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RESEARCH PAPER

The accumulation and metabolism of zidovudine in 3T3-F442A pre-adipocytes

Omar Janneh[1,2], Andrew Owen[2], Patrick G. Bray[3], David J. Back[2] and Munir Pirmohamed[2]

[1]*Department of Biomolecular and Sports Sciences, Coventry University, Coventry, UK,* [2]*Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, UK and* [3]*Molecular and Biochemical Parasitology Group, Liverpool School of Tropical Medicine, Liverpool, UK*

Background and purpose: Cultured pre-adipocytes accumulate and metabolize zidovudine (ZDV), but its mode of accumulation into these cells is unclear. We investigated the mode of accumulation of [^{3}H]-ZDV, and the impact of changes in external pH and modulators of drug transporters on its accumulation and metabolism.
Experimental approach: The initial rate and steady-state accumulation of [^{3}H]-ZDV were measured in 3T3-F442A cells. P-glycoprotein (P-gp) expression was detected by Western blotting. External pH was varied, and modulators of intracellular pH and drug transporters were used to study the mode of accumulation of ZDV. Phosphorylated ZDV metabolites were detected by high-performance liquid chromatography.
Key results: Intracellular accumulation of ZDV was rapid, reaching equilibrium within 20 min; nigericin increased accumulation by 1.9-fold, but this did not alter the generation of ZDV mono-, di- and triphosphate. The accumulation and metabolism were pH dependent, being maximal at pH 7.4 and least at pH 5.1. Monensin, carbonyl cyanide *p*-trifluoromethoxy) phenyl hydrazone, brefeldin A, bafilomycin A1 and concanamycin A increased accumulation; 2-deoxyglucose, dipyridamole, thymidine and tetraphenylphosphonium inhibited accumulation. The accumulation was saturable; the derived K_d and capacity of binding were 250 nmol per 10^6 cells and 265 nM respectively. 3T3-F442A cells express P-gp; inhibitors of P-gp (XR9576 and verapamil), P-gp/BCRP (GF120918), multidrug resistance protein (MRP) (MK571) and MRP/OATP (probenecid) increased the accumulation of ZDV. Saquinavir, ritonavir, amprenavir and lopinavir increased accumulation.
Conclusions and implications: The accumulation of ZDV in 3T3-F442A cells was rapid, energy dependent, saturable and pH sensitive. Western blot analysis showed that 3T3-F442A cells express P-gp, and direct inhibition assays suggest that ZDV is a substrate of P-gp and MRP.
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Keywords: zidovudine; intracellular accumulation; disposition; lipodystrophy; cytosolic pH modulators; P-gp

Abbreviations: Conca A, concanamycin A; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenyl hydrazone; HAART, highly active anti-retroviral therapy; mtDNA, mitochondrial DNA; TPP+, tetraphenylphosphonium chloride; ZDV, zidovudine; ZDVDP, zidovudine diphosphate; ZDVMP, zidovudine monophosphate; ZDVTP, zidovudine triphosphate

Introduction

Zidovudine (ZDV) was the first nucleoside reverse transcriptase inhibitor (NRTI) to be approved for the treatment of human immunodeficiency virus (HIV) infection. It is a monoprotic weak base (pKa 9.7), which accumulates and undergoes stepwise intracellular phosphorylation from the mono-, di- through to the active ZDV triphosphate (ZDVTP) in adipocytes (Janneh *et al.*, 2003). ZDVTP inhibits the replication of HIV by acting as a chain terminator and as an inhibitor of HIV reverse transcriptase enzyme (Lavie *et al.*, 1997).

ZDVTP (and similar drugs) also inhibit mitochondrial DNA (mtDNA) polymerase γ at high concentrations, altering mitochondrial functionality (Nolan and Mallal, 2003; Pace *et al.*, 2003; Stankov and Behrens, 2007; Stankov *et al.*, 2007; 2008; Wendelsdorf *et al.*, 2009). Although the mechanisms of ZDV-mediated mitochondrial toxicity are not completely clear (Bradshaw *et al.*, 2005; McComsey *et al.*, 2005a,b; Stankov *et al.*, 2007; Susan-Resiga *et al.*, 2007; Wendelsdorf *et al.*, 2009), it is believed that interference with mitochondrial function may at least partly alter the way the body produces, uses and distributes fat. This has been implicated in HIV-associated lipodystrophy syndrome (Stankov and Behrens,

Correspondence: Omar Janneh, Department of Pharmacology and Therapeutics, University of Liverpool, The Sherrington Buildings, Ashton Street, Liverpool, L69 3GE, UK. E-mail: o.janneh@coventry.ac.uk
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2007; Stankov *et al*., 2007; 2008; Sattler, 2008; Mercier *et al*., 2009). Other anti-retrovirals such as non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) (singly or in combination) have also been implicated in the pathogenesis of lipodystrophy. Although adipocytes metabolize ZDV and stavudine (Janneh *et al*., 2003), the effects of the drugs on adipocyte growth, adipogenesis, adipokine secretion and lipolysis remain equivocal (Janneh *et al*., 2003; Caron *et al*., 2004; Jones *et al*., 2005; Pacenti *et al*., 2006; Stankov *et al*., 2008). There is evidence that ZDV alone or in combination with other NRTIs, PIs or NNRTIs cause adipocyte mtDNA depletion and mitochondrial toxicity, and hence may mediate the pathogenesis of subcutaneous fat wasting (Le Bras *et al*., 1994; Sales *et al*., 2001; Nolan and Mallal, 2003; Wendelsdorf *et al*., 2009). However, the cellular basis of toxicity and the role of intracellular disposition of these drugs (alone and in combination) in the pathogenesis of lipodystrophy are unclear. In order to understand this, it is necessary to focus on the way the drugs access the target cells (adipocytes), and the factors that regulate the intracellular accumulation of the drugs. Data from such studies may provide clues in designing strategies to circumvent the cluster of adverse manifestations seen with HAART.

The intracellular concentration of a drug may influence drug efficacy and toxicity, and is controlled by a balance between influx and efflux, which can involve specific transporters. For the oral absorption of NRTIs, four broad categories of transporters (Na^+-dependent concentrative, Na^+-independent equilibrative, H^+/peptide and ATP-dependent efflux transporters) are important. Other nucleoside analogues (e.g. acyclovir) are transported by non-carrier-mediated diffusion or by non-nucleoside transporters (e.g. the oligopeptide transporter, PepT1; for a review, see Balimane and Sinko, 1999; Pastor-Anglada *et al*., 2005). Influx transporters, such as organic anion transporting polypeptides (OATPs), organic anion/cation transporters (OAT/OCT), the ATP binding cassette drug efflux transporters including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance protein (MRP) also impact on the transport of anti-retrovirals (Kim *et al*., 1998; Borst *et al*., 1999; Cihlar *et al*., 1999; Schuetz *et al*., 1999; Wada *et al*., 2000; Wijnholds *et al*., 2000; Chen *et al*., 2001; Huisman *et al*., 2001; 2002; Jones *et al*., 2001b; Morita *et al*., 2001; Takeda *et al*., 2002; Reid *et al*., 2003; Wang *et al*., 2003; Su *et al*., 2004; Janneh *et al*., 2005; 2008; Shaik *et al*., 2007). In previous studies, Purcet and colleagues did not detect hOATPs in T cells at both functional and mRNA levels, allowing them to rule out hOATPs in antiviral transport (Purcet *et al*., 2006). In contrast, we detected various hOATP isoforms in human lymphocytes, and data from direct inhibition assays suggest a role for these transporters in the transport of anti-retrovirals (Janneh *et al*., 2008). However, there is ample evidence of MRP- (Savaraj *et al*., 2003; Jorajuria *et al*., 2004; Eilers *et al*., 2008) and BCRP-mediated efflux of ZDV (Wang *et al*., 2003; 2004; Wang and Baba 2005; Pan *et al*., 2007). Recent evidence suggests that deletion of Bcrp1 does not play a significant role in limiting the CNS distribution of ZDV in FVB mice (Giri *et al*., 2008), but there is some evidence for the role of P-gp in the transport of ZDV (Batrakova *et al*., 1999; Leung and Bendayan, 1999).

In this study, we have investigated the mechanisms affecting the intracellular accumulation and disposition of ZDV in 3T3-F442A pre-adipocytes. Our results show that drugs that either intercalate into membranes or simply inhibit V-type ATPases, thereby increasing cytosolic pH, modulate the accumulation of ZDV. Additionally, we showed that pre-adipocytes express P-gp and that ZDV is a substrate for P-gp and MRP.

Methods

Cell culture

The 3T3-F442A pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified 10% CO_2 incubator. Pre-confluent 3T3-F442A cells were trypsinized and seeded into 24-well flat-bottomed plates at a density of 1×10^6 cells per well. The media were changed every 2–3 days, and once confluency was reached (usually after three to four media changes), the cells were utilized for the transport studies, as described below. For the phosphorylation assays, either the contents of one or two 75 cm^2 flasks were used with cell density ranging from 2.5 to 4.5 $\times$ 10^7cells.

Steady-state accumulation of ZDV and the effects of nigericin on accumulation and metabolism

Confluent pre-adipocytes (1×10^6 cells per reaction) were incubated in the absence or presence of 5 μM nigericin in serum-free DMEM media containing 32 nM [^{3}H]-ZDV. The assay was incubated at 37°C over 0–20 min, and at each time-point, the cells were washed three times in ice-cold phosphate-buffered saline (PBS) before being terminated by centrifugation (15 000× *g* for 2 min at 4°C), and the cell pellets were processed for radioactivity counting (Janneh *et al*., 2003).

pH-dependent accumulation and metabolism of ZDV

The pH-dependent accumulation (1×10^6 cells per reaction, 20 min incubation) and disposition (75 cm^2 tissue flask per reaction, 24 h incubation) of ZDV were measured by incubating the cells at 37°C, 10% CO_2 in DMEM media supplemented with 10% FCS between pH 4.0 and 7.4, and 5.1 and 7.4 respectively. Cells were harvested for scintillation counting, or extracted and separated by high-performance liquid chromatography (Janneh *et al*., 2003).

Effects of cellular acidification on the accumulation of ZDV

The cells were incubated for 20 min without or with varying concentrations of monensin, carbonyl cyanide *p*-(trifluoromethoxy) phenyl hydrazone (FCCP, dissipates H+ electrochemical gradient), bafilomycin A1, concanamycin A (Conca A), tetraphenylphosphonium chloride (TPP+) and brefeldin A. The cell pellets were washed three times in ice-cold PBS before being processed for scintillation counting.

Characterization of ZDV binding parameters and the effects of thymidine on accumulation

To determine ZDV binding parameters, 1×10^6 cells were suspended in growth medium containing 32 nM [^{3}H]-ZDV

and various concentrations of non-radioactive ZDV (0.01–300 μM). After 20 min, the reaction was terminated, and the cells were processed and counted for radioactivity as described previously (Bray *et al*., 1996). The saturable accumulation of ZDV, represented by using Scatchard plot, was calculated by subtracting non-saturable ZDV accumulation from total accumulation. The apparent affinity of binding (K_d) was determined from the reciprocal of the slope, and the amount of bound drug was given by the *X*-intercept.

As ZDV is a thymidine analogue, co-incubation of thymidine with ZDV should reduce the accumulation of ZDV. To test this, 1×10^6 cells were incubated (37°C, 20 min) in DMEM media containing 10% FCS in the presence of various concentrations of thymidine (0–1000 μM) before the assay was terminated by centrifugation as described previously (Janneh *et al*., 2003).

Western blot analysis for P-gp

Membrane protein was extracted from 30×10^6 pre-adipocytes using the method of Marshak *et al*. (1996). Protein concentration was estimated as described previously (Smith *et al*., 1985). For each sample, a volume of sample equivalent to 50 μg protein was mixed with an equal volume of loading buffer containing 10% Nupage reducing agent (Invitrogen Ltd, Paisley, UK). The samples were incubated at 70°C for 10 min before cooling to 40°C. The entire sample was then separated by electrophoresis after loading onto a 3–12% Tris–acetate gel. The gel was run at 180 V in Invitrogen Tris–acetate running buffer (Invitrogen Ltd.), and thereafter soaked in Nupage transfer buffer for 30 min before being transferred at 100 V for 1 h onto a nitrocellulose membrane. The membrane was then blocked overnight with 5% bovine serum albumin (BSA) in 0.05% Tween–Tris borate sulphate buffer (T-TBS). The membrane was then washed three times with 0.05% T-TBS before being probed with goat anti-P-gp IgG primary antibody (C-19), 1:1000 in 0.05% T-TBS buffer containing 2% BSA for 2 h. The membranes were washed four times, and then another four times at 5 min intervals in 0.05% T-TBS buffer. The membranes were further probed with horseradish peroxidase-linked anti-goat P-gp IgG secondary antibody (1:10 000) for 1 h in 0.05% T-TBS containing 2% BSA. Finally, the membranes were washed as described earlier before the protein–antibody conjugate was visualized using ECL Western blotting detection system.

Effects of inhibitors of drug transporters on the accumulation of ZDV

Initial studies investigated the effects of temperature (37, –20 and 4°C), cell viability (dead cells, pre-incubation of cells at 55°C, 10 min) versus live cells (incubation at 37°C) on the accumulation of ZDV. The effects of 1 μM XR9576, previously shown to inhibit P-gp activity (Janneh *et al*., 2005; 2007), verapamil and GF120918 (0–100 μM) (P-gp and P-gp/BCRP inhibitors, respectively, Jones *et al*., 2001a; Kawamura *et al*., 2009); probenecid and MK571 (0–100 μM) (MRP2/OATP and MRP inhibitors, respectively, Jones *et al*., 2001a; Huisman *et al*., 2002; Tahara *et al*., 2006); and PIs (0–30 μM) (substrates and inhibitors of P-gp, Gutmann *et al*., 1999; Perloff *et al*., 2000; Gupta *et al*., 2004; Hennessy *et al*., 2004; Janneh *et al*., 2007) on the accumulation of ZDV were investigated. A known potent inhibitor of nucleoside transport, dipyridamole (0–100 μM) (Leung and Bendayan, 1999) was used as an internal control to study its effects on the accumulation of ZDV.

In other experiments, the effect of 2-deoxyglucose on the accumulation of ZDV was investigated. Here, pre-adipocytes (1×10^6 cells) were incubated in HEPES Ringer buffer (100 mM HEPES, 122.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.25 mM $MgCl_2$ and 5.5 mM D-glucose or with an equimolar concentration of the non-utilizable glucose, 2-deoxyglucose, pH 7.4) containing 32 nM [^{3}H]-ZDV was used as the incubating medium. The samples were incubated and harvested for scintillation counting as described previously (Janneh *et al*., 2003).

Statistical analysis

Data were evaluated for statistical significance by analysis of variance followed by modified *t*-test (Bonferroni), where significance between means was assumed if $P < 0.05$.

Reagents and materials

[^{3}H]-ZDV (14.2 Ci·mmol^{-1}) was purchased from Moravek Biochemicals (Brea, CA, USA). Nigericin, Conca A, bafilomycin A1, monensin, TPP+, verapamil, probenecid and FCCP were purchased from Sigma Chemical Co (Poole, UK). MK571 (3-[[[3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid) was bought from Alexis Biochemicals (San Diego, CA, USA). Glaxo Wellcome (Research Triangle Park, NC, USA) and Xenova Plc (Slough, UK) donated GF120918 (*N*-[4-[2-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]-5-methoxy-9-oxo-10H-acridine-4-carboxamide) and XR9576 (tariquidar; *N*-[2-[[4-[2-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide) respectively. The PIs nelfinavir (NFV, Agouron Pharmaceuticals, San Diego, CA, USA), saquinavir (SQV, donated by Roche, Welwyn Garden City, UK), ritonavir (RTV) and lopinavir (LPV) were gifts from Abbott Laboratories (North Chicago, IL, USA); amprenavir (APV) was a gift from Glaxo Wellcome Research and Development (Hertfordshire, UK), and indinavir (IDV) was donated by Merck (Rahway, NJ, USA). CEM_{VBL} cells were a gift from Dr R. Davey (Australia) and were derived from the CEM cells treated with vinblastine (100 ng·mL^{-1}) to cause the over-expression of P-gp. These cells were used as a control line in the Western blot assay aimed at detecting P-gp in 3T3-F442A cells.

Tris–acetate gel was obtained from Invitrogen Ltd (Paisley, UK); nitrocellulose membrane was from Sigma; goat anti-P-gp IgG and primary antibody horseradish peroxidase-linked anti-goat P-gp IgG secondary antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Results

Effects of nigericin on the accumulation and metabolism of ZDV

The 3T3-F442A pre-adipocytes rapidly accumulated ZDV, reaching equilibrium within 20 min (Figure 1A). The co-incubation of 5 μM nigericin (K^+/H^+ exchanger)

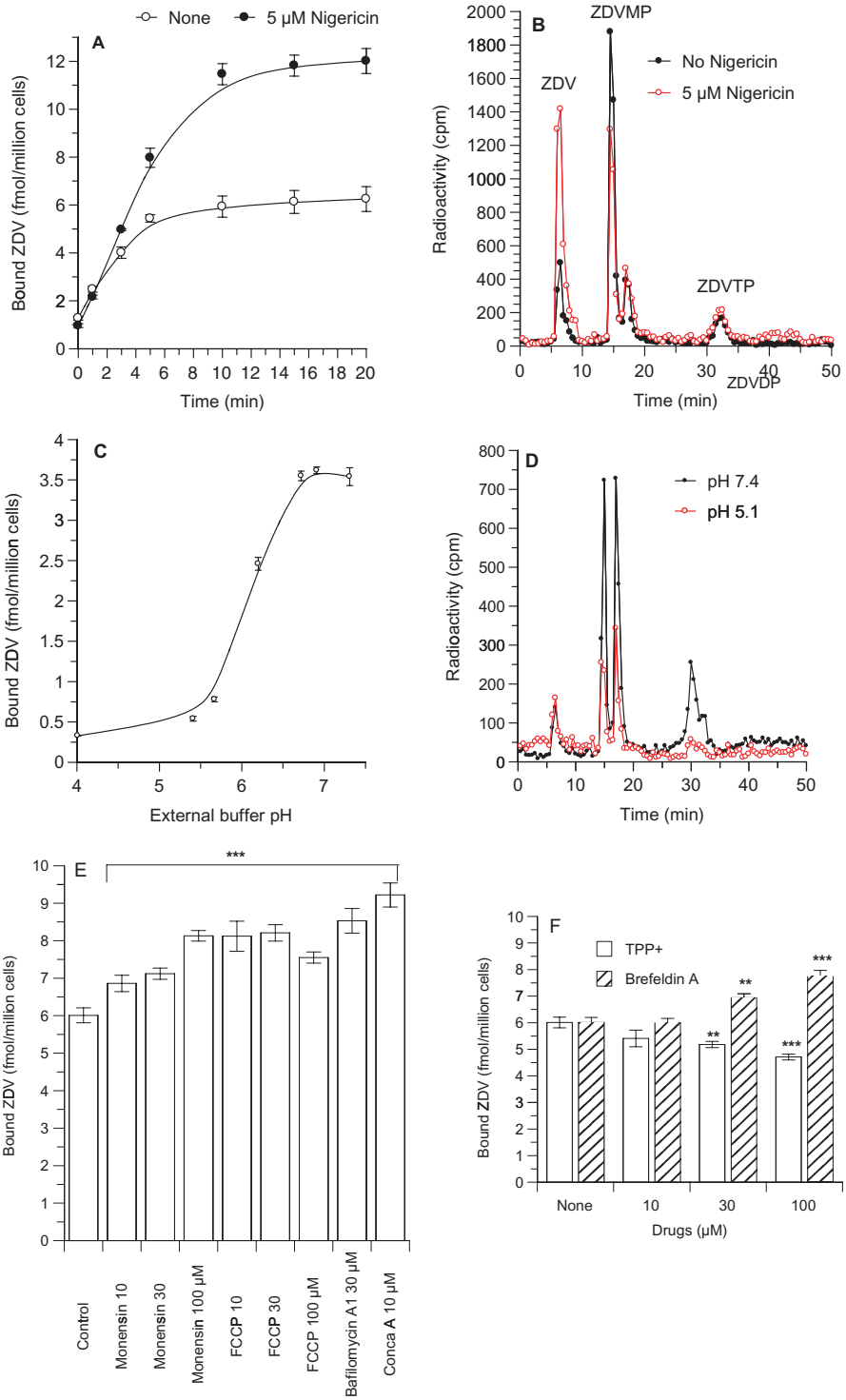

Figure 1 (A) Steady-state accumulation of ZDV in the presence and absence of 5 μM nigericin. (B) Representative trace showing the effects of 5 μM nigericin on the phosphorylation of ZDV. (C) The accumulation of ZDV in pre-adipocytes is pH dependent. (D) The phosphorylation of ZDV is pH dependent. (E) The effects of various concentrations of monensin, FCCP, bafilomycin A1 and concanamycin A (Conca A) on the intracellular concentration of ZDV. (F) The effects of various concentrations of brefeldin A and TPP^+ (tetraphenylphosphonium) on the accumulation of ZDV. Data points represent the mean ± SD, $n = 4$. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the control.

significantly increased ($P < 0.001$) the accumulation of ZDV in the cells by 1.9-fold compared to the control. Although co-administration of nigericin caused a threefold increase in ZDV, this manipulation did not increase intracellular ZDVMP and ZDVTP (Figure 1B).

pH-dependent accumulation and metabolism of ZDV

Because nigericin is known to increase cytosolic pH, the pH dependence of ZDV accumulation in 3T3-F442A pre-adipocytes, followed by metabolism studies, over two pH ranges, was investigated. Figure 1C shows that the

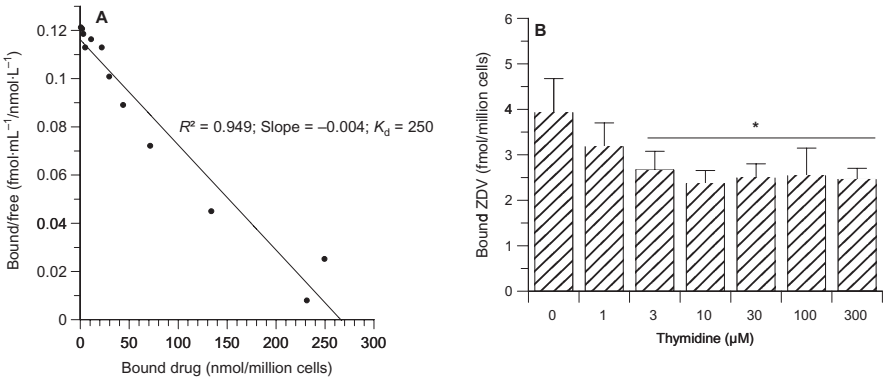

Figure 2 (A) Scatchard plot of equilibrium ZDV binding in 3T3-F442A pre-adipocytes. The derived K_d and capacity of binding values were 250 and 265 respectively. Data points represent means from four independent observations. (B) The effect of increasing concentrations of thymidine on the accumulation of ZDV in 3T3-F442A pre-adipocytes. Data points represent the mean ± SD of triplicate observations of four individual experiments. *$P \leq 0.05$, compared to the control.

accumulation of ZDV follows a pH-dependent profile being least at pH 4 and optimal at pH 7.4. The conversion of ZDV to its anabolites also followed a pH-dependent profile, being maximal at pH 7.4 and least at pH 5.1, when a 73% decrease in the production of ZDVTP was observed (Figure 1D).

Effects of changes in cytosolic pH on the accumulation of ZDV

Monensin, FCCP, bafilomycin A1 and Conca A all significantly ($P \leq 0.001$) increased the accumulation of ZDV (Figure 1E). Similarly, brefeldin A (at 30 and 100 μM), an inhibitor of V-type ATPase that causes elevation of cytosolic pH, significantly ($P < 0.01$) increased the accumulation of ZDV (Figure 1E). In contrast, TPP^+ (at 30 and 100 μM) significantly decreased accumulation by 14 and 22% ($P = 0.007$ and $P = 0.005$, respectively) compared to the control (Figure 1F).

The accumulation of ZDV is saturable and sensitive to thymidine inhibition

As shown in the Scatchard plot in Figure 2A, the accumulation of ZDV into 3T3-F442A pre-adipocytes has a derived K_d and capacity of binding of 250 nmol per 10^6 cells and 265 nM respectively. Furthermore, thymidine reduced accumulation in a concentration-dependent manner (Figure 2B).

3T3-F442A Pre-adipocytes express P-gp

The 3T3-F442A cells were shown to express P-gp, albeit at lower levels than that observed in mouse liver controls (Figure 3).

Effects of modulators of drug transporters (P-gp and MRP) and HIV PIs on the accumulation of ZDV

The accumulation of ZDV was energy dependent, decreasing the temperature below 37°C markedly decreased the accumulation of the drug, an observation which is in agreement with previous findings (Purcet *et al*., 2006). Marked differences in the accumulation of ZDV can also be seen between live and dead cells (Figure 4A). Relatively, specific inhibitors of P-gp (XR9576 and verapamil), P-gp/BCRP (GF120918), MRP

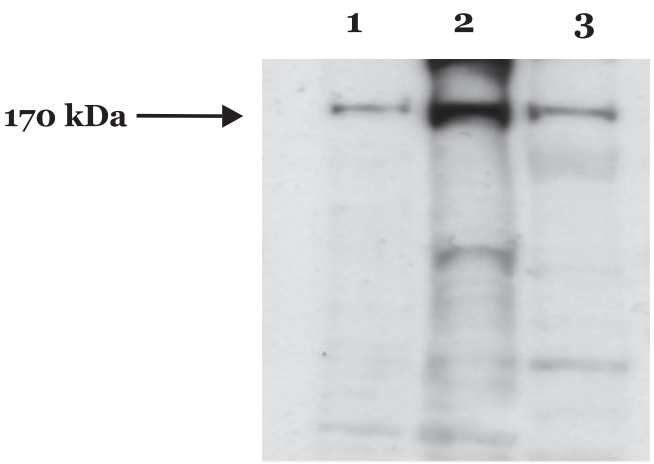

Figure 3 The expression of P-gp in 3T3-F442A pre-adipocytes. Membrane proteins were isolated from 3T3-F442A pre-adipocytes before being electrophoresed on a 3–12% Tris–acetate gel. Lane 1 represents P-gp from 3T3-F442A pre-adipocytes; lane 2, P-gp from mouse liver; lane 3, P-gp from the T lymphoblastoid cell line, CEM_{VBL}.

(MK571) and MRP/OATP (probenecid) significantly ($P \leq 0.01$) increased the accumulation of ZDV. However, given that GF120918 also inhibits BCRP (Pan *et al*., 2007; Kawamura *et al*., 2009), which transports NRTIs (Wang *et al*., 2003; 2004; Wang and Baba 2005), it is plausible that inhibition of BCRP may contribute to some of the observed effects seen with GF120918-treated samples. Dipyridamole, a classical and potent inhibitor of nucleoside transport, also significantly ($P < 0.001$) reduced accumulation (Figure 4A). Deoxyglucose at 5.5 mM significantly ($P < 0.0001$) reduced the accumulation of ZDV (Figure 4B).

Given that the current HIV treatment involves the co-administration of multiple drugs, which together may contribute to the lipodystrophy syndrome, the effects of PIs (also known substrates, inhibitors and inducers of drug efflux transporters such as P-gp, MRP and BCRP) on accumulation were investigated. Lower concentrations of some PIs (e.g. 1 and 3 μM RTV, APV and LPV; and 1–30 μM IDV) significantly ($P \leq 0.01$) increased accumulation. Conversely, NFV (1–10 μM) and SQV (1 and 3 μM) did not alter the

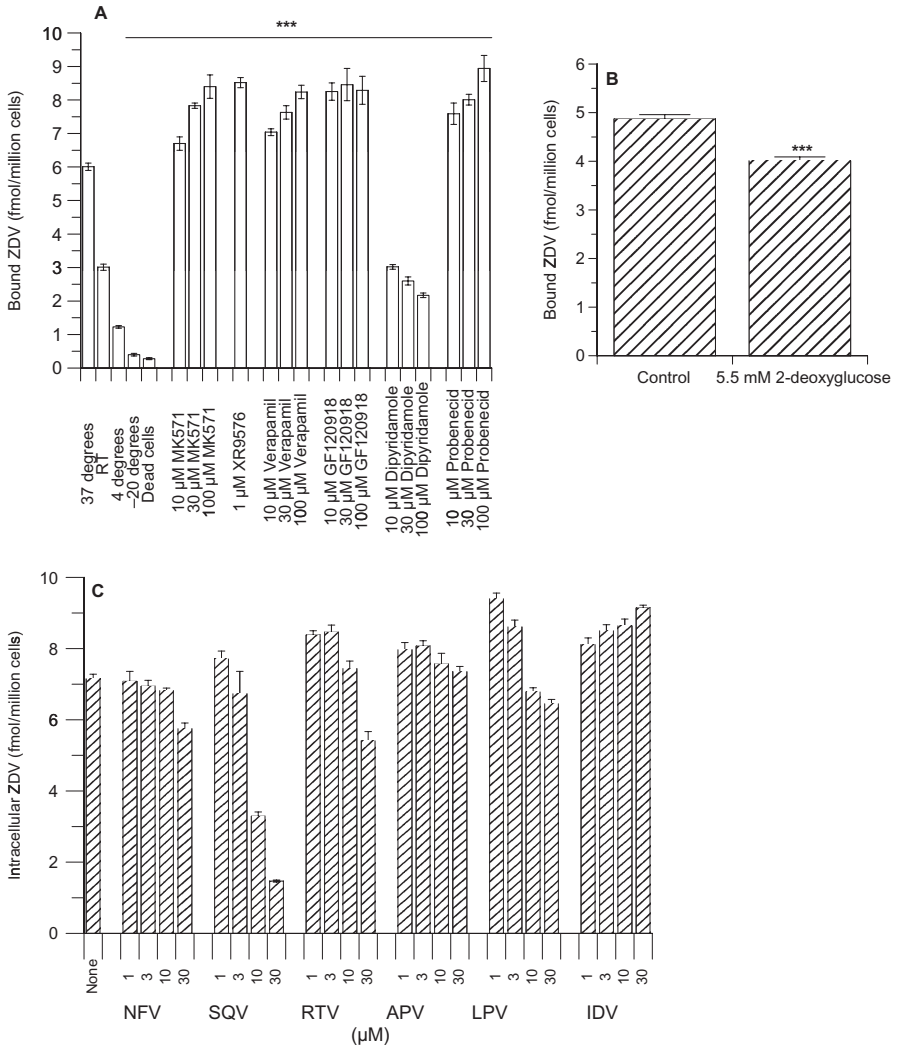

Figure 4 (A) The effects of 37, 18 and 4°C, and heat killing (dead cells) on the intracellular accumulation of [^{3}H]-ZDV. Also shown are the effects of various concentrations of MK571, XR9576 (1 μM), verapamil, GF120918, dipyridamole and probenecid on the accumulation of ZDV. (B) The effect of 5 mM 2-deoxyglucose on the accumulation of ZDV. (C) The effects of low concentrations of the HIV protease inhibitors NFV, SQV, RTV, APV, LPV and IDV on the accumulation of ZDV in 3T3-F442A pre-adipocytes. The reduction in the accumulation of ZDV caused by a high concentration of some of the PIs demonstrates that the PIs have toxic effects on the adipocytes at high concentrations. Data points represent the mean ± SD of triplicate observations from four independent experiments. For clarity in (A) and (C), the significance indicators have not been shown. Please see Results for details. In (B), ***$P < 0.001$ compared to the control.

accumulation. Higher concentrations of SQV (10 and 30 μM), NFV, RTV and LPV (30 μM) significantly ($P \leq 0.005$) decreased the accumulation.

Discussion

Although HAART has revolutionized HIV treatment, it has been shown to cause both lipid disturbances and fat redistribution. ZDV is one component of some HAART regimens, and may therefore contribute to the development of lipodystrophy (Nolan and Mallal, 2003). In order to understand the adverse metabolic effects of the drugs, it is crucial to understand the pharmacology of ZDV in adipocytes.

In the current study, using direct inhibition assays, the accumulation of ZDV in pre-adipocytes seemed to be governed by the alkalinization of cytosolic compartments. Nigericin (a carboxylic acid ionophore, uncouples K^+/H^+ bidirectionally with greater affinity for H^+) significantly increased the accumulation. These data highlight the fundamental differences in ZDV sequestration in acidic organelles compared to other drugs, such as anti-neoplastic agents, in normal and multidrug resistant cells (Willingham *et al.*, 1986). Willingham and colleagues showed that the accumulation of daunomycin and doxorubicin was lower when co-incubated with nigericin, which suggests a different mode of accumulation to ZDV. Other than differences in the cell types used, another plausible explanation is that nigericin is able to elevate calcium pools within cells (Garcia *et al.*, 1998), and it is possible that this may be linked to increased ZDV accumulation. Indeed, in some cells, the storage of calcium is dependent on mitochondria (Boncompagni *et al.*, 2009), which also accumulate and phosphorylate ZDV (Sales *et al.*, 2001).

Despite the 1.9-fold increase in the accumulation of ZDV observed with nigericin treatment, this did not cause an increase in the production of the phosphorylated metabolites of ZDV (Figure 1B), suggesting that the thymidine kinases were saturated. Characterization of the accumulation and phosphorylation of ZDV revealed that the accumulation of ZDV and the production of its phosphorylated anabolites followed a pH-dependent profile. The accumulation was maximal at pH 7.4, and least at pH 4.0. As expected, the production of ZDV anabolites was maximal at pH 7.4, being least at pH 5.1, indicating that pH 7.4 is optimal for the efficient catalytic activity of thymidine kinases.

The intracellular accumulation of ZDV was significantly increased by drugs such as monensin (inhibitor of H^+ ATPase), FCCP (a protonophoric agent), bafilomycin A1, Conca A and brefeldin A. These compounds are strong inhibitors of V-ATPases, intercalate into the membranes of acidic compartments and cause increases in internal pH. Interestingly, the lipid-soluble cation, TPP^+ (Slayman *et al*., 1994), which causes internal alkalinization via leakage of basic amino acids, Na^+ and K^+ into extracellular medium, but without titrating H^+ out of the cells, caused a significant decrease in the accumulation. Because ZDV accumulates and is metabolized in the mitochondria (Sales *et al*., 2001), it is plausible that the influence of TPP^+ on mitochondrial membrane potential (delta ψ_m), may be linked to the intracellular accumulation of ZDV. Furthermore, the recent observation that the mitochondria express human equilibrative transporter capable of transporting dideoxynucleotides (Govindarajan *et al*., 2009) and the potential inhibitory effects of TPP+ on this transporter may explain the observed effects.

These data provide evidence that the accumulation of ZDV is saturable, with a derived K_d and capacity of binding of 250 nmol per 10^6 cells and 265 nM respectively. The interaction of ZDV with thymidine kinase appears to be the rate-limiting step rather than transport into the cells, which is reduced by thymidine.

P-gp was expressed on pre-adipocytes (Figure 3) and limits the accumulation of ZDV. In agreement with data obtained on HPIs in other cell types (Jones *et al*., 2001b; Huisman *et al*., 2002), the accumulation of ZDV was energy dependent, with the co-administration of deoxyglucose significantly abrogating accumulation (Figure 4A and B). Interestingly, direct inhibition assays using inhibitors of P-gp (XR9576 and verapamil), P-gp/BCRP (GF120918), MRP/OATP (probenecid) and MRP (MK571) significantly increased the intracellular accumulation of ZDV. These data confirm previous observations that the efflux mediated by P-gp and MRP reduces the uptake of ZDV (Batrakova *et al*., 1999; Eilers *et al*., 2008). However, the observation that probenecid, which also inhibits organic anion transporters (OATPs and OATs, influx transporters), decreased the uptake of ZDV indicates that these transporters may not be involved in the accumulation of ZDV in 3T3-F442A cells. Dipyridamole, a potent inhibitor of nucleoside transport and accumulation, significantly decreased the transport of ZDV. We demonstrated that the accumulation was increased by some PIs, which are substrates and inhibitors of P-gp (Shiraki *et al*., 2000; Olson *et al*., 2002), further confirming that drugs such as RTV (as suggested by Olson *et al*., 2002), APV and LPV may actively improve sanctuary site concentrations of ZDV. Contrary to a previous report that NFV inhibits P-gp (Shiraki *et al*., 2000), in this study, it failed to increase the intracellular concentration of ZDV. High concentrations of some PIs (e.g. SQV, RTV and LPV) also caused a significant reduction in intracellular concentrations of ZDV; this is likely to be a consequence of the cytotoxic effects of the PIs (Zhang *et al*., 1999; Dowell *et al*., 2000; Lenhard *et al*., 2000; Janneh *et al*., 2003; Lagathu *et al*., 2004; Jones *et al*., 2005).

Drug transporters are expressed at numerous epithelial barriers (e.g. on hepatocytes, renal tubular cells, blood–brain barrier, epithelial cells of the intestine, macrophages and on lymphocytes) mediating the influx and extrusion of drugs and endogenous substances. Each transporter has an overlapping and broad substrate specificity. Although information regarding the expression of drug transporters in adipocytes is in its infancy, our current knowledge of transporters mediating ZDV transport in adipocytes, together with transporters yet to be investigated, is shown in Figure 5. Collectively, these transporters have the ability to pump drugs and endogenous substances bidirectionally or unidirectionally across the cell membrane.

The bidirectional transporters are able to pump substances in either direction, secreting them from the cell into the interstitial fluid, with the possibility of reabsorbing the substance back into the cell. Among these are the bidirectional OATP and OAT/OCT (from the solute carrier transporter protein, SLC21 and SLC22 gene families), which are present in the basolateral membrane. In experiments using Madin–Darby canine kidney cells stably transfected with OAT-K1 or OAT-K2 cDNA, ZDV was shown to be a substrate of OAT (Takeuchi *et al*., 2001). Future research to identify the expression in adipocytes of this and other SLC gene families, of which there are at least 46, is needed. For a review of SLC gene families, their subtypes and their potential importance in adipose tissue, see Baldwin *et al*. (2004), Gray *et al*. (2004) and Guallar *et al*. (2007).

Unidirectional pumps such as P-gp, MRP, BCRP and bile salt export protein (BSEP) are expressed on the apical membranes, and extrude drugs and endogenous substances out of the cell (Figure 5). There is also evidence of the existence of sodium hydrogen exchanger (NHE) in adipocytes (Klip *et al*., 1988). It resides in the plasma membrane and is responsible for cellular pH homeostasis, extruding metabolically generated protons for sodium ions. The micro/vesicles (called adiposomes) and plasma membrane of adipocytes also have V-type ATPases, which are involved in the biogenesis of the glucose transporter isoform 4 (GLUT4) vesicles (Malikova *et al*., 2004). Additionally, V-type ATPases function to pump protons, with the process energized by ATP hydrolysis (Karcz *et al*., 1993; 1994; Stevens and Forgac, 1997; Nelson and Harvey, 1999; Hayashi *et al*., 2000), resulting in the acidification of organelles and extracellular space. This is vital for the accumulation of nutrients and ions, and the extrusion of harmful agents (Stevens and Forgac, 1997; Nelson and Harvey, 1999). Given the effects of the proton pump inhibitors on the accumulation of ZDV (Figure 1E and F), the observed effects may be due to the effects on NHE or V-type ATPase (Figure 5). In conclusion, it is likely that proton-coupled transport proteins; cellular kinases; and drug efflux, influx proteins and passive

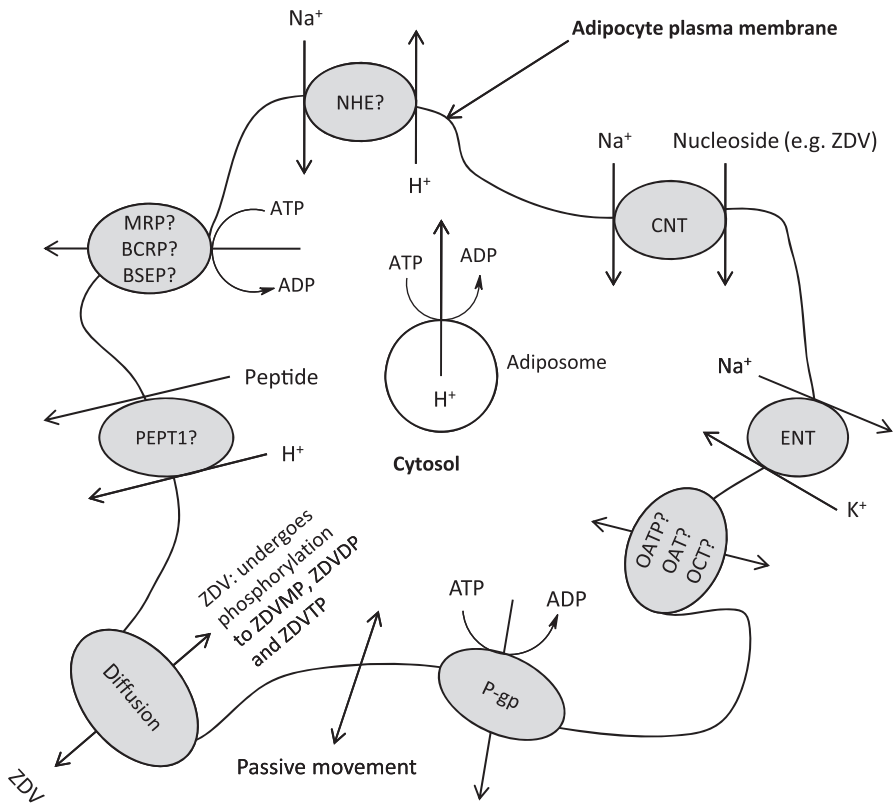

Figure 5 Schematic diagram of major transporters which may contribute to the influx and efflux of zidovudine. Transporters involved in the uptake and efflux of zidovudine have been located on adipocytes. Those known to be basolateral are the ones expressed by the SLC28 and SLC29 gene families (ENT and CNTs respectively). The apical membranes are P-gp, MRP, BCRP and bile salt export protein (BSEP). The transport mechanisms shown include four different types of processes: Na^+-dependent concentrative, Na^+-independent equilibrative, H^+/peptide and ATP-dependent efflux transporters. The Na^+/K^+-ATPase, as well as the Na^+/H^+ exchanger, are also shown because Na^+- and H^+-dependent transporters are functionally linked to these two ion carriers. OATP is from the SLC21 gene family, OAT/OCT from the SLC22 gene family and the peptide transporter-1 (PEPT1) from the SLC15 gene family. This is an idealized scheme; not all of the transporters are likely to be expressed by a single cell, and passive diffusion will also contribute to the entry of ZDV within the adipocyte. For further reading on nucleoside transporters, see Balimane and Sinko (1999), Cihlar *et al.* (1999), Wada *et al.* (2000), Morita *et al.* (2001), Takeda *et al.* (2002), Pastor-Anglada *et al.* (2005) and Guallar *et al.* (2007).

diffusion may all influence the intracellular concentration of ZDV, together with the effects of co-administered drug. Studies aimed at identifying the expression of both influx and efflux transporters in the subcutaneous and omental depots of human adipose tissue, and the mode of accumulation of anti-retrovirals in these sites, may explain the mechanistic basis of the site-specific toxicities of these drugs (alone and in combination) on adipose tissue.

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Conflict of interest

D.J.B. has received grant/research support from Abbott, Bristol-Myers Squibb, GlaxoSmithKline and Roche Pharmaceuticals, and has served as a consultant for Gilead Sciences, GlaxoSmithKline and Vertex.

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