Penetration of Zidovudine and 3'-Fluoro-3'-Deoxythymididine into the Brain, Muscle Tissue, and Veins in Cynomolgus Monkeys: Relation to Antiviral Action

E. LIUNGDAHL-STÄHLE,1* E. GUZENDA,2 D. BÖTTGER,3 B. WAHREN,1 B. ÖBERG,3 L. STÄHLE2

Department of Virology, National Bacteriological Laboratory and Karolinska Institute,1 and Department of Virology3 and Department of Pharmacology;2 Karolinska Institute, Stockholm, Sweden

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Cynomolgus monkeys had microdialysis probes implanted under ketamine anesthesia into peripheral veins, thigh muscles, and the brain in order to sample the extracellular fluid for the concentrations of unbound nucleoside analogs. A dose of 25 mg of zidovudine or 3'-fluoro-3'-deoxythymidine (FLT) per kg was administered subcutaneously to each of three animals. Relatively high antiviral concentrations of FLT and zidovudine were present in peripheral tissues and in the brain. It was found that the concentration of zidovudine in the brain was approximately one-third of that in muscle and veins; the same relation was observed for FLT. The in vivo unbound concentrations of both drugs in the brain, muscle, and venous blood exceeded those reported to inhibit human immunodeficiency virus replication in vitro. In addition, in a correlative study we found that the appearance of p24 antigen in sera of monkeys infected with simian immunodeficiency virus was significantly delayed by both compounds (15 mg/kg three times daily for 9 days after infection). Thus, we have shown that the extracellular concentrations of unbound FLT and zidovudine in the brain and peripheral tissues attained in vivo antiviral doses exceed in vitro antiviral concentrations.

The search for new drugs against the human immunodeficiency virus (HIV) is intensive, as mirrored by the frequent appearance of review articles in this field (e.g., see references 4, 7, 15, and 17). The preclinical models used in the development of new compounds primarily involve in vitro assays for HIV replication in combination with some tissue culture models to assess toxicity. Animal models of HIV infection have only recently become available, the clinically most relevant probably being infection of macaque monkeys with the simian immunodeficiency virus (SIV) (5, 13) or with HIV type 2 (HIV-2) (16). The effects of zidovudine and 3'-fluoro-3'-deoxythymidine (FLT) on HIV-1 in vitro (9) and on SIV-infected monkeys in vivo (12) were recently investigated, and it was found that FLT is approximately 10 times more potent than zidovudine. The pharmacokinetic properties of FLT are similar to those of zidovudine in monkeys, the most important difference being that FLT is not protein bound (12).

The distribution in various tissues of drugs against HIV is a factor of considerable importance. In particular, good distribution in the brain is desirable since a major clinical problem in the management of AIDS is the presence of neuropsychiatric symptoms (10). Previous studies of zidovudine distribution have used measurement of cerebrospinal fluid (CSF) in humans (8) or the intracarotid/first pass extraction technique to study blood-brain barrier permeability (21). In the present study we used the microdialysis method for measuring free (not protein-bound) extracellular tissue concentrations of drugs in the brain and other tissues (18). Microdialysis is a method to sample substances from the extracellular fluid without removing liquid volume. Instead, substances in the extracellular fluid are transported by simple diffusion to the sampling fluid flowing through the dialysis probe. The method has been used to study the blood-brain barrier distribution of other drugs (18).

MATERIALS AND METHODS

Subjects. Macaca fascicularis monkeys were housed in single cages in a biosafety level 3 facility. Each animal was found to be healthy, and those participating in the antiviral effect study were confirmed to be free of SIV antibodies and antigens as detected by enzyme-linked immunosorbent assay (11) prior to SIV inoculation.

Microdialysis. Anesthesia was induced by and maintained with ketamine given intravenously. The monkeys were placed in a stereotaxic instrument. In each monkey two microdialysis probes (CMA/10; CMA Microdialysis AB, Stockholm, Sweden) with a diffusible area of 10.0 by 0.5 mm (length by outer diameter) were implanted bilaterally into the striatum through a guide cannula which was secured to the skull bone by means of an acrylic dental cement. Two microdialysis probes were also implanted in muscle tissue in the thigh, and two probes were implanted into a dorsal superficial vein of the lower limb. Duplicate probes were used to prevent loss of data. Dialysates were collected in 20-min fractions and covered with tube caps. After implantation, each microdialysis probe was perfused for 60 min with degassed Ringer solution (ACO, Stockholm, Sweden) at a constant flow rate of 2.0 μl/min. Next, to determine the recovery over the dialysis membrane in vivo, each probe was perfused with a 10 μM solution of zidovudine or FLT at the same rate for 60 min and then the perfusion medium was switched back to Ringer solution for a washout period. The loss of drug to the tissue by diffusion over the dialysis membrane was measured as the difference between the drug concentration in the perfusion medium (10 μM) and the drug concentration in the dialysate. The proportion of drug lost over the dialysis membrane was used as an estimate of the in vivo recovery (18). After the washout period of 60 min,
between recovery determination and systemic drug administration, the concentration of the antiviral compound in the dialysate was below the limit of detection. Each monkey was then injected with 25 mg of drug per kg subcutaneously (s.c.) into the neck, and 20-min fractions were collected for 180 min.

**SIV infection and antigen assay.** M. fascicularis monkeys were infected with SIV_{SM} and treated s.c. with antiviral compounds or saline every 8 h for 7 to 10 days. Blood samples were drawn before any treatment and at days 4, 6, 8, 10, 13, 16, 20, 30, and 41. SIV_{SM} was obtained from H. McClure and P. Fultz at the Yerkes Regional Primate Research Center, Atlanta, Ga.

SIV p24 antigen was determined by taking advantage of cross-reactivity with SIV of antibodies to HIV-1 in a commercial kit (HTLV-III EIA; Abbott Laboratories, Chicago, Ill.) modified as described elsewhere (11).

**Antiviral drugs.** 3'-Azido-3'-deoxythymidine (zidovudine) was obtained from Burroughs Wellcome, Dartford, United Kingdom, and FLT was obtained from Medivir AB, Huddinge, Sweden. The compounds were dissolved in sterile Ringer solution.

**HPLC analysis of zidovudine and FLT.** Determination of the concentrations of the antiviral compounds was made by isocratic high-performance liquid chromatography (HPLC) separation on a C_{18} column. A mobile phase consisted of 0.05 M NH_{4}H_{2}PO_{4} buffer (pH 6.0) with 20% methanol (FLT, ~180-s retention time from the solvent front) or 40% methanol (zidovudine, ~60-s retention time from the solvent front). The validity of the zidovudine assay was investigated by dividing some samples from the microdialysis experiment and analyzing them with both HPLC and a radioimmunoassay (Institute of Isotopes of the Hungarian Academy of Sciences, Budapest, Hungary) (3). A CMA 200 refrigerated autoinjector (CMA Microdialysis AB) was used to inject the samples into the HPLC column, resulting in a very high reproducibility (<0.5% coefficient of variation for all concentrations tested). The sensitivity, defined as three times the noise level, of the assays was below 30 nM for the samples, which in terms of extracellular concentrations is below 300 nM. No extracellular concentration was below 1 μM. The method was linear in the concentration range studied.

**Radioimmunoassay.** Free zidovudine in the dialysate samples was determined by using a test from Institute of Isotopes of the Hungarian Academy of Sciences (3). The assay is based on competition between 125I-labelled zidovudine and unlabelled zidovudine from the samples for binding to antizidovudine serum. Six microdialysis samples from the thigh muscle were examined in the radioimmunoassay to determine accuracy and showed close agreement with the levels obtained by HPLC (r = 0.993) (Fig. 1).

**Experimental design and statistical analysis.**

(i) **Distribution of zidovudine and FLT.** The distribution of zidovudine (n = 3) and FLT (n = 3) was studied as simple time-response curves. The samples were collected for 20 min and are therefore represented in the graphs as the midpoint of that time period (i.e., 10, 30, . . . 170 min after administration), which allows accurate estimation of kinetic parameters such as half-life (19). Because microdialysis samples are the time integral of the concentration during the sampling interval, the area under the concentration-time curve from 0 to 180 h (AUC_{0-180}) is directly obtained by summing the nine products of concentrations times sampling interval (19). Data are presented as means and standard errors of the means. The comparison between FLT and zidovudine with respect to AUC was made by Student’s t test.

(ii) **Antiviral effects of zidovudine and FLT.** The antiviral effects of zidovudine (n = 4) and FLT (n = 4) in SIV-infected monkeys were assessed as the delay in appearance of p24 antigen compared to that in saline-treated controls (n = 8). Statistical significance was assessed by the Mann-Whitney U test with α = 0.05.

**RESULTS**

**Kinetics and distribution of FLT.** The time-concentration curves for FLT for each monkey are given in Fig. 2. The variation between blood probes was smaller for FLT than for zidovudine (Table 1). The maximum concentration of FLT appeared earlier than that of zidovudine in all tissues (Fig. 2). For muscle the mean maximum concentration in the dialysate (C_{max}) was 44.6 ± 1.2 μM. The time to maximum concentration in the dialysate (T_{max}) for extracellular concentrations in the brain varied from 70 to 130 min, with a C_{max} of 12.8 ± 3.5 μM, but was consistently found to be 50 min for muscle and veins. The brain/muscle AUC_{0-180} ratio was 0.30 ± 0.05.

**Kinetics and distribution of zidovudine.** The time-concentration curves for zidovudine for each monkey are given in Fig. 3. The mean C_{max} in muscle was 72.0 ± 40.2 μM, with a T_{max} of 77 ± 12 min (Table 1). The brain/muscle AUC_{0-180} ratio was 0.33 ± 0.05. The variation between probes was small for the brain and muscle but considerably larger for blood (Table 1). This was probably because of fluctuations in blood flow around the intravenous probes. The T_{max} for zidovudine varied more than for FLT, with values for the muscle tissue being the most stable.

**Delay of viral replication in SIV-infected monkeys.** The serum antigen levels in SIV-infected monkeys treated with FLT, zidovudine, or vehicle are shown in Fig. 4. Both zidovudine and FLT delayed the appearance of viral antigen, and the peak levels were lower than those for the controls. With the stated doses of drug and the infective dose of SIV, the antiviral treatments did not prevent viral replication, in spite of the fact that treatment started prior to infection.
DISCUSSION

In the present study we showed that the extracellular concentrations of zidovudine and FLT in blood, muscle, and the brain in vivo are well within the concentration ranges at which antiviral effects can be observed both in vivo (12, 13) and in vitro systems (1, 14). With an s.c. injection of 25 mg of antiviral compound per kg, the concentrations detected in microdialysis samples during the experiment always exceeded the HIV inhibitory concentrations found in cell culture (0.005 to 0.1 μM) (1, 9, 14). Furthermore, in the in vivo SIV model both drugs displayed a significant antiretrovirus efficacy (Fig. 4).

The present study was in part undertaken to clarify the discrepancy observed in rat studies between those using microdialysis (20) and whole-tissue measurements (6, 21) and also the discrepancy between human (8) and rat (6) CSF

<table>
<thead>
<tr>
<th>Antiviral compound</th>
<th>Blood $C_{\text{max}}$ (μM)</th>
<th>$T_{\text{max}}$ (min)</th>
<th>Muscle $C_{\text{max}}$ (μM)</th>
<th>$T_{\text{max}}$ (min)</th>
<th>Brain $C_{\text{max}}$ (μM)</th>
<th>$T_{\text{max}}$ (min)</th>
<th>Brain AUC/ muscle AUC</th>
</tr>
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<tbody>
<tr>
<td>FLT</td>
<td>48.5 ± 9.4a</td>
<td>63 ± 12</td>
<td>44.6 ± 1.2</td>
<td>50 ± 0</td>
<td>12.8 ± 3.5</td>
<td>103 ± 30</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>52.3 ± 26.5</td>
<td>83 ± 30</td>
<td>72.0 ± 40.2</td>
<td>77 ± 12</td>
<td>24.3 ± 13.7</td>
<td>103 ± 42</td>
<td>0.33 ± 0.05</td>
</tr>
</tbody>
</table>

* Mean ± standard error of the mean.
data. Hence, a discussion of methodology and pharmacokinetic principles is necessary.

First, it should be pointed out that in the study of the kinetics and distribution of drugs like zidovudine and FLT it is desirable to measure the concentrations of the triphosphate of the drug in the different retrovirus-infected cells in vivo. From a practical point of view this is, however, very difficult. The best alternative is to measure the concentrations of nucleosides available to the cells for uptake and subsequent phosphorylation. In vivo this is the extracellular concentration, which has the same role in vivo as the concentration in the medium of in vitro cell cultures. Whole-tissue measurements are accompanied by several difficulties such as differences in phosphorylation capacity between cell lines and the relative proportions of the unchanged drugs and the phosphorylated forms (free or bound to cellular elements or the extracellular fluid matrix). Thus, we believe that sampling of the extracellular fluid by, e.g., microdialysis provides more relevant information than whole-tissue measurements.

In the present study we found that extracellular concentrations of zidovudine in the brain are about one-third of unbound concentrations in plasma. Collins et al. (2) reported that levels in CSF in rhesus monkeys are about one-fifth of total levels in plasma which, when corrected for plasma protein binding (12), closely agree with the present findings. Interestingly, it has been found that levels of zidovudine in CSF in humans are about 50% of total levels in plasma 4 h after administration (i.e., almost 100% of the free concentration in plasma) (8). This figure is taken as an approximate average from a study (8) in which single measurements of zidovudine in CSF were taken after various modes of administration in a small number of patients. Hence, 50% is not a figure of considerable accuracy (as opposed to microdialysis data, in which the whole time-concentration curve is available for each tissue studied). In rats we found, by microdialysis, that the free extracellular concentration is about a quarter of the free concentration in plasma (20). Together, these data suggest that the extracellular concentration of zidovudine in the brain is highest in humans, intermediate in monkeys, and lowest in rats. The similarity between the distribution of zidovudine and FLT in rats and monkeys suggests that the distribution of both drugs should also be similar in humans.

It remains to be discussed why whole-tissue data for rats differ from extracellular data. Galinsky et al. (6) reported a 2.3% concentration of zidovudine in brain tissue compared with the total concentration in plasma. This would be 4 to 5% of the free (unbound) concentration in plasma. However, the brain extracellular volume is only about 20% of the total brain tissue volume. Thus, assuming that the intracellular concentration of zidovudine is low (phosphorylated forms may be prevalent in high concentrations), the data of Galinsky et al. would suggest an extracellular fluid concentration of 20 to 25%, which is in good agreement with our findings (18). Our data suggest that zidovudine in unchanged (unphosphorylated) forms exists mainly extracellularly. We have no information yet on whole-tissue levels of FLT.

The free concentration of zidovudine in blood decreases more slowly than that of FLT and remains at 50 μM after 3 h. The reason for this is at present unclear, but we cannot exclude that ketamine interacts with the nucleoside analogs to prolong the half-life and to increase levels due to a lack of glucuronidation.

Thus, we conclude that the concentrations of zidovudine and FLT in the extracellular fluid can be estimated by microdialysis and that the information obtained can be used to relate in vitro data to the present findings. More specifically, a dose of 25 mg/kg, slightly above the 15 mg/kg which is active against SIV in monkeys, yields FLT concentrations in the brain of about 10 μM and zidovudine concentrations of 8 to 30 μM. These drugs are active in cell cultures at concentrations below 1 μM. Thus, the concentrations of both drugs attained in the peripheral tissues and in the brain after administration of in vivo antiviral doses are clearly within the concentration range that is antiviral in cell cultures. However, the sensitivity of a given infected organ or cell line has to be assessed separately since the rate of uptake and phosphorylation may vary considerably.

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REFERENCES


FIG. 4. Comparison between mean SIV antigen values for infected monkeys treated with FLT (circles; 15 mg/kg/day three times, n = 4), zidovudine (triangles; 15 mg/kg/day three times, n = 4), and saline (controls) (broken line; n = 8). Antiviral compounds were administered for 10 days. Bars show standard errors of the means.