, dNMP, dNDP and dNTP for A, C, T and G) metabolite pools within the mitochondria in our model. The rate at which AZT changes over time is described by:

$$d[AZT]/dt = NucsideIn - NucsideOut - NucKin - NucTidase \quad (A6)$$

where $NucsideIn$ is the rate of AZT (or deoxynucleoside) transport into the matrix space, $NucsideOut$ is the rate of AZT (or deoxynucleoside) transport out of the matrix space, $NucKin$ is the rate of the AZT nucleoside kinase (TK2) reaction, and $NucTidase$ is the rate of the AZTMP nucleotidase (deoxynucleotidase-2) reaction. Four similar equations are used to model the rate of change of the concentration of the four natural deoxynucleosides in the mitochondrion (see [18] for details).

The rate at which the mitochondrial AZTMP concentration changes over time is described by the following equation:

$$d[AZTMP]/dt = NucKin - NucTidase + NMPKrev - NMPKfwd \quad (A7)$$

where $NMPKfwd$ and $NMPKrev$ are the rates of formation of AZTDP and AZTMP by the forward and reverse reactions

respectively of the nucleoside monophosphate kinase (NMPK) enzyme. Again, analogous equations are used for the four natural deoxynucleotides.

The rate at which AZTDP changes over time is described by the equation:

$$\begin{aligned} d[AZTDP]/dt = & NMPK_{fwd} - NMPK_{rev} + NDPK_{rev} \\ & - NDPK_{fwd} + DNCdNDP_{in} \\ & + DNCdNDP_{out} \end{aligned} \quad (A8)$$

where $NDPK_{fwd}$ and $NDPK_{rev}$ are the rates of formation of AZTTP and AZTDP by the forward and reverse reactions respectively of the NDPK enzyme, and $DNCdNDP_{in}$ and $DNCdNDP_{out}$ are the rates of AZTDP transport into and out of the matrix space respectively by the DNC.

The rate at which the AZTTP pool changes over time is described by the equation:

$$\begin{aligned} d[AZTTP]/dt = & NDPK_{fwd} - NDPK_{rev} \\ & + DNCdNTP_{in} - DNCdNTP_{out} \\ & - (R_{poly} \times fT)(rAZT/rT_{sum}) \end{aligned} \quad (A9)$$

where $DNCdNTP_{in}$ and $DNCdNTP_{out}$ are the rates of DNC transport of AZTTP into and out of the matrix space respectively, and fT is the fraction of T to be polymerized on a particular strand of mtDNA. AZTTP and dTTP compete for incorporation into mtDNA, so the $(rAZT/rT_{sum})$ term in the equation is present here, but is not included in equations describing dATP, dCTP and dGTP pools [18].

Differential equation for the four mitochondrial AZT pools

The full differential equations for the mitochondrial AZT metabolite pools are listed below as an example. The equations for the thymidine pools have been shown previously [18].

$$\begin{aligned} \frac{d[AZT]}{dt} = & \frac{V_{max,1}[AZT]_{cyto}}{K_{m,1}\left(1 + \frac{[dA]_{cyto}}{K_{i,1,1}} + \frac{[dC]_{cyto}}{K_{i,3,1}} + \frac{[dT]_{cyto}}{K_{i,4,1}}\right) + [AZT]_{cyto}} + \frac{-V_{max,1}[AZT]}{K_{m,1}\left(1 + \frac{[dA]}{K_{i,1,1}} + \frac{[dC]}{K_{i,3,1}} + \frac{[dT]}{K_{i,4,1}}\right) + [AZT]} \\ & + \frac{-V_{max,2}[AZT]^h}{K_{m,2}^h\left(1 + \frac{[dTTP]}{K_{i,dTTP}} + \frac{[dCTP]}{K_{i,dCTP}} + \frac{[dC]}{K_{i,dC}} + \frac{[dT]}{K_{i,dT}}\right) + [AZT]^h} + \frac{V_{max,3}[AZTMP]}{K_{m,3}\left(1 + \frac{[dTMP]}{K_{i,4,3}}\right) + [AZTMP]} \end{aligned} \quad (A10)$$

$$\begin{aligned} \frac{d[AZTMP]}{dt} = & \frac{V_{max,2}[AZT]^h}{K_{m,2}^h\left(1 + \frac{[dTTP]}{K_{i,dTTP}} + \frac{[dCTP]}{K_{i,dCTP}} + \frac{[dC]}{K_{i,dC}} + \frac{[dT]}{K_{i,dT}}\right) + [AZT]^h} + \frac{-V_{max,3}[AZTMP]}{K_{m,3}\left(1 + \frac{[dTMP]}{K_{i,4,3}}\right) + [AZTMP]} \\ & + \frac{0.5V_{max,4}[AZTDP]}{K_{m,4}\left(1 + \frac{[dTMP]}{K_{i,4,4}} + \frac{[dTDP]}{K_{i,4,4}} + \frac{[AZTMP]}{K_{i,4}}\right) + [AZTDP]} \\ & + \frac{-V_{max,4}[AZTMP]}{K_{m,4}\left(1 + \frac{[dTMP]}{K_{i,4,4}} + \frac{[dTDP]}{K_{i,4,4}} + \frac{[AZTDP]}{K_{i,4}}\right) + [AZTMP]} \end{aligned} \quad (A11)$$

$$\begin{aligned} \frac{d[AZTDP]}{dt} = & \frac{V_{max,4}[AZTMP]}{K_{m,4}\left(1 + \frac{[dTMP]}{K_{i,4,4}} + \frac{[dTDP]}{K_{i,4,4}} + \frac{[AZTDP]}{K_{i,4}}\right) + [AZTMP]} + \frac{-0.5V_{max,4}[AZTDP]}{K_{m,4}\left(1 + \frac{[dTMP]}{K_{i,4,4}} + \frac{[dTDP]}{K_{i,4,4}} + \frac{[AZTMP]}{K_{i,4}}\right) + [AZTDP]} \\ & + \frac{0.75V_{max,5}[AZTTP]}{K_{m,5}\left(1 + \frac{[dTDP] + [dTTP] + [dCDP] + [dCTP]}{K_{i,4,5}} + \frac{[dADP] + [dGDP] + [dATP] + [dGTP]}{K_{i,2,5}} + \frac{[ADP] + [ATP]}{K_{i,NDP}} + \frac{[AZTDP]}{K_{i,5}}\right) + [AZTTP]} \\ & + \frac{-V_{max,5}[AZTDP]}{K_{m,5}\left(1 + \frac{[dTDP] + [dTTP] + [dCDP] + [dCTP]}{K_{i,4,5}} + \frac{[dADP] + [dGDP] + [dATP] + [dGTP]}{K_{i,2,5}} + \frac{[ADP] + [ATP]}{K_{i,NDP}} + \frac{[AZTTP]}{K_{i,5}}\right) + [AZTDP]} \\ & + \frac{V_{max,6}[AZTDP]_{cyto}}{K_{m,6}\left(1 + \frac{[dCDP]_{cyto}}{K_{i,3,6}} + \frac{[dGDP]_{cyto}}{K_{i,2,6}} + \frac{[dADP]_{cyto}}{K_{i,1,6}} + \frac{[dTDP]_{cyto}}{K_{i,4,6}} + \frac{[dCTP]_{cyto}}{K_{i,3,7}} + \frac{[dGTP]_{cyto}}{K_{i,2,7}} + \frac{[dATP]_{cyto}}{K_{i,1,7}} + \frac{[dTTP]_{cyto}}{K_{i,4,7}} + \frac{[AZTTP]_{cyto}}{K_{i,7}}\right) + [AZTDP]_{cyto}} \\ & + \frac{-V_{max,6}[AZTDP]}{K_{m,6}\left(1 + \frac{[dCDP]}{K_{i,3,6}} + \frac{[dGDP]}{K_{i,2,6}} + \frac{[dADP]}{K_{i,1,6}} + \frac{[dTDP]}{K_{i,4,6}} + \frac{[dCTP]}{K_{i,3,7}} + \frac{[dGTP]}{K_{i,2,7}} + \frac{[dATP]}{K_{i,1,7}} + \frac{[dTTP]}{K_{i,4,7}} + \frac{[AZTDP]}{K_{i,7}}\right) + [AZTDP]} \end{aligned} \quad (A12)$$

$$\begin{aligned}
\frac{d[AZTTP]}{dt} = & \frac{V_{\max,5}[AZTDP]}{K_{m,5} \left(1 + \frac{[dTDP] + [dTTP] + [dCDP] + [dCTP]}{K_{i,4,5}} + \frac{[dADP] + [dGDP] + [dATP] + [dGTP]}{K_{i,2,5}} + \frac{[ADP] + [ATP]}{K_{i,NDP}} + \frac{[AZTTP]}{K_{i,5}} \right) + [AZTDP]} \\
& + \frac{-0.75V_{\max,5}[AZTTP]}{K_{m,5} \left(1 + \frac{[dTDP] + [dTTP] + [dCDP] + [dCTP]}{K_{i,4,5}} + \frac{[dADP] + [dGDP] + [dATP] + [dGTP]}{K_{i,2,5}} + \frac{[ADP] + [ATP]}{K_{i,NDP}} + \frac{[AZTTP]}{K_{i,5}} \right) + [AZTTP]} \\
& + \frac{V_{\max,6}[AZTTP]}{K_{m,6} \left(1 + \frac{[dCDP]}{K_{i,3,6}} + \frac{[dGDP]}{K_{i,2,6}} + \frac{[dADP]}{K_{i,1,6}} + \frac{[dTDP]}{K_{i,4,6}} + \frac{[dCTP]}{K_{i,3,7}} + \frac{[dGTP]}{K_{i,2,7}} + \frac{[dATP]}{K_{i,1,7}} + \frac{[dTTP]}{K_{i,4,7}} + \frac{[AZTDP]}{K_{i,6}} \right) + [AZTTP]} - (R_{\text{poly}} \times fT) \\
& + \frac{-V_{\max,6}[AZTTP]_{\text{cyto}}}{K_{m,6} \left(1 + \frac{[dCDP]_{\text{cyto}}}{K_{i,3,6}} + \frac{[dGDP]_{\text{cyto}}}{K_{i,2,6}} + \frac{[dADP]_{\text{cyto}}}{K_{i,1,6}} + \frac{[dTDP]_{\text{cyto}}}{K_{i,4,6}} + \frac{[dCTP]_{\text{cyto}}}{K_{i,3,7}} + \frac{[dGTP]_{\text{cyto}}}{K_{i,2,7}} + \frac{[dATP]_{\text{cyto}}}{K_{i,1,7}} + \frac{[dTTP]_{\text{cyto}}}{K_{i,4,7}} + \frac{[AZTDP]_{\text{cyto}}}{K_{i,6}} \right) + [AZTTP]_{\text{cyto}}}
\end{aligned} \quad (A13)$$

$V_{\max}$, K_m and K_i terms given in matrix (row, column) form specify the values in Tables 2 (K_m and K_i) and 3 ($V_{\max}$) of [18]. $V_{\max}$, K_m and K_i terms followed by one number (column) are listed in

Table 1. Values for K_i terms followed by an inhibitor name can be found by identifying that inhibitor in the middle column of Table 1 of [18].

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