Transport Characteristics of the Anti-human Immunodeficiency Virus Nucleoside Analog, Abacavir, into Brain and Cerebrospinal Fluid

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Received March 26, 2001; accepted May 14, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The role of the blood–brain and blood–cerebrospinal fluid (CSF) barriers in the distribution of anti-human immunodeficiency virus (HIV) drugs is integral to the design of effective treatment regimens for HIV infection within the brain. Abacavir (formerly 1592U89) is a nucleoside analog reverse transcriptase inhibitor, which has activity against HIV. The ability of this drug to reach the brain at therapeutic concentrations has been explored by means of an established bilateral in situ brain perfusion model in combination with high-performance liquid chromatography analysis in the anesthetized guinea pig. The influence of other drugs on the entry of abacavir into the brain was also investigated and is of special significance with the use of three of more anti-HIV drugs as the recommended treatment for HIV infection. The results of this study indicate that intact [14C]abacavir can cross the blood–brain and blood–CSF barriers and enter the brain and cisternal CSF. Further studies, at a perfusion time of 10 min, revealed that the uptake (R

The free movement of drugs into the brain is restricted by the presence of the blood–brain and blood–CSF barriers. The blood-brain barrier (BBB) is located at the level of the cerebral capillary endothelial cells and is associated with the presence of tight junctions, metabolic enzymes, and the lack of intracellular vesicles and endothelial fenestrae (Brightman and Reese, 1969). It protects the brain from many toxins and pathogens that are present within the blood. The blood-CSF barrier is formed by the choroid plexuses and arachnoid membrane. The choroid plexuses consist of a single layer of epithelial cells enclosing a vascular core. It is the epithelial cells that, as well as being responsible for secreting the majority of CSF (Csern, 1971), are joined together by a sealing ring of tight junctions and thus block the free paracellular movement of molecules (Brightman and Reese, 1969; Smith and Shine, 1992). Tight junctions are also located between the cells of the arachnoid membrane (Nabeshima et al., 1975; Smith and Shine, 1992).

HIV-1 is thought to enter the central nervous system (CNS) early in infection and can persist in the brain throughout the course of the disease (Resnick et al., 1988; Sawchuk and Yang, 1999; Ellis et al., 2000). The brain provides a sanctuary site for virus replication, protecting it from plasma levels of anti-HIV drugs due to the presence of the blood-brain and blood-CSF barriers (Cohen, 1998; Schrager and D’Souza, 1998; Marra and Booss, 2000). A clearer understanding of the ability of anti-HIV drugs to cross these barriers and enter the brain is of major concern if we are to 1) eradicate this viral reservoir and prevent peripheral reinfec-

This work was supported by grants from Glaxo Wellcome Research and Development and The Wellcome Trust. S.A.T. is a Wellcome Research Career Development Fellow.

ABBREVIATIONS: CSF, cerebrospinal fluid; BBB, blood-brain barrier; HIV, human immunodeficiency virus; RTI, reverse transcriptase inhibitor; AZT, azidodeoxythymidine; CNS, central nervous system; NBMPR, 6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine; HPLC, high-performance liquid chromatography.
such as acquired immunodeficiency syndrome dementia complex, associated with HIV infection (Dore et al., 1999; Gartner, 2000; Major et al., 2000).

The objective of this present study is to investigate the ability of the anti-HIV nucleoside analog, abacavir [(-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol; 1592U89], to cross the blood-brain and blood-CSF barriers and enter the brain tissue. The bilateral in situ brain perfusion technique in anesthetized guinea pigs, in combination with capillary depletion analysis, is a well established method and has been used to explore the movement of other anti-HIV nucleoside reverse transcriptase inhibitors (RTIs) into the CNS (Thomas and Segal, 1996, 1997a, 1998). It also has the advantage that it explores the movement of molecules into the brain and CSF simultaneously and allows any differences between drug distribution into these different regions to be observed. This is of immense importance as clinical studies are necessarily confined to measuring drug concentrations within human CSF (Tashima, 1998). Unfortunately, the concentration of a drug in the CSF does not reflect the concentration of a drug in the brain tissue (Groothuis and Levy, 1997; Thomas and Segal, 1998).

The animal brain perfusion model also has the advantage that it allows specific solute concentrations in the artificial plasma to be manipulated, which is essential if the mechanisms of transport of a particular molecule into the CNS are to be elucidated. This is of interest if we are to understand potential drug interactions at the transport level of the brain barriers and has become more of a concern with the current recommendations for the treatment of HIV infection, which involves using three or more anti-HIV drugs in parallel (International AIDS Society-USA, 2000). If combinations of anti-HIV drugs interact competitively for transport sites at the level of the blood-brain and blood-CSF barriers in vivo, it is likely that at least one of the drugs would not reach its therapeutic concentration and therefore would no longer effectively suppress viral replication. This failure would allow the development of drug-resistant variants of HIV, which is a major concern in the design of effective long-term treatment regimens for HIV infection. A recent study has suggested that both abacavir and azidothymidine (AZT; zidovudine) can interact with a thymidine transporter identified in a continuous rat microglia cell line (MLS-9) (Hong et al., 2000). To address these issues at the level of the brain barriers in vivo, the mechanism of abacavir entry into the CNS was also investigated using the brain perfusion model.

**Experimental Procedures**

**Bilateral In Situ Brain Perfusion.** All experimental procedures were carried out within the guidelines of the Animals (Scientific Procedures) Act, 1986, United Kingdom. The bilateral in situ brain perfusion technique in anesthetized guinea pigs has been previously described and will only be briefly detailed here (Thomas and Segal, 1996). Adult Dunkin-Hartley guinea pigs (250–350 g) were anesthetized (0.32 mg/kg i.m. fentanyl, 10 mg/kg fluanisone, 5 mg/kg midazolam) and heparinized (10,000 U/kg i.p.). The common carotid arteries were cannulated with silicon tubing connected to a perfusion system and the jugular veins were sectioned with the start of perfusion. The perfusion fluid consisted of thrice-washed ovine erythrocytes suspended in a saline-dextran (mol. wt. = 70,000) medium to a hematocrit of 20% (Thomas and Segal, 1996). The fluid was gassed with 95% O2 and 5% CO2 and maintained at 37°C using a water bath. The pH of the oxygenated perfusion fluid was 7.4. The perfusion fluid was passed, by means of a peristaltic pump, through a filter and bubble trap before entering each carotid artery at a flow rate of 3.7 ml/min. Perfusion pressure was maintained at approximately 100 mm Hg. [14C]Abacavir was introduced via a side arm into the perfusion medium at a flow rate of 0.5 ml/min and achieved a final concentration in the inflowing perfusion medium of 0.86 μM. After the set perfusion period (2.5–30 min) a cisterna magna CSF sample was taken and the animal was decapitated. The perfusion fluid containing the radiolabeled abacavir was collected from the individual carotid canulae at the end of each time point to serve as a reference. The brain was removed and the individual cerebral hemispheres and cerebellum homogenized separately. Part of the homogenate (three samples per cerebral hemisphere and two per cerebellum), the CSF and 100 μl of perfusate samples were prepared for radioactive scintillation counting by the addition of 0.5 ml of tissue solubilizer (Soluisol; National Diagnostics, Hull, UK). The samples were left to dissolve over 24 h and then, with the addition of 3.5 ml of scintillation fluid (Uniscint BD; National Diagnostics), taken for radioactive counting on a LKB-Wallac 1219 Rackbeta liquid scintillation counter (EG and G Wallac, Milton Keynes, UK). This spectrometer has a dual multichannel analyzer that automatically corrects the counts per minute for chemiluminescence. Counting error was ±3% for each sample. The corrected counts per minute were then converted to disintegrations per minute by the use of internally stored quench curves.

In a separate series of single-time point (10 min) experiments the CNS uptake of [14C]abacavir (0.86 μM) was examined in the presence of different concentrations of unlabeled abacavir (6.5, 50, 150, 200 μM). The effects of 100 μM unlabeled abacavir was also investigated at 5 and 20 min. The effects of 100 μM adenine, a substrate for the nucleoside transporter, and 10 μM 6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine (NBMPR), a nucleoside transport inhibitor, on the uptake of radiolabeled abacavir were also investigated after a perfusion period of 10 min (Plagemann et al., 1988; Washington and Giaconini, 1995).

**Capillary Depletion Analysis.** Measurement of the cerebral vascular component to total brain uptake of radiolabeled abacavir was performed using a capillary depletion step. Cerebral hemisphere samples (0.5 g) were homogenized in 3.5 ml of physiological buffer kept on ice (10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, and 10 mM D-glucose). The sample was further diluted with 4 ml of ice-cold 26% dextran (mol. wt. = 70,000) and the sample rehomogenized. Two aliquots of homogenate were taken and centrifuged at 5400g for 15 min in a centrifuge (Heraeus Instruments, Brentwood, UK). The capillary depleted supernatant was then separated from the vascular enriched pellet and prepared for radioactive scintillation counting as described above.

**Expression of Results.** The amount of radioactivity in the brain and CSF (disintegrations per minute per unit weight) was expressed as a ratio of that found in the perfusate and termed Rbrain and RCSF, respectively. Blood-to-brain and blood-CSF transfer constants (Kin; μl/min/g) were determined by single-time point analysis by means of the following equation (Williams et al., 1996):

\[ K_{in} = \frac{R_{brain}}{T} \]

where T is the length of perfusion in minutes. To calculate the blood-to-brain transfer constants the brain uptake values (Rbrain) were first corrected for vascular space by subtracting the mannitol uptake value. It must be noted that this correction factor is not the same as the initial volume of distribution (V1), which may also include the capillary endothelial cell volume. As such a Kin value determined in this manner may be an overestimation by <0.1%. However, it must also be considered that a CNS-to-blood efflux...
Entry of $^{14}$C]abacavir into the brain and CSF was examined by means of the in situ brain perfusion technique in anesthetized guinea pigs and compared with results for the plasma space molecule mannitol (Fig. 1). The uptake of $^{14}$C]abacavir into the cerebrum rose from 3.0 ± 1.3% at 2.5 min to 21.6 ± 5.1% at 30 min and was significantly greater than that measured for mannitol at all measured time points (Mann-Whitney rank sum test, $p < 0.005$). The whole brain was also separated into two compartments, a capillary endothelial cell enriched pellet and the brain tissue (Fig. 2). This capillary depletion analysis identifies the fraction of drug that has been trapped within the cerebrovascular endothelial cell and therefore has not actually entered the central nervous system. A small percentage of radiolabeled drug was detected in the capillary endothelial cell enriched pellet (<2.4%), but the majority of the radioactivity detected in the whole brain was located in the brain tissue (Fig. 2).

The uptake of abacavir into the CSF was 0.6 ± 0.3% at 2.5 min and rose to 12.6 ± 2.3% at 30 min. The brain and CSF data illustrated in Fig. 1 was found to be unsuitable for regression analysis by testing the residuals for homoscedasticity (Spearman rank correlation, $p < 0.01$). Thus, single-time uptake analysis to determine a unidirectional transfer constant was performed at 10 min. The rate of transfer of $^{14}$C]abacavir into the brain (9.3 ± 0.6 µl/min/g) after considering mannitol/vascular space (0.8 ± 0.1%) was significantly greater than that into the CSF (4.5 ± 1.4 µl/min/g; Students' $t$ test, $p < 0.05$). The negligible difference between the uptake of $^{14}$C]abacavir into the whole brain and the brain tissue compartment (Fig. 2) would suggest that any accumulation of $^{14}$C]abacavir within the capillary endothelial cells does not significantly contribute to the calculated $K_{in}$ value.

Figure 3 illustrates the uptake of radiolabeled abacavir into the cerebrum, cerebellum, and CSF in the presence of varying concentrations of unlabeled abacavir at a perfusion period of 10 min. As can be seen the uptake of $^{14}$C]abacavir was not significantly affected by the presence of 6.8, 50, 150, or 200 µM unlabeled abacavir. Studies were also performed at a perfusion period of 5 and 20 min and an unlabeled abacavir concentration of 100 µM and further confirmed that there was no significant self-inhibition of radiolabeled drug uptake (Fig. 4). Figure 4 also shows the data obtained after part of the cerebrum was taken for capillary depletion analysis. In addition, cross-competition studies indicated that $^{14}$C]abacavir was not significantly taken into the brain by
either an NBMPR-sensitive, nucleoside transport system or a nucleobase (adenine) transport system (Fig. 5).

Figure 6 illustrates the extraction of [14C]abacavir in the perfusion medium before (arterial inflow) and after (venous outflow) it had passed through the cerebral circulation. As can be seen the majority of the radioactivity was eluted as a single peak, with the retention times matching each other as well as radioactive standards for this nucleoside analog. Small radioactive peaks were detected in the solvent front (~2.0%) at 11.8 min (~2.4%) and at 12.9 min (~6.7%) in the samples taken from the venous outflow. The extraction of intact [14C]abacavir in the brain was important in determining whether the multiple-time uptake data actually represented intact drug and not free radioactive counts (Fig. 7). Due to the high level of radioactivity detected in the brain after 10 min (Fig. 1) it was possible to examine the brain for [14C]abacavir after a brain perfusion period of this length. After this period most of the radioactivity eluted at a retention time of 16 min 50 s, which matched the radioactive abacavir standard.

The octanol/saline partition coefficient for [14C]abacavir was 6.6 ± 0.2 (n = 3). The percentage of protein bound [14C]abacavir in the perfusion medium, which contained ovine erythrocytes, was determined to be 10.8 ± 1.0% (n = 6), with >89% remaining in the free form.

Discussion

Abacavir is a carbocyclic nucleoside analog RTI, which has been shown to be safe and well tolerated by HIV-infected patients (Saag et al., 1998; Hughes et al., 1999; Kumar et al., 1999). Studies have shown that abacavir can enter rat brain and monkey CSF (Daluge et al., 1997); however, this is the first study to examine the mechanisms of abacavir transport into both mammalian brain and CSF. This is important when we consider the complex relationship between drug concentrations in the brain and CSF and also that several anti-HIV drugs are now given in parallel to patients, so abacavir might compete with other drugs for transport into the brain.

Figures 1, 2, 6, and 7 illustrate that intact [14C]abacavir can cross the BBB and confirms a study that found intact abacavir in rat brain 2 h after intraperitoneal administration.
Abacavir Entry into the Brain and Cerebrospinal Fluid

20 minutes

Fig. 4. Effect of 100 μM unlabeled abacavir on the uptake of [14C]abacavir after a perfusion period of 5 and 20 min. Uptake is expressed as the percentage ratio of tissue to plasma radioactivities ($R_{\text{tissue}}$; ml/100 g). Values are the mean ± S.E.M. for three to six animals. Unpaired Student’s $t$ test showed that there was no difference in the uptake of [14C]abacavir in the absence or presence of abacavir.

5 minutes

Fig. 5. Effect of specific transport inhibitors on the brain uptake of [14C]abacavir (each group represents $n = 3–4$). Unpaired Student’s $t$ test revealed that there was no significant difference between the control ([14C]abacavir alone) and test inhibitor groups.

(Daluge et al., 1997). Figure 6 also confirms that there was little dissociation of the 14C label from abacavir during its passage through the cerebral circulation and that intact drug was presented to the luminal membranes of the barriers.

Abacavir (300 mg) is administered to HIV-infected patients twice daily (Kumar et al., 1999). Furthermore, the mean human plasma abacavir concentration after this dose has been administered is twice the 50% inhibitory concentration in HIV-1 clinical isolates for over 6 h ($IC_{50} = 0.26 \mu$M; Daluge et al., 1997; Kumar et al., 1999). The transfer constant for the cerebrum uptake of [14C]abacavir was 9.3 μl/min/g and was used to calculate a theoretical flux for abacavir entry into the mammalian brain (4.8 pmol/min/g) and estimate that it would take ~55 min for abacavir to reach an effective brain concentration (i.e., 0.26 μM). It is important to note that this modeling does not consider abacavir distribution into target cells within the brain, kinetics of intracellular phosphorylation, disposition of the active metabolite (carbovir triphosphate; Faletto et al., 1997), metabolism in pathways other than carbovir triphosphate production, and removal of abacavir from the brain. These factors could reduce the antiviral effect of abacavir within the brain and increase the estimated time taken for abacavir to reach its $IC_{50}$ value.

The CSF uptake of [14C]abacavir was 0.6 ± 0.3% at 2.5 min, appeared to plateau between 10 to 20 min, and then at 30 min (12.6 ± 2.3%) was double that of 20 min (Fig. 1). This increase is possibly a consequence of the CSF sink action, whereby diffusion of abacavir from the brain results in drug accumulation in the CSF. The effect of time on the uptake of
abacavir could not be detected and would suggest that a transfer constant of 4.5 ± 1.4 μl/min/g. These results indicate that [14C]abacavir can cross the blood-CSF barrier at a greater rate than that for mannitol (p < 0.05; 0.6 ± 0.1 μl/min/g). These results are in agreement with earlier studies that found abacavir in the CSF of monkeys 1 h after oral administration; the abacavir levels being 16 to 20% of that found in the plasma (Daluge et al., 1997). Clinical studies revealed a CSF to plasma abacavir ratio of 18%, −2 h after administration of 200 mg of abacavir to HIV-infected patients who were receiving the drug three times daily (McDowell et al., 1999). Abacavir has also been detected in human CSF after a single 600-mg oral dose (McDowell et al., 1999).

The brain uptake of abacavir after considering vascular space was significantly greater than uptake into CSF. Although it is appreciated that in vivo studies the separation of uptake across either the BBB or the blood-CSF barrier is not possible, it would appear unlikely that the blood-CSF route would produce the abacavir levels observed in the brain. In brief, this is related to the differing structural characteristics of the brain barriers (Bouldin and Krigman, 1975), the smaller surface area of the choroid plexuses versus the cerebral capillaries, the inefficient ability of certain drugs in the CSF to reach deep brain sites by the process of diffusion (Grothuis and Levy, 1997), and the sink action nature of the CSF to brain extracellular fluid (for review, see Thomas and Segal, 1998). If the CSF route was acting as the predominant route of brain entry you would expect a higher concentration of [14C]abacavir in the CSF and a brain level that followed the CSF concentration.

Figures 3 and 4 revealed that a saturable uptake of [14C]abacavir could not be detected and would suggest that a transport system is not involved in [14C]abacavir entry into the brain or CSF. This is in agreement with Mahony et al. (1995) who showed that abacavir entered human erythrocytes and CD4+ cells by passive diffusion. The multiple-time uptake studies for [14C]abacavir show a nonlinear uptake with time (Figs. 1 and 2), which due to the nonsaturable nature of abacavir uptake (Fig. 3), would suggest that an efflux process is involved in the overall movement of [14C]abacavir into the brain. At the earlier time points influx is greater than efflux, but after 15-min influx and efflux become equal, hence the reduced uptake rate.

Abacavir is ultimately anabolized to carbovir triphosphate, the potent inhibitor of HIV reverse transcriptase (Paletto et al., 1997). Carbovir can permeate erythrocyte membranes by facilitated diffusion; primarily by the nucleobase carrier and secondarily by the nucleoside transporter (Mahony et al., 1992). Nucleoside carriers have been identified at the BBB and the blood-CSF barriers of the guinea pig (Thomas and Segal, 1996, 1997b). Nucleobase carriers have been identified at the dog BBB (Drewes and Gilboe, 1977) and rabbit choroid plexus (Washington and Giacomini, 1995). The absence of an effect by unlabeled abacavir and the nucleoside transporter inhibitor (NBMPR) on the CNS uptake of [14C]abacavir suggests that the movement of intact abacavir and not its metabolite, carbovir, has been measured (Figs. 3–5). This was also indicated by the cross-competition study with the nucleobase transporter substrate adenine (Fig. 5). In addition, HPLC analysis would confirm that the brain uptake of intact radiolabeled abacavir has been examined (Fig. 7). Furthermore, these results suggest that abacavir would not compete with other anti-HIV drugs at the transport level of the brain barriers for entry into the brain. It is possible, however, that other drugs do affect the brain removal of abacavir. As stated earlier this study does support the concept of an efflux of abacavir out of the CNS. Abacavir and AZT can interact with a sodium-dependent nucleoside transporter of the N3 (cib) type identified in a continuous rat microglia cell line (MLS-9) (Hong et al., 2000). However, it is thought that if a sodium-dependent nucleoside transporter is present at the guinea pig blood-brain and blood-CSF barriers, it is of the N2 (cit) type (Thomas and Segal, 1997b). The differing substrate selectivities of the N2 and N3 transporters may explain the absence of abacavir interaction with the nucleoside transporter previously suggested at the guinea pig brain barriers. In addition, a clinical study has indicated that coadministration of AZT or 3TC with abacavir does not significantly affect abacavir pharmacokinetics (Wang et al., 1999).

Abacavir and AZT have been shown to enter rat brain and monkey CSF to a similar extent (Daluge et al., 1997). However, in this present investigation the transfer constant for [14C]abacavir uptake into the brain was greater than that previously determined for [3H]AZT (11 times) and [3H]D4T (27 times) (Thomas and Segal, 1997a, 1998). The ability of substances to cross cell membranes is partly related to lipophilicity and the greater ability of abacavir, compared with the other nucleoside analogs, to enter the brain could be related to its higher lipophilicity as measured by its octanol-saline partition coefficient. However, another influence on the ability of drugs to enter the brain is drug binding to plasma proteins. The protein binding aspect of this present study quantified the percentage of unbound abacavir that was present in the perfusion medium (i.e., >89%) and cannot be related to human plasma protein binding studies. It has been estimated that 50% of abacavir is protein bound in human plasma (McDowell et al., 1999) and that AZT is not highly protein bound (Dudley, 1995). This difference may explain the discrepancy observed between the studies comparing CNS uptake of abacavir and AZT. An investigation into the spread of viral infection in the CNS using a severe combined immunodeficiency mouse model of HIV-1 encephalitis, however, has shown that abacavir and lamivudine (3TC) are more effective in decreasing viral replication compared with the other nucleoside reverse transcriptase inhibitors, including AZT (Limoges et al., 2000). Although the difference measured in this HIV-1 encephalitis study could be due to peripheral pharmacokinetics, it may also be related to the ability of the drugs to reach the brain.

Overall, this present study suggests that the nucleoside RTI abacavir can reach the mammalian brain. These data would also suggest that abacavir entry into the CNS would be unaffected by the presence of other anti-HIV drugs. However, it is not known whether combinations of anti-HIV drugs would affect the removal of abacavir from the brain. These results suggest that abacavir might be an important addition to a treatment regimen for HIV infection within the brain.

References