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Equilibrium distribution of HIV antiviral drugs into human peripheral blood mononuclear cells (PBMC) is controlled by free drug concentration in the extracellular medium

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Abstract

Effect of protein binding on the equilibrium distribution of selected HIV antiviral drugs into isolated human peripheral blood mononuclear cells (PBMC, mainly lymphocytes) was investigated. Human PBMC from a single healthy human donor were isolated, purified, and cryopreserved. Uptake of non-peptide HIV-1 protease inhibitors PNU-96988 and PNU-103017 by these cells in vitro was evaluated as a function of increasing concentration of human serum in the cell incubation media. Both PNU-96988 and PNU-103017 were extensively bound to serum proteins. Uptake/efflux kinetics were very rapid such that accumulation by the cells was thermodynamically, not kinetically, controlled. Accumulation by human PBMCs in vitro was directly proportional to the free and not the total drug concentration in the media. For comparative purposes, the serum protein binding effect on the distribution of two HIV reverse transcriptase (RT) inhibitors, delavirdine (RESCRIPTOR) and zidovudine (AZT), was also evaluated. Like the HIV-1 protease inhibitors, delavirdine was found to be extensively associated with serum proteins and its accumulation by human PBMCs in vitro to be proportional to the free and not total drug concentration. In contrast, AZT was not bound to serum proteins to any significant extent. The uptake of this drug by human PBMCs in vitro was independent of serum concentration. However, the intrinsic cellular accumulation of PNU-96988, PNU-103017 and delavirdine were all greater than AZT. Thus, the extent to which drugs uptake by cells is affected by serum appears proportional to the binding affinity of the serum proteins for the drug. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The rapid spread of the acquired immunodeficiency syndrome (AIDS) [1] epidemic has stimulated efforts to discover therapeutic agents to

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control the replication of the causative virus, human immunodeficiency virus (HIV). The viral enzyme, HIV protease, plays a critical role in maturation and infectivity of the human immunodeficiency virus (HIV), and therefore, compounds which inhibit this enzyme represent a potential therapy for AIDS [1,2]. Recent advances have resulted in an increasing number of HIV protease inhibitors undergoing clinical evaluations and approved for treatment of AIDS [3]. PNU-96988 and PNU-103017 are representative non-peptide HIV-1 protease inhibitors under investigation as a potential oral treatment for acquired immune deficiency syndrome (AIDS) [4,5]. These drugs are extensively bound to serum proteins [6].

Reduced in vitro antiviral potency associated with increasing amounts of fetal bovine (FBS) or human serum (HS) in cell culture media has been reported for several extensively serum protein-bound HIV antiviral drugs (both reverse transcriptase (RT) inhibitors and protease inhibitors) [7–10]. In contrast, serum protein has been shown to have relatively little effect on the in vitro antiviral activity of drugs which are poorly bound to serum proteins, such as zidovudine (AZT) [7,11] or dideoxyinosine (ddI) [10].

We have previously evaluated the serum protein binding of PNU-96988, PNU-103017 and several analogs [6]. The high-affinity interaction of PNU-96988 and similar analogs with serum protein(s) has been shown to markedly reduce cellular transmembrane permeation [12], accumulation [13], and cytotoxicity [13] of these drugs.

The goal of the present study was to evaluate the extent to which human serum protein binding of the protease inhibitors PNU-96988 and PNU-103017 in the extracellular medium affects their distribution into normal human lymphocytes in vitro. These cells are a primary target for HIV viral infection and the virus is known to replicate in these cells. For comparative purposes we have also evaluated the effects of serum protein binding on the distribution of two HIV reverse transcriptase inhibitors, delavirdine and AZT.

2. Experimental

2.1. Chemicals and reagents

[^{14}C]PNU-96988 (3-(α-ethylbenzyl)-6-(α-ethylphenethyl)-4-hydroxy-2H-pyran-2-one), specific activity 114.76 μCi mg^{-1}, radiochemical purity 98% (Lot 26471-JPM-138A), [^{14}C]PNU-103017 (4-cyano-*N*-[3-[cyclopropyl(5,6,7,8,9,10-hexahydro-4-hydroxy-2-oxo-2H-cycloocta[*b*]pyran-3-yl] phenyl]benzenesulfonamide), specific activity 94 μCi mg^{-1}, radiochemical purity 96% (Lot 27983-JPM-71C), [^{14}C]delavirdine, specific activity 34.7 μCi mg^{-1}, radiochemical purity >99% (Lot 25853-EHC-148A) were prepared at Pharmacia & Upjohn (Kalamazoo, MI). [2-^{14}C]3′-Azido 3′-deoxythymidine (AZT), specific activity 205.8 μCi mg^{-1}, radiochemical purity 99.9% (Lot 114-078-055) was obtained from Moravek Biochemicals (Breca, CA). [^{14}C]Sucrose, specific activity 475 mCi mmol^{-1}, was obtained from NEN™ Life Science (Boston, MA). The position of ^{14}C labels in each of the drugs is indicated with an * in the structures shown in Fig. 1. The racemic mixtures of chiral PNU-96988 and PNU-103017 were used in the studies. RPMI 1640 w/L-glutamine cell culture medium was obtained from Gibco-BRL (Rockville, MD). The medium was buffered at pH 7.4 with the addition of 10 mM Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; 5 ml of a Gibco 1 M Hepes solution (Cat. No. 15360-015) was added to 500 ml medium). The medium was supplemented with 2 mM additional L-glutamine by addition of 5 ml of a 200 mM solution of L-glutamine in 0.85% saline (BioWhittaker, Walkersville, MD) to 500 ml of the RPMI 1640. Additionally, 5 ml of antibiotic–antimycotic mixture obtained from Gibco (100×, liquid; Cat. No. 600-5240AG, Lot 14K8228) was added to 500 ml of the RPMI 1640. Sterile fetal bovine serum (FBS) was obtained from Gibco-BRL (Cat. No. 200-6140AJ, Lot No. 33P6022). Sterile, pooled, human serum (HS) was obtained from Bayer (Kankakee, IL; sterile filtered human serum, Pentex, Cat. No. 82-320-1, Lot 613B). Serum complement was heat inactivated by heating the serum at 56°C

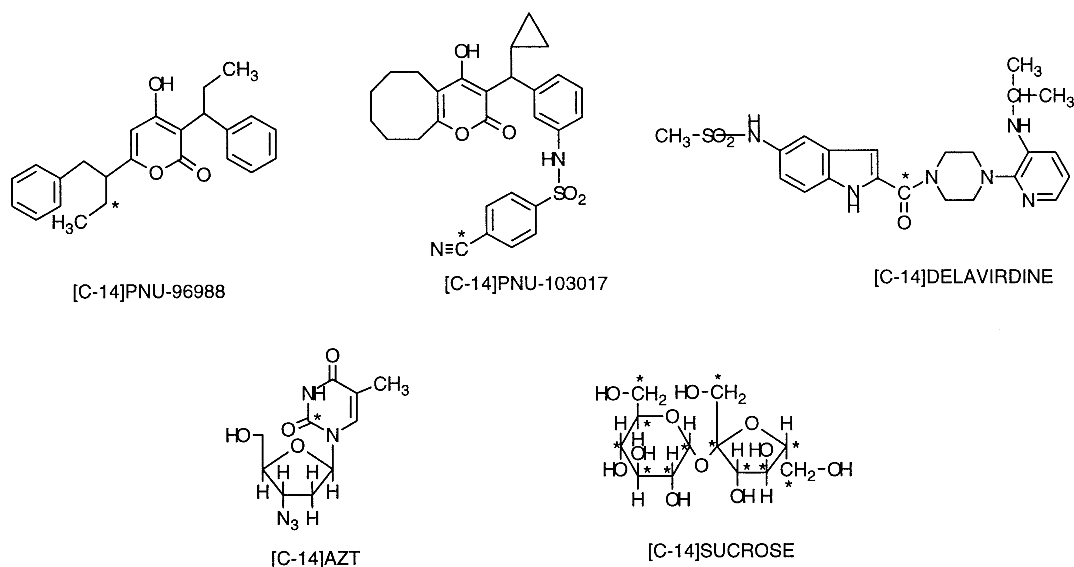

Fig. 1. Structures of compounds used in cell distribution studies. Position of radiolabel is indicated with an *.

for 45 min. The same lots of sera were used for all protein binding studies to eliminate lot-to-lot serum variability.

2.2. Isolation of human peripheral blood mononuclear cells (PBMC)

A cell suspension highly enriched in human PBMCs from a single healthy volunteer (male, age 21, non-smoker, no medication for 14 days, no alcohol for 2 days, HIV negative, hepatitis B negative, normal blood cell counts and chemistry) was obtained from the Leukopheresis program at Pharmacia & Upjohn Research Clinic in Kalamazoo, MI [14]. Whole blood was withdrawn continuously from a 16-gauge fistula needle placed in the brachial vein of the left arm of a human volunteer. The blood was mixed with citrate anticoagulant and passed through a Fenwal CS-3000 Blood Cell Separator (Fenwal division of Baxter Scientific; Dearfield, IL). The blood cell separator was programmed to collect an enriched PBMC fraction and return red blood cells and plasma through an 18-gauge angiocath (teflon catheter) into the patient's right arm. The entire process took about 2.5–3 h, during which 5 l of the patient's blood was processed. At the conclusion of the procedure, a 100-ml suspension of cells (leukopack), highly enriched in PBMCs, was obtained. The cell suspension was placed on ice and transported to the laboratory for further purification.

2.3. Cell purification by density gradient centrifugation

The cell suspension obtained from the leukophoresis program was further purified in the laboratory by density gradient centrifugation. Aliquots (20 ml) of the cell suspension were added to four separate 50-ml centrifuge tubes and diluted with 15 ml of sterile Dulbecco's phosphate-buffered saline (PBS). A serological pipet was used to carefully layer 12 ml of lymphocyte separation medium (Organon–Teknika; Durham, NC) under the diluted cell suspensions (35 ml). The tubes were placed in a Damon/IEC tabletop centrifuge (with swinging bucket rotor, $r_{max} = 18.3$ cm) and centrifuged at $800 \times g$ (2000 rpm) for 20 min. After this centrifugation step the bulk of the upper layer was aspirated and discarded. A sterile pipet was used to transfer the narrow white cell layers to each of four separate 50-ml centrifuge tubes diluted to 50 ml with the addition of sterile

PBS. The cells were sedimented by centrifugation at $450 \times g$ (1500 rpm) for 15 min. The supernatant was discarded. Each cell pellet was resuspended in 5 ml of PBS. Two 5-ml cell suspensions were combined to yield 2×10 ml cell suspensions from the original 4×5 ml. The cells were washed again by sedimenting at $450 \times g$ (1500 rpm) for 10 min. Again, the supernatant was discarded. The cells were resuspended in 2×5 ml PBS. The two tubes were then combined to give one tube of 10 ml. This was added to 40 ml PBS to give a total volume of 50 ml. The cells were counted both manually (2.73×10^7 cells ml^{-1}) using a hemacytometer and with a Coulter Counter ZM (Attn = 8, 3.11×10^7 cells ml^{-1}). Cell viability by trypan blue and ethidium bromide exclusion assays was estimated to be about 90 and 97%, respectively. The isolation and purification yielded 1.6×10^9 total cells.

2.4. Cryopreservation and thawing of PBMC cells

A cryopreservation medium containing RPMI 1640 (with 10 mM Hepes, 2 mM L-glutamine, and antibiotic/antimycotic), 10% (v/v) DMSO, and 20% (v/v) fetal bovine serum (FBS) was prepared. The cell suspension from the above purification was centrifuged at $450 \times g$ (1500 rpm) for 10 min to sediment the cells. The supernatant was discarded. The cells were resuspended in 45 ml of cryopreservation medium. Aliquots (1.5 ml) of the cell suspension were dispensed into Corning 2-ml cryopreservation vials. The vials were capped and placed in a closed styrofoam container. The styrofoam container was placed in a −20°C freezer for 2 h to allow slow cooling of the cells. The container was then placed at −70°C. After overnight storage at −70°C the vials were removed from the styrofoam container and transferred to a −135°C freezer for storage. Cryopreserved cells were rapidly thawed by immersion in a water bath at 37°C for 2–3 min. The cells were washed and resuspended accordingly. Viability after thawing was > 82% by the trypan blue dye exclusion assay.

2.5. Preparation of 40 μM drug stock solutions in cell medium

Concentrated stock solutions (2 mM) of [^{14}C]U-96988 and [^{14}C]U-103017 inhibitors were prepared in 0.1 M NaOH, taking advantage of the high solubility of these analogs at high pH due to the acidic 4-hydroxyl functional group. Stock solutions (40 μM) of each drug in RPMI cell medium were prepared with the addition of 300 μl of 2 mM stock solution (0.1 M NaOH) to 15 ml of serum-free RPMI 1640 cell medium. The addition of the 0.1 M NaOH to the buffered cell medium did not exceed its buffering capacity as evidenced by lack of change in pH. A delavirdine stock solution (2 mM) was prepared in 0.1 M HCl taking advantage of the enhanced solubility of this basic drug in acid. A 40 μM solution of delavirdine in RPMI 1640 was prepared by the addition of 300 μl of the stock to 15 ml of serum-free RPMI 1640 cell medium. The addition of dilute acid did not change the pH of the buffered cell medium. A 40 μM stock solution of [^{14}C]AZT was prepared as follows. The ethanol was removed from 0.66 ml of an ethanolic solution of AZT (50 μCi ml^{-1}, 55 mCi mmol^{-1}). The material was then directly dissolved in 15 ml of serum-free RPMI 1640 cell medium.

2.6. Liquid scintillation measurement of drug concentration

Drug concentrations for the radiolabeled drugs used in these studies were measured by liquid scintillation counting (LSC). Aliquots (100 μl) were added to 10 ml of Ultima Gold liquid scintillation cocktail and counted by LSC on a Packard (Meriden, CT) Tri-Carb® Liquid Scintillation Analyzer Model 1900CA for 10 min. Conversion of dpm to drug concentration was done based on the known specific activity (μCi mg^{-1} drug), molecular weight of the drug, and aliquot volume.

2.7. Kinetics of [^{14}C]PNU-96988 accumulation by PBMC: time to equilibrium

A series of incubation media containing a total U-96988 concentration of 10 μM and radioactivity of 0.41 μCi ml^{-1}, but different human serum concentration and thus different free PNU-96988 concentration were prepared. Two complete sets of incubation mixtures were prepared: one that

contained PBMC cells and one without PBMC cells. Appropriate volumes of either 10% HS or 100% HS were added to Corning polypropylene 2-ml cryotubes. A volume of 1.5 ml of 40 μM [^{14}C]PNU-96988 (1.63 μCi ml^{-1}) stock solution in RPMI 1640 was added to each tube. The indicated volume of serum-free RPMI 1640 cell medium was added (determined so that total volume was 6 ml after all components were added). A volume of 1.5 ml of a PBMC cell stock solution in serum-free RPMI 1640 cell medium (cell density $= 16 \times 10^6$ cells ml^{-1}) was added to each tube for the set of tubes containing cells. A volume of 1.5 ml of RPMI 1640 cell medium in place of cell stock solution was added to the control set of tubes.

Each incubation mixture was incubated at 37°C (5% CO_2) for fixed time periods ranging from 0.5 to 6 h (0.5, 1, 2, 4 and 6 h). After incubation the individual incubations were mixed gently by inversion and 0.5-ml aliquots withdrawn in duplicate and transferred to polypropylene tubes. The aliquots were centrifuged on a Fisher Microcentrifuge Model 59A (with swinging bucket rotor at a setting of 7) to sediment the cells. The centrifugation step was also done for the controls to eliminate the possibility that some of the radioactivity that settled during sedimentation of cell mixtures was undissolved drug and not actually associated with cells. After pelleting, the supernatant was aspirated to waste, taking care to remove all medium without disturbing the cell pellets. A volume of 0.5 ml of Dupont Solvable™ tissue and gel solubilizer (Dupont NEN Research Products, Cat. No. NEF-910; 0.5 M solution) was added to each tube. Each tube was vortex-mixed and heated at 37°C for 30 min. The total volume of each tube was transferred to a corresponding scintillation vial. A volume of 10 ml of Ultima Gold liquid scintillation cocktail was added, and each sample was counted by LSC for 10 min using a Packard Tri-Carb® liquid scintillation analyzer Model 1900CA.

2.8. Measurement of the equilibrium distribution of test compounds

The equilibrium distribution of [^{14}C]PNU-96988, [^{14}C]PNU-103017, [^{14}C]AZT, and [^{14}C]delavirdine into human PBMC was measured as a function of human serum concentration. The design of these experiments was similar to the U-96988 kinetic experiment (above), except that incubations were carried out at only one time point (30 min), 0.5 ml of 40 μM drug stock solution in RPMI 1640, a total volume of 2 ml as used, and triplicate 0.5-ml aliquots of the incubation mixtures were analyzed.

2.9. Determination of unbound (free) drug concentration

2.9.1. Ultrafiltration

[^{14}C]PNU-96988, [^{14}C]PNU-103017, and [^{14}C]AZT protein binding experiments were carried out by centrifugal ultrafiltration through Amicon Centrifree™ devices. Amicon devices were loaded with 1 ml of serum/cell medium pre-equilibrated at 37°C. A Beckman GS-6R centrifuge with GA-10 fixed 35° angle rotor was used. The rotor was pre-equilibrated at 37°C. Ultrafiltration devices were centrifuged between 1500 and 3000 rpm ($309-1240 \times g$ at $r_{max} = 123$ mm) for 5–10 min depending on serum concentration and, thus, viscosity of the particular sample. Centrifugation time and speed were adjusted such that no more than 200 μl ($<20\%$ of total volume) of ultrafiltrate was collected. Drug adsorption to the ultrafiltration devices was checked for each individual drug using a 10-μM solution of drug in 67 mM, pH 7.4 phosphate buffer, containing no serum. Recovery $>95\%$ was considered acceptable. Recovery $<95\%$ indicated that the specific drug underwent significant adsorption to the device and ultrafiltration was not the method of choice for free drug determination.

2.9.2. Equilibrium dialysis

[^{14}C]Delavirdine protein binding measurements were carried out by equilibrium dialysis using a Dianorm Equilibrium Dialyzer (DIAN-20) and Dianorm membranes (10000 molecular weight cut-off). Dianorm semi-micro teflon cells were loaded with 1 ml of serum sample on one side and 1 ml of RPMI 1640 or dialysis buffer on the other side of the membrane. Dialysis buffer was prepared by dissolving an appropriate amount of

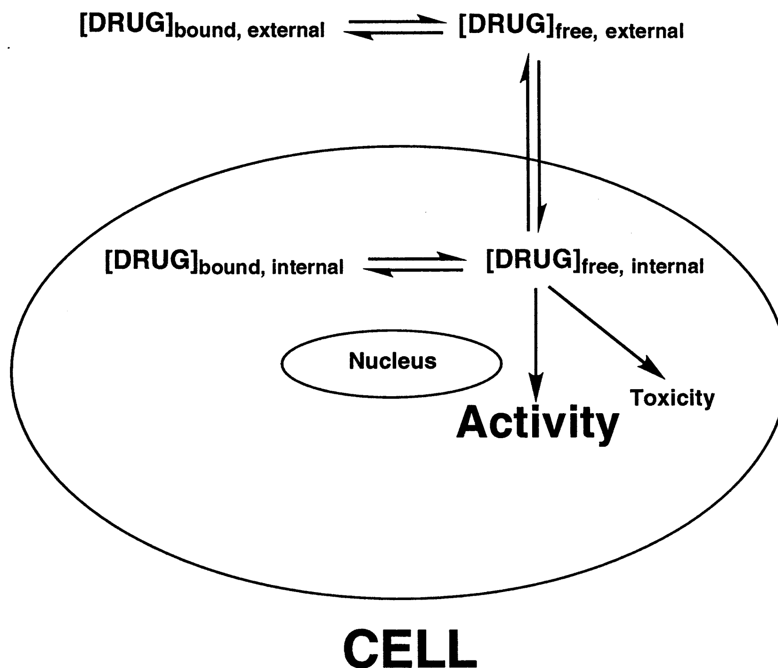

Fig. 2. Equilibrium of a drug between intracellular and extracellular space.

$(NH_4)_2HPO_4$ in Milli-Q water to prepare a 0.02 M solution, adding 6.76 g of sodium chloride/l to increase osmolarity, and adjustment of the pH to 7.4 with H_3PO_4. All dialysis experiments were carried out at 37°C for 4 h. Prior to the loading of cells, all apparatus, serum, and buffer were equilibrated at 37°C. Duplicate cells were run for each serum sample. Prior to dialysis serum solutions were sampled by removing two 100-μl aliquots to measure the total drug concentration. Sampling of cells post-dialysis was done by removing a single 500-μl aliquot from the buffer compartment, and a single 100-μl aliquot from the serum compartment. Equilibrium dialysis binding calculations were carried out as previously described [15].

3. Results and discussion

3.1. Choice of cell type for accumulation/distribution studies

Many cell types have been shown to be infected by the HIV virus. However, the primary targets for viral infection appear to be lymphocytes (specifically a subtype of T cells with CD4 receptors), monocytes, and macrophages. Collectively, lymphocytes and monocytes in the blood are referred to as peripheral blood mononuclear cells (PBMC). Adequate penetration of an antiviral drug into these cells and accessibility to its target site, the virus particle, are thus likely the necessary attributes for an effective HIV antiviral agent. Hence they were selected for this investigation. Two primary factors limiting the passive uptake of molecules into cells include the molecule's intrinsic ability to permeate the cell membrane and the binding of the molecule to components external to the cell. As is illustrated in Fig. 2, it is generally accepted that only free (unbound) drugs are able to penetrate cells by passive diffusion. Reversible binding of drugs to components such as serum proteins in the external medium reduces the amount of drug available to diffuse into the cell. In addition, binding to intracellular sites other than the target site can reduce activity. Most studies that have investigated the effect of serum protein binding on cell uptake

involve the uptake of drugs into red blood cells (RBC). Plasma protein binding has been shown to significantly reduce the uptake of various drugs into red blood cells [16–18]. Polarity, hydrophobicity, charge as well as other structural considerations influence the intrinsic permeation of a drug into a cell. Molecules which are not protein bound, but have very poor intrinsic permeability (e.g. sucrose), do not significantly distribute into or accumulate within cells.

3.2. Effect of human serum on uptake of HIV antiviral agents by human lymphocytes in vitro

Several approaches were considered for the physical separation of the cells from the incubation media to enable quantitation of drug which distributed into the cells. Of primary importance was the observation that drug efflux out of the cells was very fast, and washing of the cells to remove any intercellular trapped drug after pelleting caused complete loss of intracellular drug. Sedimentation of cells in each incubation mixture, aspiration of the supernatant, and direct scintillation counting of the cell pellets (without a wash step) was found to give the most reproducible results. Although, in the absence of a wash step, physically trapped intercellular radioactivity was present in the cell pellet, it did not pose a significant problem considering the experimental design. Since all incubation solutions (regardless of serum concentration) had identical total drug and radioactivity concentrations, this type of physical entrapment should have been equivalent for all samples and represent a constant absolute bias (or offset). Since it is the change in total cell radioactivity versus percent serum, and not the absolute cell radioactivity, which are important for interpretation of these experiments, a constant bias did not significantly affect the conclusions of this investigation.

Quantitation of cellular uptake by monitoring changes in the cell media concentration was not a practical alternative. The PBMC density (4–5×10^6) in the incubation was chosen to be comparable to that in normal human whole blood. Because the amount of total radioactivity taken up by this number of cells is very small ($\ll 0.5\%$) relative to the total radioactivity in the incubation medium, a measurable change in radioactivity of the media was not observed under the conditions of our experiments.

3.3. Time to uptake/efflux equilibrium

Human PBMC were incubated at a cell density of about 4×10^6 cells ml^{-1} with [^{14}C]PNU-96988 at a total drug concentration of 10 μM containing four different human serum concentrations: 0, 0.5, 10 and 50% (v/v) HS. This cell density was to approximate the total white blood cell density of whole human blood (4–12×10^6 cells ml^{-1}). Each mixture was prepared to contain an identical radioactive concentration (0.41 μCi ml^{-1}) which was verified by LSC. Distribution of radiolabeled U-96988 into cells was measured at 0.5, 1, 2, 4 and 6 h. The results in Table 1 demonstrate that distribution equilibrium for PNU-96988 was attained before the first time point (30 min). Similar in vitro studies with human H-9 T cells have shown that uptake equilibrium is reached in < 5 min under these same conditions [12]. Based on these results, an equilibration time of 30 min was used for all other uptake experiments. Note that although each incubation mixture contained identical total concentrations of PNU-96988 and cells, the amount of drug which distributed into the cells markedly decreased with increasing serum concentration, consistent with cell accumulation controlled by free, not total, drug concentration.

3.4. Measurement of the equilibrium distribution

The equilibrium distribution of [^{14}C]PNU-96988, [^{14}C]PNU-103017, [^{14}C]AZT, and [^{14}C]delavirdine into human PBMC was measured as a function of human serum concentration. Human PBMC were incubated at a cell density of about 4×10^6 cells ml^{-1} with each HIV antiviral drug at a total drug concentration of 10 μM containing eight different human serum concentrations: 0, 0.1, 0.5, 1, 2, 5, 10 and 50% (v/v) HS. Distribution of the radiolabeled

Table 1
[^{14}C]PNU-96988 Cell uptake/efflux kinetics

Time (h)	Radioactivity in cell pellet (dpm pellet–background (dpm ± S.D. ($n=2$)))			
	0% HS	0.5% HS	10% HS	50% HS
0.5	6117 ± 707	2423 ± 1044	599 ± 169	183 ± 130
1	7299 ± 675	3786 ± 79	691 ± 230	276 ± 147
2	6487 ± 457	2855 ± 347	704 ± 193	194 ± 223
4	5390 ± 240	2639 ± 802	753 ± 311	109 ± 112
6	6107 ± 397	3083 ± 491	578 ± 137	212 ± 97

Each incubation medium contained 10 μM, 7.4×10^{6} dpm ml^{-1} [^{14}C]PNU-96988 and 3.04×10^{6} cells ml^{-1}.
Aliquots of 0.5 ml of each incubation mixture were sedimented in duplicate to give cell pellets containing about 1.5×10^{6} cells/pellet. Total radioactivity per cell pellet values listed in the table were corrected for background radioactivity in the corresponding control incubation of the same serum concentration (see details in Section 2).

drugs into cells was measured after an equilibration time of 30 min at 37°C. The level of total PNU-96988 and PNU-103017 associated radioactivity in the PBMC pellets (and thus also in individual cells) was found to drop steeply with increases in the serum concentration in the cell medium (Table 2; Fig. 3A). As illustrated in Fig. 3B and tabulated in Table 3, the free drug concentration of these drugs in PBMC incubation mixtures (in cell media and thus external to the cells) was found to decrease drastically with increases in the human serum concentration. Thus differences in radioactivity in cell pellets can only be correlated with changes in the serum concentration of the media and thus the unbound (free) concentration of drug available.

The marked decrease in total cell-associated radioactivity directly paralleled the serum protein binding curves for the drugs. In Fig. 3C, plots of the cellular uptake data with the x-axis transformed from %HS to the corresponding free drug concentration are shown. These plots reveal that the amount of PNU-96988 or PNU-103107 taken up into the PBMC is correlated with the free drug concentration of either drug in the external medium. The decrease in cell uptake as serum increased is greater for PNU-103017 than PNU-96988, consistent with the greater binding affinity of PNU-103017 for serum proteins [6].

Similar to the protease inhibitors, uptake of the RT inhibitor, delavirdine, by human PBMC was decreased significantly with increasing concentrations of human serum in the extracellular medium (Table 2). Free drug concentration versus serum binding relationship was determined by equilibrium dialysis. The distribution of delavirdine into human PBMC was proportional to the free drug concentration in the extracellular medium (Table 3). The effect appeared less than for PNU-96988, probably due to the lower serum protein binding affinity of human serum proteins for delavirdine as compared to PNU-96988.

The free drug versus human serum concentration relationship for AZT is given in Table 3. Note the lack of change in unbound drug concentration with increasing concentrations of human serum up to 50%. This result is consistent with the low extent of binding of this drug which has been reported [6,11]. Variation in human serum concentrations (0–50% HS) also had no effect on the distribution of AZT into human PBMC (Table 2). Upon comparison of each of the antiviral drugs on a molar basis it was clear that very little PNU-96988 or PNU-103017 accumulates in cells in the presence of 50% HS (Table 2). On the other hand, a small but measurable level of AZT was taken up by the cells virtually independent of serum concentration. Although cellular levels of delavirdine decrease with increasing serum, significant accu-

Table 2
[^{14}C]PNU-96988, [^{14}C]PNU-103017, [^{14}C]AZT, and [^{14}C]delavirdine PBMC cell in vitro accumulation/distribution results vs human serum concentration in the incubation media

% HS	Drug in cell pellet (dpm pellet–background (dpm ± S.D. ($n = 3$)) (pmol cell^{-1} ± S.D.))			
	[^{14}C]PNU-96988	[^{14}C]PNU-103017	[^{14}C]AZT	[^{14}C]Delavirdine
0	10053 ± 600 ($4.4 \pm 0.3 \times 10^{-5}$)	7638 ± 746 ($2.7 \pm 0.3 \times 10^{-5}$)	3891 ± 485 ($1.2 \pm 0.1 \times 10^{-5}$)	6258 ± 553 ($6.0 \pm 0.5 \times 10^{-5}$)
0.1	7164 ± 344 ($3.1 \pm 0.2 \times 10^{-5}$)	5740 ± 384 ($2.1 \pm 0.1 \times 10^{-5}$)	2362 ± 423 ($0.7 \pm 0.1 \times 10^{-5}$)	6128 ± 1444 ($5.9 \pm 0.1 \times 10^{-5}$)
0.5	4340 ± 937 ($1.9 \pm 0.4 \times 10^{-5}$)	3798 ± 526 ($1.4 \pm 0.2 \times 10^{-5}$)	1592 ± 293 ($0.5 \pm 0.1 \times 10^{-5}$)	5233 ± 421 ($5.0 \pm 0.4 \times 10^{-5}$)
1	3314 ± 147 ($1.4 \pm 0.1 \times 10^{-5}$)	2229 ± 316 ($0.8 \pm 0.1 \times 10^{-5}$)	1793 ± 382 ($0.5 \pm 0.1 \times 10^{-5}$)	5008 ± 315 ($4.8 \pm 0.3 \times 10^{-5}$)
2	2340 ± 416 ($1.0 \pm 0.2 \times 10^{-5}$)	903 ± 200 ($0.3 \pm 0.1 \times 10^{-5}$)	1784 ± 452 ($0.5 \pm 0.1 \times 10^{-5}$)	3254 (3.1×10^{-5})
5	1273 ± 245 ($0.6 \pm 0.1 \times 10^{-5}$)	768 ± 91 ($0.3 \pm 0.03 \times 10^{-5}$)	1497 ± 222 ($0.5 \pm 0.1 \times 10^{-5}$)	3148 ± 1159 ($3.0 \pm 1.1 \times 10^{-5}$)
10	1252 ± 206 ($0.5 \pm 0.1 \times 10^{-5}$)	703 ± 121 ($0.2 \pm 0.04 \times 10^{-5}$)	2261 ± 432 ($0.7 \pm 0.1 \times 10^{-5}$)	2299 ± 535 ($2.2 \pm 0.5 \times 10^{-5}$)
50	535 ± 219 ($0.2 \pm 0.1 \times 10^{-5}$)	371 ± 93 ($0.1 \pm 0.03 \times 10^{-5}$)	1500 ± 566 ($0.5 \pm 0.1 \times 10^{-5}$)	3834 ± 775 ($3.7 \pm 0.7 \times 10^{-5}$)

Incubation medium contained 10 μM of corresponding drug and cells: [^{14}C]PNU-96988 (83 960 dpm (100 μl)$^{-1}$; cell density, 5.0×10^6 cells ml^{-1}); [^{14}C]PNU-103107 (87 190 dpm (100 μl)$^{-1}$; cell density, 5.3×10^6 cells ml^{-1}); [^{14}C]AZT (124 682 dpm (100 μl)$^{-1}$; cell density, 5.4×10^6 cells ml^{-1}); [^{14}C]delavirdine (18 067 dpm (100 μl)$^{-1}$; cell density, 5.0×10^6 cells ml^{-1}).
Aliqouts of 0.5 ml of each incubation mixture were sedimented in duplicate to give cell pellets. Total radioactivity and pmol cell^{-1} values listed in the table were corrected for background radioactivity in the corresponding mock control incubation of the same serum concentration (see details in Section 2).

mulation of delavirdine in human PBMC was observed in vitro even in 50% HS. This result is consistent with both the greater free fraction of delavirdine in 50% HS and its greatest intrinsic buffer–cell partition coefficient (T.J. Raub, unpublished data).

Absolute determination of the amount of drug in the cells was difficult to ascertain. Because of the absence of a wash step, part of the radioactivity in cell pellets was physically trapped cell medium in the intercellular space. The assumption was made for the PNU-96988/PNU-103017 incubation mixtures containing 50% HS that a negligible level of drug exists intracellularly, and that pelleted radioactivity is intercellularly entrapped. With this assumption, an upper limit on the extracellular volume of the cell pellets was estimated using the radioactive concentrations of the cell media used. The extracellular volume estimated in this way was 0.64 ± 0.26 μl. Sucrose is frequently used as an extracellular volume marker, although it can be taken up by cells to a limited extent by endocytotic processes [19]. The extracellular volume of our pellets was measured with radiolabeled sucrose in an experiment similar to those measurements of the equilibrium distribution for test compounds. The extracellular volume was estimated to be less than 1.6 ± 0.1 μl. It is not known what percentage of this apparent volume is represented by volume taken up by endocytosis. In Table 2 the antiviral drugs evaluated are compared for their relative ability to accumulate with cells in the absence of serum. Note that the uptake of AZT by cells was relatively low (in absence or presence of serum). The other drugs evaluated had normalized *intrinsic* (i.e. 0% serum) accumulations as reported in Table 4.

3.5. Extrapolation to in vivo situations

There are more than 2×10^{12} total lymphocytes in the human body, but only about 1% of these are present in blood at any time [20]. The majority of lymphocytes are present in the lymphatic system (within lymph in lymph ducts and lymphoid

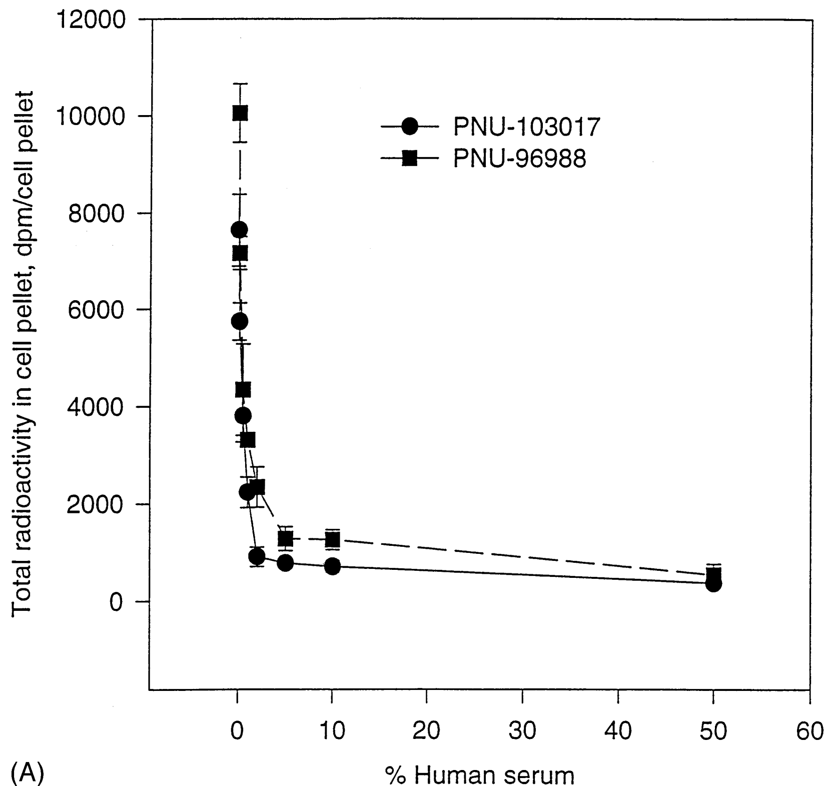

Fig. 3. Association of [^{14}C]PNU-96988 and [^{14}C]PNU-103017 to human PBMC. (A) Association of [^{14}C]PNU-96988 and [^{14}C]PNU-103017 to human PBMC versus % serum in cell medium ($n = 3$ replicate determinations per data point; error bars $\pm$ 1 S.D.; background radioactivity from control samples (i.e. no cells) was subtracted from all data points illustrated). (B) Serum protein binding of [^{14}C]PNU-96988 and [^{14}C]PNU-103017: free versus %HS (determined by ultrafiltration, $n = 2$ replicate determinations per data point, error bars $\pm$ 1 S.D., recovery from ultrafiltration membrane of [^{14}C]PNU-96988 was shown to be $> 98\%$ with no evidence of non-specific adsorption). (C) Total radioactivity in cell pellet versus free [^{14}C]PNU-96988 and [^{14}C]PNU-103017.

organs: lymph nodes, the thymus, spleen, appendix). It has been demonstrated that significant HIV infection exists in the lymphatic system during what was previously thought to be a latent period of the disease, based on the lack of detectable virus in the blood [21,22]. Adequate distribution of an HIV antiviral drug in the lymphatic system may thus be important. In general, it is known that highly protein-bound drugs tend to distribute more poorly into the lymphatic and other body compartments [23]. Lymphatic cannulation techniques for continuous collection of lymph from laboratory animals have been described which allow the monitoring of the lymph to plasma ratio of drugs [24,25]. It may be possible to use such a technique to attempt to monitor the amount of a radiotracer (or metabolites) taken up in vivo by lymph and lymphocytes.

4. Conclusions

Equilibrium distribution of PNU-96988 and PNU-103017 into human white blood cells (specifically PBMC) in vitro was markedly reduced as the serum concentration of the matrix in which the cells were suspended was increased. The total amount of drug which distributed into the cells was directly proportional to the free and not the total drug concentration external to the cells. If the antiviral action of these drugs occurs within cells or virion particles, these results suggest that

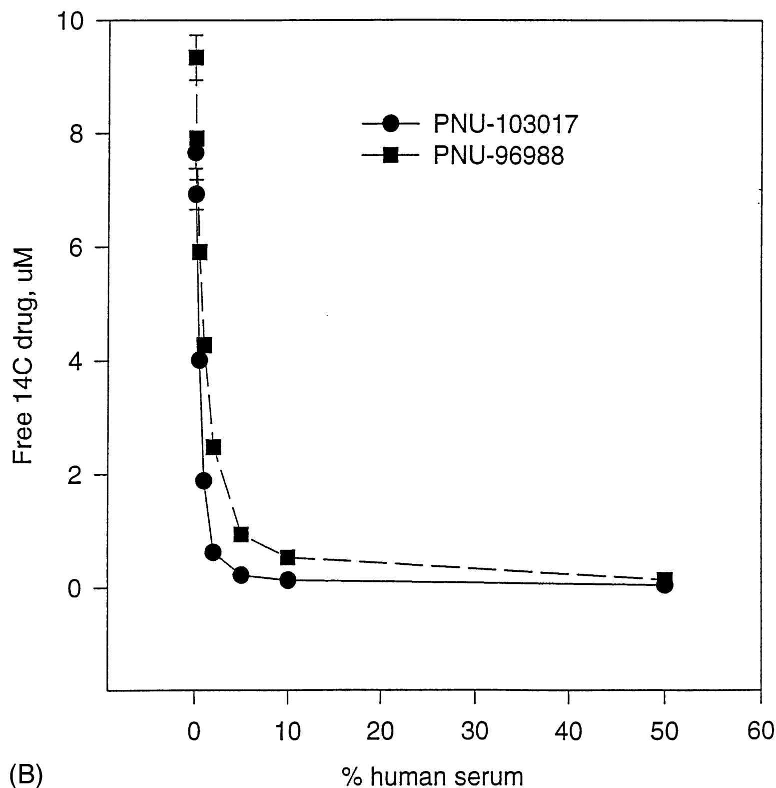

Fig. 3. (*Continued*)

it may be the free drug concentration which is important for activity. Meaningful comparison of the potency of inhibitors in systems with differing serum concentrations (for example the in vitro antiviral assay with 10% FBS, and the in vivo situation in human blood with 100% HS) should likely be made on the basis of comparison of *free*, not *total*, drug concentrations. In the absence of any in vivo animal pharmacology data, these results suggest that (all other factors equal) much higher total drug levels may need

Table 3
Human serum (HS) protein binding of selected HIV antivirals: free drug as a function of % HS in RPMI cell medium

% HS	% Free drug PNU-96988	PNU-103017	Delavirdine	AZT
0	103.0 ± 5.1	94.2 ± 4.9	98.6 ± 7.3	103.1 ± 3.1
0.1	87.9 ± 1.7	85.5 ± 7.2	87.7 ± 2.4	100.6 ± 4.3
0.5	65.6 ± 2.8	49.0 ± 2.6	82.1 ± 1.6	101.0 ± 5.5
1	46.1 ± 0.8	21.8 ± 0.4	63.8 ± 0.9	100.2 ± 4.4
2	27.2 ± 1.4	7.5 ± 0.3	52.4 ± 2.0	100.0 ± 4.9
5	10.3 ± 0.4	2.6 ± 0.2	29.8 ± 2.4	99.1 ± 3.7
10	5.9 ± 0.2	1.5 ± 0.1	17.4 ± 0.04	98.3 ± 2.8
50	1.5 ± 0.1	0.7 ± 0.1	5.4 ± 0.18	97.6 ± 4.3

Table 4
Intrinsic accumulation of HIV antiviral drugs into human peripheral blood mononuclear cells (PBMC) in the absence of serum

Drug	Level per cell (pmol $cell^{-1}$) at 0% HS	Normalized (to AZT) amount per cell at 0% HS
PNU-96988	4.4×10^{-5}	4
PNU-103017	2.7×10^{-5}	2
Delavirdine	6.0×10^{-5}	5
AZT	1.2×10^{-5}	1

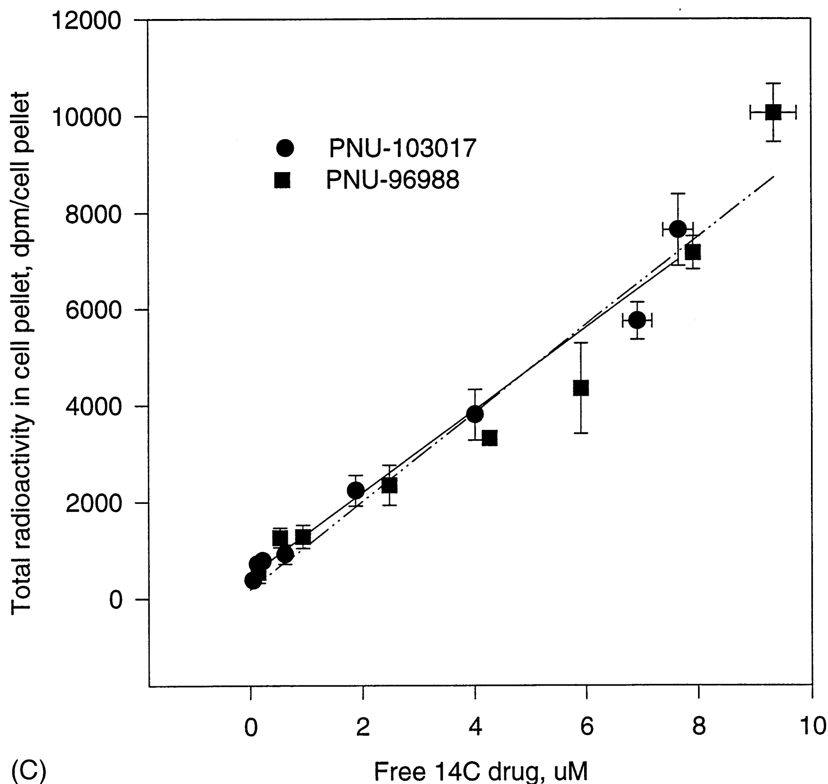

Fig. 3. (*Continued*)

to be achieved in vivo in the clinic (i.e. 100% HS) to achieve free drug levels (and presumably bioactivity) similar to that achieved in in vitro antiviral assays. Delavirdine, an HIV reverse transcriptase inhibitor, was bound to serum proteins with a lower affinity than PNU-96988. Consistent with this, serum protein effects on the uptake by cells in vitro were less for delavirdine than for PNU-96988. At the opposite extreme, AZT did not bind to serum proteins to any significant extent (at serum concentrations up to 50%), and serum protein had no apparent effect on the accumulation of AZT into cells in vitro. Thus the extent to which drugs uptake by cells was affected by serum appeared to be proportional to the binding affinity of the serum proteins for the drug.

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