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Metabolism of deoxypyrimidines and deoxypyrimidine antiviral analogs in isolated brain mitochondria

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Abstract

The goal of this project was to characterize deoxypyrimidine salvage pathways used to maintain deoxynucleoside triphosphate pools in isolated brain mitochondria and to determine the extent that antiviral pyrimidine analogs utilize or affect these pathways. Mitochondria from rat brains were incubated in media with labeled and unlabeled deoxynucleosides and deoxynucleoside analogs. Products were analyzed by HPLC coupled to an inline UV monitor and liquid scintillation counter. Isolated mitochondria transported thymidine and deoxycytidine into the matrix, and readily phosphorylated both of these to mono-, di, and tri-phosphate nucleotides. Rates of phosphorylation were much higher than rates observed in mitochondria from heart and liver. Deoxyuridine was phosphorylated much more slowly than thymidine and only to dUMP. AZT, an antiviral thymidine analog, was phosphorylated to AZT-MP as readily as thymidine was phosphorylated to TMP, but little if any AZT-DP or AZT-TP was observed. AZT at $5.5 \pm 1.7 \mu\text{M}$ was shown to inhibit thymidine phosphorylation by 50%, but was not observed to inhibit deoxycytidine phosphorylation except at levels $> 100 \mu\text{M}$. Stavudine and lamivudine were inert when incubated with isolated brain mitochondria. The kinetics of phosphorylation of thymidine, dC and AZT were significantly different in brain mitochondria compared to mitochondria from liver and heart.

Keywords

Nucleoside reverse transcriptase inhibitors (NRTIs); brain mitochondria; thymidine kinase 2; mitochondrial toxicity

INTRODUCTION

Our objective in this study was to characterize the mitochondrial deoxypyrimidine salvage pathways used to maintain deoxynucleotide triphosphate (dNTP) pools in brain mitochondria and potentially used to phosphorylate selected anti-viral nucleoside analogs including the thymidine analogs zidovudine (AZT) and stavudine (d4T), as well as the deoxycytidine analog, lamivudine (3TC). The central nervous system displays a relative resistance to nucleoside analogs such as AZT in both treatment and toxicity when compared to other organ systems. This may be related to efflux transporters in the blood brain barrier that reduce the CNS concentration of AZT compared to serum (Busidan, Shi et al. 2001). These metabolic pathways are increasingly relevant not only to the treatment of HIV/AIDS, but also to targeting the role of mitochondrial dysfunction in developing potentially new nucleoside analog treatment options for primary neoplasms of the CNS (Ordys, Launay et al. 2010).

AZT was originally developed for treatment of neoplasms, but was the first drug to show efficacy in the treatment of AIDS, for which it has now been used for the past 20 years. Stavudine and lamivudine were subsequently developed and used in AIDS therapy. These analogs are administered as pro-drugs. The deoxynucleosides are taken up by cells and phosphorylated by host enzymes to the tri-phosphate form which inhibits the viral reverse transcriptase. Members of this class of drugs are referred to as nucleoside analog reverse transcriptase inhibitors (NRTIs). AZT in the early days of single regimen high dose therapy was most often associated with myopathy and cardiomyopathy followed by cytopenias and hepatic toxicity. In present treatment highly active antiretroviral therapy (HAART) (Tozser 2001) is utilized that involves combination therapy in which several different NRTIs, such as AZT and 3TC, are given at lower doses than in monotherapy, together with a viral protease inhibitor, such as ritonavir, in a drug cocktail. While the most recent international guidelines on AIDS therapy recommend tenofovir/emtricitabine or abacavir/lamivudine as the NRTIs of choice in the HAART regimen (Hammer, Eron et al. 2008), zidovudine is still

routinely used in many settings and stavudine is still employed in some resource limited settings. While HAART has revolutionized AIDS treatment it is life-long and is still associated with a myriad of toxicities that vary according to the NRTIs used. For example, zidovudine/lamivudine is associated with a fat redistribution syndrome called lipodystrophy, while tenofovir is associated with renal dysfunction. Other toxicities include cardiovascular complications, hepatic dysfunction and hyperlactatemia (Tozser 2001). In a transgenic AIDS mouse model of the disease, treatment with HAART reproducibly led to a cardiomyopathy (Lewis, Haase et al. 2001) that was not observed in the model without treatment. On the other hand clinical reports of neurotoxic seizures, neuropsychiatric symptoms, and neuropathies are less common than reports of these other organ toxicities. However, clinically detected neurotoxic effects may also be more common than reported. Psychiatric and cognitive symptoms, often attributed to the neurotoxic effects of HIV itself, may be multi-factorial. Neurotoxicity has also been observed in infants secondary to in utero exposure of AZT in pregnant mothers, but it has not occurred at a level to cause significant concern in clinical practice (Blanche, Tardieu et al. 1999; Calamandrei, Valanzano et al. 2002; Divi, Einem et al. 2010). However, the response of disease burden to AZT in AIDS patients, including measurement of viral load, has also not been as good in the central nervous system as other organs (Letendre, Marquie-Beck et al. 2008), which may be a function of the ability of CNS cells to accumulate and phosphorylate NRTIs to their active tri-phosphates.

In tissues that display toxic effects to NRTI treatment, it has generally been accepted that morphologically abnormal, dysfunctional mitochondria generating too little ATP for cellular survival were at fault. Since depletion of mitochondrial DNA has often been associated with tissue toxicity, and direct inhibition of purified DNA polymerase γ by a variety of NRTI-triphosphates has been demonstrated (Lewis, Simpson et al. 1994), the prevailing hypothesis for mitochondrial toxicity has been the inhibition of mitochondrial replication by the triphosphate forms of the antiviral nucleoside analogs (Simpson, Chin et al. 1989; Arnaudo, Dalakas et al. 1991; Lewis, Gonzalez et al. 1992; Lewis, Simpson et al. 1994). However, while there is a good correlation between the toxicity of many NRTIs and the ability of NRTI-triphosphates to inhibit polymerase γ , this does not fit very well for AZT and alternative mechanisms of toxicity have been proposed (McKee, Bentley et al. 2004; Lund, Peterson et al. 2007; Susan-Resiga, Bentley et al. 2007; Morris 2009). For example, the concentration of AZT-TP required for a 50% inhibition of polymerase γ is $>100 \mu\text{M}$ (Hanes and Johnson 2007), a value much higher than other less toxic NRTIs (Lee, Hanes et al. 2003). Because of the well-known bottleneck in AZT-MP phosphorylation (Lavie, Schlichting et al. 1997), the level of AZT-TP in replicating tissue is several orders of magnitude below $100 \mu\text{M}$. In non-replicating tissues such as the adult heart, where significant toxicity was observed in early monotherapy regimens, AZT-TP has not been detected (McKee, Bentley et al. 2004; Lynx, Bentley et al. 2006; Susan-Resiga, Bentley et al. 2007), and if present is at an even lower concentration. Alternatively, AZT-MP has been shown to inhibit the exonuclease activity of polymerase γ , but at a concentration of 1-2 mM (Lim and Copeland 2001). This is an exceedingly high level, and it is not yet clear if levels approaching this can be reached in the heart or other tissues that are made up of mostly non-replicating cells. On the other hand, we have demonstrated that the pro-drug AZT is a potent competitive inhibitor of thymidine phosphorylation (Lynx and McKee 2006) in isolated heart (McKee, Bentley et al. 2004) and liver (Lynx, Bentley et al. 2006) mitochondria, as well as in the isolated perfused heart (Susan-Resiga, Bentley et al. 2007) and in a number of different cultured cell lines (Lynx, Kang et al. 2008). The inhibitory values observed in these studies provide perhaps a more realistic cause of toxicity than those discussed above for AZT-TP and AZT-MP. From this work we have proposed an alternative mechanism to account for the observed AZT-linked mtDNA depletion, in which inhibition of thymidine phosphorylation leads to a decrease in the TTP pool, which unbalances the dNTP pool

supplying replication. Either the limiting level of TTP or the unbalanced pools, or both, would lead to mitochondrial DNA replication errors and deletions that would in turn culminate in the observed mitochondrial DNA depletion. We have demonstrated that AZT depletes the TTP pool in the perfused rat heart to 50% of control in just 30 min of perfusion (Morris 2009). Additional support for this hypothesis comes from mutations characterized in humans that cause a partial deficiency in the nuclear encoded mitochondrial thymidine kinase 2 (TK2). Individuals with this disorder were shown to have a severe myopathy that is lethal in childhood and is associated with muscle mtDNA depletion (Saada, Shaag et al. 2001; Saada, Shaag et al. 2003). More recently, humans with TK2 mutations have been shown to have serious neurological phenotypes as well (Gotz, Isohanni et al. 2008). In fact, recent work has shown that the brain, perhaps more than any other tissue, is dependent upon TK2 for the dNTP salvage pathway. TK2 has also been shown to be essential for neuronal functioning. A knockout mouse strain deficient in TK2 died within the first few weeks of life with skeletal muscle, heart, liver, and spleen showing progressive depletion of mtDNA (Zhou, Solaroli et al. 2008). Further analysis of this strain demonstrated the mice had a severe ataxic phenotype with histopathological neuronal degeneration of cerebellum associated with depletion of mt-DNA and reduced electron transport chain complexes in the brain (Zhou, Solaroli et al. 2008; Bartesaghi, Betts-Henderson et al. 2010). A knockin mutant mouse model harboring a mutant mouse TK2 analogous to the mutant human TK2 (H121N) developed fatal encephalomyopathy in the second week of life associated with severe mt-DNA depletion in the brain and heart. Interestingly, the heart and muscle, but not the brain compensated for the loss of mt-DNA by increasing mitochondrial transcription (Dorado, Area et al. 2011). Further evidence that these dNTP salvage pathways are essential to balancing dTNP pools in the mitochondria is found in MNGIE and animals models, in which asymmetry in dNTP pools leads to loss of synthesis of mt DNA and subsequently, cell death (Ferraro, Pontarin et al. 2005; Lopez, Akman et al. 2009). These data strongly support the critical nature of TK2 activity in the brain and point out the importance of investigating the effects of AZT and other antivirals on this activity.

A fundamental problem with studying neurotoxicity is that the mechanisms for maintaining CNS homeostasis with blood still remain imperfectly understood (Suzuki, Terasaki et al. 2001; Varatharajan and Thomas 2009). In these investigations we bypassed these mechanisms by using isolated rat brain mitochondria to characterize the mitochondrial phosphorylation pathways of thymidine, deoxycytidine, deoxyuridine, zidovudine, stavudine, and lamivudine and to study the competitive effects of these deoxynucleosides on each other's metabolism.

Material and Methods

Brain Removal and Mitochondrial Isolation

Non-synaptosomal mitochondria were isolated from freshly removed brains from adult (250-400 g) male Harlan Sprague Dawley rats. The animal protocol used in this study was approved by the University of Notre Dame IACUC. The rats were injected intraperitoneally with 0.1 ml heparin sulfate, (10mg/ml) to prevent clotting of the blood as the brain was removed. After waiting at least ten minutes to allow heparin to take effect, a lethal dose of sodium pentobarbital was injected (1-1.2 ml, 50 mg / ml). Once the rat was unresponsive the skull was opened and the brain including, cerebrum, cerebellum, and part of the upper spinal cord was removed with a sterile spatula and placed into a beaker containing ice cold isotonic buffer (220 mM mannitol, 70 mM sucrose, 5mM MOPS, pH 7.4) (MSM). The brain was blotted, weighed, and minced and the mince washed once in the MSM to remove blood. Mitochondria from the rat brain were isolated by a modification of the method used to isolate heart mitochondria (McKee, Grier et al. 1990). The minced brain(s) were incubated for 30 seconds with nagarse (subtilisin, Sigma) 0.4 mg/ml in MSM +0.05 mM EDTA at 10

ml/g brain. An additional 10 ml of MSM plus bovine serum albumin (0.2%) was added per g of brain mince and homogenized with a rotating Teflon pestle for 3×5 seconds, to break the cells. The homogenate was poured into 50 ml centrifuge tubes and spun at $10,000 \times g$ for 10 min to quickly separate the nargarse from the homogenized brain. The supernatant was discarded and the pellet resuspended in MSM+BSA, 10 ml/g brain, and centrifuged at $1,700 \times g$ for 10 minutes to remove cellular debris. The resulting supernatant was decanted and saved and the pellet resuspended in MSM+BSA (10ml/g brain) and centrifuged again at $1,700 \times g$ for 10 minutes to recover residual mitochondria. The supernatant was decanted, combined with the previous saved supernatant and centrifuged at $7,650 \times g$ for 10 minutes to pellet mitochondria. The supernatant was discarded and the mitochondrial pellet resuspended in MSM at 5 ml/g brain and the slow and high speed spins are repeated once more and the pellet is resuspended in a small amount of MSM and placed on ice. The protein content of the pellet was measured by the method of Lowry and diluted to 20 mg protein/ml in MSM.

Mitochondrial Intactness

The intactness of the brain mitochondrial preparation was determined by measuring the respiratory control ratio (RCR). The RCR was determined by comparing oxygen consumption in mitochondria actively synthesizing ATP from ADP (State 3) to oxygen consumption in the absence of ATP synthesis (no ADP, State 4). High ratios are evidence of an intact inner membrane. RCR was determined as described previously with heart mitochondrial (McKee, Bentley et al. 2004) in the presence of 10 mM glutamate, 2.5 mM malate, and 0.5-1.0 mg mitochondrial protein using a Yellow Springs Instruments oxygen electrode and chamber. In later studies, mitochondrial RCRs were measured in a similar manner using the Oroboros Oxygraph Y2K (Innsbruck, Austria). This method required far less mitochondria (0.05-0.2 mg) and was conducted in 2 ml of MiR05 buffer as described (Mario Fasching 2010). The average RCR value brain mitochondrial preparations were between 4.5 and 5. Preparations with RCR values RCRs less than 4 were discarded.

Determining the Rate of Deoxynucleoside Phosphorylation in Isolated Brain Mitochondria

Mitochondria were incubated at a final concentration of 4 mg protein /ml in media described previously (McKee, Grier et al. 1990) with labeled and unlabeled deoxynucleosides and deoxynucleoside analogs at concentrations and specific radioactivities noted in figure legends. Samples were taken at various times of incubation as noted in the figures and figure legends. Samples were prepared and analyzed as described below.

Sample Preparation and Analysis

Aliquots of the mitochondrial incubation (200 μ l) were removed at specific time points and combined with an equal volume of 10% trichloroacetic acid to lyse mitochondria and precipitate the protein and nucleic acids. This mixture was placed on ice for 10 minutes, followed by centrifugation in a microfuge for 2 minutes. A measured amount of the acid soluble extract was removed and placed into a tube containing sufficient AG-11A8 ion exchange resin to neutralize the TCA (250 mg of resin/250 μ l of acid soluble extract). The extract-resin mixture was vortexed for 30 seconds. Samples were often frozen at $-80^{\circ}C$ at this point. Neutralization of the acid was confirmed using pH test strips and once the pH was 6.5 or greater, the extract was filtered through a Whatman syringeless filter with a 0.45 μ m pore. The amount of radioactivity in each sample was determined by counting aliquots in Aquasure with a Beckman Liquid Scintillation counter (LS6500). Quantitation of labeled deoxynucleosides and phosphorylated products were analyzed by HPLC on an Alltech nucleoside-nucleotide reverse phase column coupled to an inline UV monitor and liquid scintillation counter as previously described (McKee, Bentley et al. 2004).

Data analysis

HPLC Data—Peaks were identified by co-elution with standards and quantitated by peak integration (Breeze Systems, Waters Corp. Boston, MS). Results were expressed in DPM on HPLC chromatograms and converted in all other figures to pmol product / mg mitochondrial protein by dividing by the specific radioactivity of the deoxynucleoside used and the content of mitochondrial protein in the incubation. Results at each time point and concentration were plotted as the mean and standard error of the mean.

Kinetic Data—In the kinetic experiments (Figures 3 and 4), velocity of phosphorylation was determined at increasing deoxynucleoside concentrations (0.4 - 200 μ M). Velocity was determined as the best-fit slope through 0, 30, and 60 min time-points (Sigma-Plot 9.01, Systat Software, San Jose, Ca). The resulting velocities were plotted with deoxynucleoside concentration in three different ways. 1) A Michaelis-Menton plot of velocity against substrate concentration, with the best fit of the data to the Michaelis-Menton equation ($V = V_{max} * S / (K_m + S)$) shown (Figure 3 Panels A and C, Figure 4, Panel A) (Sigma-Plot 9.01). 2) To detect potential negative cooperativity, the Michaelis-Menton equation was rearranged to the Eadie-Hofstee equation in which $V = K_m V / [S] + V_{max}$ and velocity is plotted against velocity / substrate concentration (Figure 3, Panels B and E, and Figure 4, Panel B). In the absence of cooperativity the data should show a straight line. 3) To further address cooperativity in multisubunit enzyme systems, the thymidine data were plotted as a Hill plot in which $\log [V / (V_{max} - V)] = n \log S \log K$ with $\log [V / (V_{max} - V)]$ plotted against $\log [\text{substrate}]$ and the best fit to the line shown (Figure 3, Panels C and F, Figure 4, Panel C). A Hill plot slope = 1 indicates no cooperativity, > 1, positive cooperativity, < 1, negative cooperativity.

Results

Extent of Pyrimidine Deoxynucleoside Phosphorylation in Isolated Brain Mitochondria

The extent of phosphorylation of the naturally occurring pyrimidine deoxynucleosides thymidine, dC, and dU was determined by incubating isolated brain mitochondria for 3 hrs with the appropriate [3 H]-deoxynucleoside or analog as described In the Material and Methods. The HPLC chromatograms of the observed products are shown in Figure 1 with all of the peaks identified by comparison to standards. The small peak coming off between 0 and 3 minutes in the [3 H]-thymidine incubation (Figure 1, Panel A) is in the void volume and is observed in zero time samples as well. It is apparent that isolated brain mitochondria contain all of the enzymes necessary to readily convert thymidine (Panel A) and dC (Panel B) to the mono-, di-, and tri-phosphates with consumption of the parent deoxynucleoside. These enzymes includes TK2 (Wang, Munch-Petersen et al. 1999; Wang and Eriksson 2000), TMP kinase (thymidylate kinase) (Chen, Lin et al. 2008), demonstrated earlier in our laboratory in heart and liver mitochondria (McKee, Bentley et al. 2004; Lynx, Bentley et al. 2006) and a diphosphokinase. A small amount of thymidine is converted to thymine indicating the presence of a low activity thymidine phosphorylase. A small amount of dC was also deaminated to dU (Figure 1, Panel B), suggesting the presence of dC deaminase. Interestingly, [3 H]-dU was converted only to dUMP, and at much slower rate than either thymidine or dC (Figure 1, Panel C). Breakdown to uracil was not detected. Slow conversion of dU to dUMP only was also noted in isolated heart mitochondria, while phosphorylation of dU was not detected in perfused heart (Morris 2009).

In a similar experiment brain mitochondria were incubated with the [3 H]-deoxynucleoside analogs, AZT, 3TC, or d4T for 3 hr. The results for AZT are shown in Figure 1, Panel D. Both 3TC and d4T were completely inert during the incubation and only the parent peak was observed for both (data not shown), indicating that these drugs are either not transported into

the matrix; or, as suggested by others (Wang, Su et al. 2000), not good substrates for TK2, or for thymidine phosphorylase. Conversely, AZT was readily converted to AZT-MP during the 3 hr incubation, with the AZT-MP peak becoming larger than the parent AZT peak (Figure 1, Panel D). This is quite different from liver and heart mitochondria in which AZT remains the dominate peak even after 3 hours of incubation. Interestingly we have observed a small but detectable peak corresponding to AZT-DP in one sample, but not in the replicate experiments, suggesting that this peak is right at the limits of detection. An AZT-DP peak was never observed in heart or liver mitochondria (McKee, Bentley et al. 2004; Lynx, Bentley et al. 2006). Breakdown of AZT to thymine was not detected.

Time-Course of Phosphorylation for Thymidine, dC, dU, and AZT

The time-course of phosphorylation in isolated brain mitochondria for 1 μ M levels of the naturally occurring pyrimidine deoxynucleosides and AZT is shown in Figure 2. All three phosphorylated forms as well as the sum of all three phosphorylated forms are plotted for thymidine, dC, and AZT. For dU, the only phosphorylated form detected was dUMP. After a lag time of 15-30 min, total phosphorylation for thymidine (Figure 2, Panel A, TNP), dU (Figure 2, Panel C, dUMP), and AZT (Figure 2, Panel D, AZT-NP) are relatively linear for the next 60-90 min followed by a plateau. A lag-time was not apparent for phosphorylation of dC (Figure 2, Panel B), which remained linear throughout the 3 hr incubation. Thymidine and dC reached similar levels of 160 pmol / mg mitochondrial protein at 3 hr, which accounted for 65% conversion of the parent compound to a phosphorylated product. Phosphorylation of dU was much slower, reaching a level of 85 pmol / mg mitochondrial protein (about half the rate of thymidine and dC phosphorylation). For thymidine and dC the major phosphorylated products were the mono- and tri-phosphate. The tri-phosphate form decreases somewhat in the 3rd hour with an increase in the di-phosphate form consistent with a fall in mitochondrial energy charge in the last hour of incubation. A small amount of radioactivity appears to be incorporated into mitochondrial DNA for thymidine and dC (data not shown), but this is negligible relative to the total pools.

The time course of AZT phosphorylation of brain mitochondria is shown in figure 2, Panel D. Phosphorylation of AZT to AZT-MP was linear for about 150 minutes, reaching a plateau of 125 pmol / mg mitochondrial protein, or about 55% of the parent AZT peak. In one experiment a small peak of AZT-DP was detectable but was not observed in others.

Kinetics of Phosphorylation of Thymidine, dC, and AZT

To determine the kinetics of thymidine and dC phosphorylation, mitochondria were incubated with increasing concentrations of [³H]-thymidine, dC, or AZT and the rate of total phosphorylation determined over a time-course of 1 hour for each concentration (TNP, dCNP, and AZT-NP (the best-fit slopes of the lines in Figure 2, Panels A, B, and D, respectively). The resulting slopes were plotted as velocity versus substrate concentration for thymidine, dC and AZT respectively. The curves represents the best fit of the data to the Michaelis-Menton equation (thymidine, Figure 3, Panel A), dC (Figure 3, Panel D), and AZT (Figure 4, Panel A). The apparent K_m s for thymidine and dC phosphorylation in brain mitochondria were $10.1 \pm 2.3 \mu$ M (Figure 3, Panel A), and $33 \pm 11 \mu$ M (Figure 3, Panel C), respectively, suggesting that TK2 appears to show a preference for binding thymidine. The maximum velocities obtained for thymidine and dC phosphorylation were quite similar, at 750 ± 48 and 888 ± 100 pmol/mg/hr respectively. These values are substantially higher than values previously observed in heart and liver for thymidine phosphorylation suggesting that TK2 is much more active in the brain.

The kinetics of AZT phosphorylation yielded a K_m of $8.3 \pm 1.8 \mu$ M (Figure 4, Panel A), which was very comparable to K_m s observed in heart at 7.5 (McKee, Bentley et al. 2004)

and liver at 6.3 μM (Lynx, Bentley et al. 2006). The V_{max} of AZT phosphorylation in brain mitochondria was $330 \pm 18 \text{ pmol mg}^{-1} \text{ hr}^{-1}$, about half the rate of thymidine and dC. However, as noted above for thymidine and dC phosphorylation, brain mitochondrial phosphorylation of AZT was also much faster than observed in heart ($20 \text{ pmol mg}^{-1} \text{ hr}^{-1}$) (McKee, Bentley et al. 2004), or liver ($69 \text{ pmol mg}^{-1} \text{ hr}^{-1}$)

Thymidine phosphorylation by purified recombinant TK2 has been associated with negative cooperativity (Wang, Munch-Petersen et al. 1999; Barroso, Elholm et al. 2003). To detect this possibility, the kinetic data were re-plotted as Eadie-Hofstee (Figure 3, Panel B, thymidine; Panel E, dC) and Hill (Figure 3, Panel C, thymidine; Panel F, dC) plots. The presence of bi-phasic curves and Hill plots with slopes less than 1 for both thymidine and dC were indicative of negative cooperativity for both thymidine and dC phosphorylation. Negative cooperativity of thymidine phosphorylation was also shown in isolated heart (McKee, Bentley et al. 2004) and liver (Lynx, Bentley et al. 2006) mitochondria as well as in the perfused heart (Susan-Resiga, Bentley et al. 2007). However, dC phosphorylation was not reported in previous work to be associated with negative cooperativity (Wang, Munch-Petersen et al. 1999; Barroso, Elholm et al. 2003)

In a manner similar to thymidine and dC phosphorylation, Eadie-Hofstee (Figure 4, Panel B), and Hill (Figure 4, Panel C) plots were constructed for AZT phosphorylation. A biphasic curve was not observed and the slope of the Hill plot was close to 1, indicating that phosphorylation of AZT by TK2 did not display negative cooperativity. This was different from results reported for recombinant TK2 (Wang, Munch-Petersen et al. 1999) and to results observed in heart (McKee, Bentley et al. 2004) and liver (Lynx, Bentley et al. 2006) mitochondria in which negative cooperativity was clearly observed.

AZT Inhibition of Thymidine, dC, and dU phosphorylation

As AZT, thymidine, dC, and dU are all phosphorylated by the same matrix TK2 enzyme, the effect of AZT on the phosphorylation rate of the others was determined. A dose-response plot of the effects of AZT on 1 μM amounts of thymidine, dC, and dU phosphorylation in brain is shown in Figure 5. The concentration of AZT that causes a 50% reduction of total phosphorylation in brain mitochondria for each deoxynucleoside was $5.5 \pm 1.7 \mu\text{M}$ for thymidine, 268 μM for dC, and $1.0 \pm 0.1 \mu\text{M}$ for dU. Clearly, AZT, as a thymidine analog was much more potent in inhibiting thymidine and dU, while having little effect on the phosphorylation rate of dC. This is similar to the effect of AZT observed for thymidine phosphorylation in liver mitochondria (Lynx, Bentley et al. 2006) and in perfused heart (Susan-Resiga, Bentley et al. 2007) and heart mitochondria (McKee, Bentley et al. 2004). The increased potency of AZT on dU phosphorylation was also noted in heart mitochondria (McKee, E. E., personal observation). The lack of AZT inhibition of dC phosphorylation was noted in perfused heart, as well as heart and liver mitochondria (Morris, LaClair et al. 2010). Finally, as noted in the introduction, efflux transporters in the blood brain barrier keep AZT at lower levels in the CNS than in other tissues, such that AZT is less likely to inhibit TK2 in the brain. This may account for the lower levels of toxicity noted.

The Effect of d4T and 3TC on Thymidine and dC phosphorylation

The NRTI drugs d4T and 3TC are also analogs of thymidine and deoxycytidine, respectively. As noted earlier, while a small amount of phosphorylated 3TC was observed in the perfused heart (Morris, LaClair et al. 2010), phosphorylated products were not detected for either one in brain, heart, or liver mitochondria. To determine if either of these analogs interfered with phosphorylation of thymidine or dC, brain mitochondria were incubated with varying concentrations of these analogs (0-200 μM) exactly as described for AZT. In two independent experiments neither of these analogs affected the phosphorylation of thymidine

or deoxycytidine in brain mitochondria (data not shown), a result identical with the observations in perfused heart and heart mitochondria (Morris, LaClair et al. 2010). Others have suggested that these analogs may be poor substrates for TK2 (Eriksson, Kierdaszuk et al. 1991). Alternatively, it is possible that they are not transported into the matrix.

Effect of dC on thymidine phosphorylation and of thymidine on dC phosphorylation

Thymidine and dC are both phosphorylated by TK2, but differentially affected by AZT. As a result, it seemed important to determine the effect of thymidine and dC on each other's phosphorylation. As shown in Figure 6, an IC_{50} value of $8.8 \pm 3.9 \mu M$ was determined for the effect of dC on thymidine phosphorylation (Figure 6, Panel A), while in the reverse experiment, thymidine had very little effect on dC phosphorylation with an $IC_{50} > 400 \mu M$ (Figure 6, Panel B). These data are opposite that reported in competition studies on the purified enzyme (Munch-Petersen, Cloos et al. 1991).

DISCUSSION

The goal of this project was to identify deoxypyrimidine salvage pathways used to maintain dNTP pools in intact non-synaptosomal brain mitochondria, and the extent to which these pathways are used or inhibited by certain classical deoxypyrimidine nucleoside analogs such as AZT, d4T, and 3TC compared to other organ systems. In this investigation brain mitochondria demonstrated a robust salvage pathway for [3H]-thymidine and [3H]-deoxycytidine that generated all three phosphorylated forms and was 5-6 times more active at $1 \mu M$ concentrations than the mitochondrial salvage pathways in heart (McKee, Bentley et al. 2004) and liver (Lynx, Bentley et al. 2006). This demonstrates that isolated brain mitochondria, like heart and liver mitochondria are able to transport thymidine and deoxycytidine across the inner membrane into the matrix and contains all of the enzymes necessary for conversion to the triphosphate. This includes the presence of a mitochondrial UMP/CMP kinase which has been characterized in humans (Xu, Johansson et al. 2008), and the presence of a mitochondrial thymidylate kinase, an activity that has been demonstrated in heart and liver mitochondria (McKee, Bentley et al. 2004; Lynx, Bentley et al. 2006), and recently described in human cells (Chen, Lin et al. 2008).

Incubation of brain mitochondria with [3H]-deoxyuridine lead to the synthesis of dUMP at a much slower rate than the conversion of thymidine to TMP. Uracil and other phosphorylated forms were not detected. As a mitochondrial dUTP pyrophosphorylase has been identified (Spector and Boose 1983; Ladner, McNulty et al. 1996), it is possible that dUMP was phosphorylated to dUTP and then quickly dephosphorylated back to dUMP such that intermediates were not detected. Preliminary data has suggested that dUTPase is active in rat brain mitochondria (McCann and McKee, personal observations). While conversion of dUMP to TMP was not detected in brain mitochondria, a pathway for the conversion of dUMP to TMP has been identified in mammalian mitochondria from Chinese hamster ovary (CHO) cells (Anderson, Quintero et al. 2011). When [3H]-deoxyuridine was incubated with isolated heart mitochondria, a slow formation of dUMP was also observed; however, labeled dUMP could not be detected in hearts perfused with [3H]-deoxyuridine (Morris 2009). Since deoxyuridine was shown to be taken up intracellularly by the perfused heart, and since deoxyuridine is known to be a good substrate for purified TK2 (Wang and Eriksson 2000), these data raised the possibility that deoxyuridine transport into the matrix of heart mitochondria may be limiting. The small amount of dUMP formed in isolated heart and/or brain mitochondria might be accounted for by a small loss of membrane intactness in the in vitro preparations. The final disposition of dUMP synthesized by brain mitochondria is unknown and is under investigation.

The antiviral analogs 3TC and d4T were not reactive in isolated brain mitochondria, nor did they have any effect on the salvage pathways of thymidine or deoxycytidine. Identical findings were observed in isolated heart and liver mitochondria and support the finding that these two drugs are not very good substrates for purified TK2 (Wang and Eriksson 2000). On the other hand, phosphorylation of AZT to AZT-MP was extremely active, 20-30 fold higher at 1 μ M levels than in either liver (Lynx, Bentley et al. 2006) or heart (McKee, Bentley et al. 2004) mitochondria respectively. The main difference in this finding is that at 1 μ M concentrations of thymidine and AZT, heart and liver mitochondrial preferred thymidine to AZT by a factor of 4-5 to 1. In brain mitochondria at the same concentrations, there was only a slight preference for thymidine. While a substantial amount of AZT-MP was synthesized in brain mitochondria, a barely detectable peak that may have been AZT-DP was observed in only one experiment. In the balance of experiments neither AZT-DP nor AZT-TP were detected. The absence of AZT-TP confirms this laboratory's earlier findings in other tissues that toxicity is not mediated by AZT-TP inhibition of DNA polymerase γ (McKee, Bentley et al. 2004; Lynx, Bentley et al. 2006; Susan-Resiga, Bentley et al. 2007).

The kinetics of thymidine phosphorylation (Figure 3, Panels B and C), displayed apparent negative cooperativity as has been previously noted in isolated liver and heart mitochondria (McKee, Bentley et al. 2004; Lynx 2009), isolated perfused heart (Susan-Resiga, Bentley et al. 2007), and in recombinant purified TK2 (Wang, Munch-Petersen et al. 1999). Previous work in these same systems has also shown that the kinetics of phosphorylation of AZT displayed apparent negative cooperativity, but this was not observed in the present study. Further, the kinetics of phosphorylation of deoxycytidine (Figure 3, Panels E and F) clearly displayed negative cooperativity in this study, whereas this has not been previously noted for deoxycytidine phosphorylation in other kinetic studies. Finally the data in Figure 6 indicate that dC is a much stronger competitive inhibitor of thymidine phosphorylation than thymidine is of dC phosphorylation. This is the opposite of results on the purified enzyme (Munch-Petersen, Cloos et al. 1991) and differ from results obtained in isolated heart mitochondria in which neither deoxynucleoside was very effective at inhibiting the other's phosphorylation (Morris, LaClair et al. 2010). These data taken together suggest that the deoxypyrimidine phosphorylation activity in brain mitochondria is more active and differs in significant ways from the activities described in other tissues and in purified recombinant TK2.

The increase in phosphorylation activity in brain mitochondria may simply reflect increased expression of TK2 in the brain. This high activity pathway probably accounts for the fact noted in the introduction that the TK2 knock in mutant mice appeared to die from CNS toxicity. A recent study has shown that the tissue distribution of purified TK2 activity in rats can be quite variable, with brain having significantly more activity than heart and liver (Wang and Eriksson 2010), supporting the finding observed here. However, this does not address the significant differences in the kinetic details of deoxypyrimidine phosphorylation and inhibition of phosphorylation observed in intact brain mitochondria compared to mitochondria from heart or liver, or to purified rat liver, or human recombinant TK2. One possible mechanism accounting for tissue specific differences may follow from the work of Wang et al. (Wang, Munch-Petersen et al. 1999) that showed that the mRNA coding for TK2 has the ability to form multiple transcripts, depending on the tissue. Thus, variable splicing could lead to tissue specific isoforms, or multiple isoforms of TK2 that could have different kinetic properties. Alternatively, differences in the kinetics and inhibition of deoxypyrimidine phosphorylation in brain mitochondrial might be related to the complex and incompletely understood regulation of TK2 (for review, (Munch-Petersen 2010)). First, no successful crystallization of the enzyme has been done (Barroso, Elholm et al. 2003). It is not yet clear if the enzyme exists as a monomer or a homo-multimer. This clearly calls into question the interpretation of negative cooperativity, which requires that the enzyme be at

least a dimer, such that when thymidine binds to one subunit binding site the binding affinity of thymidine to the second site is reduced. Others have suggested that TK2 may exist in two conformational forms in equilibrium, in which each form has a different affinity for binding thymidine (Radivoyevitch, Munch-Petersen et al. 2011). The equilibrium of the two forms could be regulated by binding of substrate or feedback inhibitors such as TTP and dCTP. Wang et al. (Wang, Sun et al. 2011) have shown that the kinetics of thymidine and deoxycytidine phosphorylation by purified rat liver TK2 and recombinant human TK2 differ considerably depending on whether the enzyme has TTP bound or not. The recombinant enzyme bound TTP in a 1:1 stoichiometry, and in this form AZT inhibited thymidine phosphorylation, but surprisingly stimulated deoxycytidine phosphorylation. Removal of TTP from the recombinant enzyme by incubation with deoxynucleosides and ATP, led to an enzyme in which AZT inhibited both thymidine and deoxycytidine phosphorylation, albeit at different levels. Stimulation of deoxycytidine phosphorylation by AZT has not been shown in the studies here with intact brain mitochondria, or in earlier studies with intact heart and liver mitochondria, or in the perfused heart. The degree to which TTP, or dCTP is bound by TK2 in intact mitochondrial systems as the one described here is not known, nor is the role (if any) of the mitochondrial matrix environment or inner membrane transport processes on TK2 regulation known. It is possible that these features may account for regulatory differences between tissues or when compared to purified TK2 enzymes. While this study was limited to intact non-synaptosomal brain mitochondria, we are presently obtaining additional information on the kinetics of deoxypyrimidine phosphorylation in membrane free mitochondrial extracts.

Lastly, it is possible that the deoxypyrimidine phosphorylation kinetics observed in brain mitochondrial might be related to a novel phosphorylating enzyme. In this regard there has been a recent report of TK2-like activity purified from mitochondrial free cytosol in a variety of rat tissues (Wang and Eriksson 2010). Additional work in attempting to fractionate the mitochondrial phosphorylation activity will help address this issue.

While neurotoxicity is not listed as a particularly major side-effect of NRTI's, it is worth noting that between 25-60% of HIV patients have some psychiatric or cognitive symptoms which is generally determined to be secondary to the neurotoxicity of the HIV itself. However, there may also be some multi-factorial element to this in which the NRTI's play a role. The degree to which TK2 activity is critical in supplying TTP is dependent on the extent that cells can use alternative pathways for TTP synthesis. Alternative sources of dCTP and TTP synthesis include *de novo* synthesis of UMP and/or salvage of uridine or cytidine to UMP and CMP with subsequent reduction via ribonucleotide reductase of UDP and CDP to dUDP and dCDP. The dUDP formed must be further converted to dUTP, quickly cleaved to dUMP and then converted to TMP by thymidylate synthase (Ferraro, Pontarin et al. 2005). A more likely route is the conversion of dCMP to dUMP by deamination. Salvage of deoxyuridine to dUMP would follow the final steps of this pathway. While it is thought that the mammalian brain has a limited capacity for *de novo* UMP synthesis (Redzic, Malatiali et al. 2009), the salvage and conversion of ribonucleosides via ribonucleotide reductase, and/or deoxyuridine to TMP and dCMP remain additional mechanisms for TTP synthesis (Suleiman and Spector 1982) that is unexplored in the brain. In the adult perfused rat heart, previous work in this laboratory has shown that mitochondrial TTP and dCTP pools are maintained solely through salvage of thymidine and deoxycytidine (Morris 2009; Morris, LaClair et al. 2010) via mitochondrially localized salvage pathways (McKee, Bentley et al. 2004; Morris, LaClair et al. 2010). Previous work has demonstrated not only that TK2 is essential for neuronal functioning, but that it is essential for the dNTP salvage pathway and maintenance of mtDNA (Zhou, Solaroli et al. 2008; Bartesaghi, Betts-Henderson et al. 2010). A recent study of TK2 knockout mice suggests that while TK 2 dependent tissues such as heart and muscle may be

able up regulate compensatory mechanisms, no such mechanisms were found in the brain and the mice died of a fatal encephalomyopathy at 10-13 days of age (Dorado, Area et al. 2011).

As AZT has been shown to inhibit thymidine phosphorylation in the brain, studying the mechanisms of dNTP pool balance in the CNS remain important. Further, studying the metabolism of deoxypyrimidine analogs in brain may help in identifying new targets in treatment options not only for HIV in the CNS, but also for primary neoplasms, such as gliomas (Pai, Pai et al. 1998). Recently, there has been an increasing awareness that mitochondrial metabolism has been overlooked in glioma research. Treatment options for gliomas have not improved in recent decades, and prognosis for those diagnosed with glioblastoma remains poor. Mitochondrial metabolic dysfunction remains an important, but still imperfectly understood, feature of not only of gliomas but also of a variety of neurodegenerative diseases (Ordys, Launay et al. 2010).

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Drug Abbreviations

AZT	3'-azido-3'-deoxythymidine, zidovudine
d4T	2',3'-didehydrodideoxy-thymidine, stavudine
3TC	2'-deoxy-3'-thiacytidine, lamivudine
NRTIs	nucleoside analog reverse transcriptase inhibitors

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Figure 1. Tritium Labeled HPLC Chromatograms of Pyrimidine Deoxynucleosides and AZT
Isolated non-synaptosomal brain mitochondria were incubated for 3 hr with the appropriate [^3H]-deoxynucleoside or AZT. Samples were prepared for HPLC separation and peaks were quantitated as DPMs by an in-line scintillation counter as described in Materials and Methods. **Panel A:** Representative chromatogram (n=3) of brain mitochondria incubated 3 hr with [^3H]-thymidine (~5550 DPM / pmol, 1 μM). The small amount of radioactivity observed between 0 and 3 min of retention time was observed in the zero time control. **Panel B:** Representative chromatogram (n=3) of brain mitochondria incubated 3 hr with [^3H]-dC (~5550 DPM / pmol, 1 μM). **Panel C:** Representative chromatogram (n=3) of brain mitochondria incubated 3 hr with [^3H]-dU (~5550 DPM / pmol, 1 μM). **Panel D:** Representative chromatogram of brain mitochondria incubated 3 hr with [^3H]-AZT (~5550 DPM / pmol, 1 μM). This was the only chromatogram of the 3 that suggested the presence of AZT-DP.

Figure 2. Time-Course of Phosphorylation of [^3H]-Thymidine, dC, dU, and AZT in Isolated Brain Mitochondria

Isolated non-synaptosomal brain mitochondria were incubated for varying lengths of time up to 3 hr with the appropriate [^3H]-deoxynucleoside and samples prepared and analyzed as described in Materials and Methods. DPM data from the HPLC chromatograms were converted to pmol / mg mitochondrial protein as described in Materials and Methods and are expressed as the mean and standard error of the mean for each time point ($n = 3$). **Panel A:** Brain mitochondria incubated up to 3 hr with [^3H]-thymidine (4500~6000 DPM / pmol, 1 μM) TNP is the sum of TMP + TDP + TTP and represents total thymidine phosphorylation. **Panel B:** Brain mitochondria incubated up to 3 hr with [^3H]-dC (4700~5650 DPM / pmol, 1 μM) dCNP is the sum of dCMP + dCDP + dCTP and represents the total of dC phosphorylation. **Panel C:** Brain mitochondria incubated up to 3 hr with [^3H]-dU (~5200-6600 DPM / pmol, 1 μM). **Panel D:** Brain mitochondria incubated up to 3 hr with [^3H]-AZT (4440-5700 DPM / pmol, 1 μM). AZT-NP is the sum of AZT-MP and a very small amount of AZT-DP (not usually present).

Figure 3. Kinetics of Thymidine and dC Phosphorylation in Isolated Brain Mitochondria
Isolated non-synaptosomal brain mitochondrial were incubated with increasing concentrations of [³H]-thymidine as described in Materials and Methods (Panels A-C) or [³H]-dC (Panels D-F). Panel A: For thymidine and dC concentrations from 0.4 – 20 μM the specific radioactivity was ~8,000 DPM / pmol, while thymidine and dC concentrations of 80 and 200 μM had specific radioactivities of ~5,000 and 2,500 DPM / pmol respectively. Data are expressed in all panels as the mean and SEM, n = 3. **Panels A and C:** Michaelis-Menton plot as described in Materials and Methods for thymidine and dC phosphorylation respectively. **Panels B and D:** Eadie-Hofstee plots as described in the Materials and Methods for thymidine and dC phosphorylation respectively. **Panels C and F:** Hill plots as described in Materials and Methods for thymidine and dC phosphorylation respectively.

Figure 4. Kinetics of AZT Phosphorylation in Isolated Brain Mitochondria

Isolated non-synaptosomal brain mitochondrial were incubated with increasing concentrations of [³H]-AZT (0.4-200μM) exactly as was described for thymidine in Figure 3. For AZT concentrations from 0.4 – 20 μM the specific radioactivity was ~8,000 DPM / pmol, while AZT concentrations of 80 and 200 μM had specific radioactivities of ~5,000 and 2,500 DPM / pmol respectively. Data are expressed in all panels as the mean and SEM, n = 3. **Panel A:** Michaelis-Menton plot as described in Materials and Methods. **Panel B:** Eadie-Hofstee plot as described in Material and Methods. **Panel F:** (Inset): Hill plot as described in Material and Methods.

Figure 5. Effect of AZT on Phosphorylation of Thymidine, dC, and dU

Non-synaptosomal brain mitochondria were incubated with [^3H]-thymidine, [^3H]-dC, or [^3H]-dU (all at $1\ \mu\text{M}$, ~ 5000 - $6000\ \text{DPM} / \text{pmol}$) and increasing concentrations of AZT ($0 - 200\ \mu\text{M}$) for 2 hr. The rate of the control reaction was set at 100% and the rates of each AZT treated sample expressed as a percent of control. Values represent the mean and SEM of 3 observations.

Figure 6. Effect of Thymidine on [3H]-dC Phosphorylation and of dC on [3H]-Thymidine Phosphorylation

Panel A: Non-synaptosomal brain mitochondria were incubated for 2 hr as described in Materials and Methods with [3H]-thymidine (1 μM , 5000-6000 DPM / pmol) and varying concentrations of dC (0-200 μM). Total phosphorylation rates of the control mitochondria (0 dC) were set for each experiment at 100% and the rates of total phosphorylation in the presence of varying dC concentrations expressed as a % of control. Data shown are the means and SEM of three experiments. **Panel B:** Brain mitochondria were incubated for 2 hr as described in Materials and Methods with [3H]-dC (1 μM , 5000-6000 DPM / pmol) and varying concentrations of thymidine (0-200 μM). Total phosphorylation rates of the control mitochondria (0 thymidine) were set for each experiment at 100% and the rates of total phosphorylation in the presence of varying thymidine concentrations expressed as a % of control. Data shown are the means and SEM of three experiments.