Comparative Brain Exposure to (-)-Carbovir After (-)-Carbovir or (-)-6-Aminocarbovir Intravenous Infusion in Rats

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Purpose. Evaluate the ability of (-)-6-aminocarbovir ((-)-6AC) to improve the CNS exposure to (-)-carbovir ((-)-CBV). ***Methods.*** Activation of (-)-6AC *in vitro* was assessed by incubations of rat brain tissue homogenates. The *in vivo* brain exposure to (-)-CBV was then examined in rats after iv infusions of either (-)-CBV (n = 4) or (-)-6AC (n = 5). The drugs were infused to steady-state via the jugular vein. At the end of the infusion, a bolus of [^{3}H]inulin was injected via the femoral vein in order to obtain an estimate of the brain vascular space. ***Results.*** (-)-6AC was converted to (-)-CBV by incubations of rat brain tissue homogenates. After iv infusion of (-)-CBV, the brain/blood concentration ratio of (-)-CBV was 0.032 ± 0.009. The brain/blood concentration ratio of (-)-CBV after iv infusion of (-)-6AC was 0.080 ± 0.020. ***Conclusions.*** (-)-6AC improved the brain delivery of (-)-CBV, although the absolute exposure of the brain tissue to (-)-CBV was still quite low.

KEY WORDS: brain uptake; (-)-carbovir; (-)-6-aminocarbovir; prodrug.

INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) dementia complex is frequently observed in the late stages of an infection with human immunodeficiency virus type 1 (HIV-1) (1). A direct effect of HIV-1 on the brain is generally recognized as the cause of this serious neurological dysfunction (1). The virus present in the central nervous system (CNS) may also serve as a reservoir for persistent infection (2). Therefore, anti-AIDS agents must penetrate the blood-brain barrier in order to achieve effective therapy.

Several clinically available anti-AIDS drugs have been studied for their capability to penetrate the blood-brain barrier. The brain/blood concentration ratio of zidovudine (AZT), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxyinosine (ddI) were reported to be in the range of 0-0.28 in rats (3-7), indicating limited penetration of nucleosides into the CNS. Ester prodrugs (8,9) and chemical delivery systems (9-11) have exhibited some success in increasing the exposure of the CNS to AZT in animals. A recent study demonstrated a dramatic improvement of the brain delivery of ddI in rats by 6-halo-dideoxypurine prodrugs (12,13).

Carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine (carbovir, CBV, figure 1) was the most effective and selective *in vitro* inhibitor of HIV replication among carbocyclic purine analogs evaluated (14). The prodrug (-)-carbocyclic 2',3'-didehydro-2',3'-dideoxy-6-deoxy-6-aminoguanosine ((-)-6-aminocarbovir, (-)-6AC, figure 1) was originally designed to improve the bioavailability of (-)-CBV (15), the active enantiomer of CBV (16). Although the bioavailability of (-)-CBV was increased two- to three-fold by the prodrug (15), the question remained as to whether (-)-6AC could effectively deliver (-)-CBV to the CNS.

The goals of the present study were to determine if (-)-6AC could be converted to (-)-CBV by rat brain tissue. (-)-6AC was then evaluated for its ability to enhance the exposure of the CNS to (-)-CBV *in vivo*.

MATERIALS AND METHODS

Chemicals. (-)-CBV, (-)-6AC, and the internal standard for the HPLC assay, carbocyclic 2',3'-dideoxy-6-deoxyguanosine, were synthesized as reported previously (17,18). [^{3}H]inulin and H_2O_2 (30%) were purchased from Sigma (St. Louis, MO). The radiochemical purity of [^{3}H]inulin exceeded 97%, as determined by gel filtration. Triethylamine (TEA) and trichloroacetic acid (TCA) were purchased from Aldrich Chemical (Milwaukee, WI). Hexanesulfonic acid (HSA) was obtained from Eastman Kodak (Rochester, NY). Phosphoric acid (70%) was purchased from Fisher Scientific (Eden Prairie, MN). HPLC-grade methanol and perchloric acid (70%) were obtained from Mallinckrodt (Paris, KY). All other chemicals were reagent grade or better.

In Vitro Bioconversion of (-)-6AC in Brain Tissue. Three experiments were carried out. Three male Sprague-Dawley rats were used in each experiment. After anesthesia with sodium pentobarbital (50 mg/kg, ip), the rats were decapitated and the brains were excised. Three brains were pooled and homogenized with 3 volumes of ice-cold phosphate-buffered saline (PBS, pH 7.4). The homogenate was centrifuged at 10,000 × g at 4°C for 60 min. The supernatant was then centrifuged at 100,000 × g at 4°C for 60 min. Four ml of the final supernatant were pre-incubated for 5 min at 37°C and 100 µl (-)-6AC (400 µg/ml) were added to start the incubation. Triplicate samples of 100 µl each were taken at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, and 360 min. Samples were pipetted into microcentrifuge vials on ice, to which 400 µl internal standard solution (0.8 µg/ml) and 10 µl TCA (60%) had been added previously. Samples were immediately vortexed. After centrifuging at 13,000 × g for 6 min in a Fisher (Pittsburgh, PA) Model 235B microcentrifuge, 25 µl of saturated $NaHCO_3$ were added to 400 µl of the supernatant to neutralize the sample. The samples were stored at -20°C until assay. (-)-6AC was also incubated with PBS as a control.

Brain Uptake Experiments. Nine male Sprague-Dawley rats (weighing 270 ± 40 g, Biolabs, St. Paul, MN) were used in this study. The rats were anesthetized with sodium pentobarbital (50-60 mg/kg, ip), then injected with atropine sulfate (320 µg/kg, ip). Cannulas were surgically implanted into the right jugular and right femoral veins of each rat 24-48 hr

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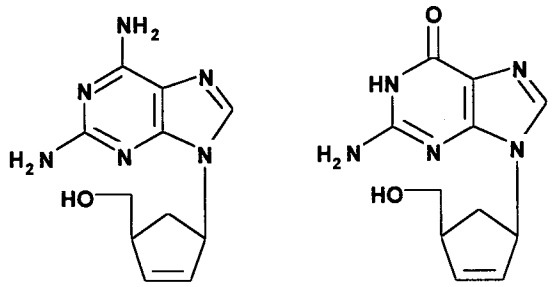

6-Aminocarbovir **Carbovir**

Fig. 1. Structures of 6-aminocarbovir (6AC) and carbovir (CBV).

before drug treatment. The cannulas were threaded through a subcutaneous tunnel, and a swivel and tether were used to enclose the distal end of the cannula that emerged from the skin between the scapulae. This arrangement facilitated free movement of the rat during the study. The animals were housed in plastic metabolic cages (Nalgene, Rochester, NY) in a room with a 12-hr dark/light cycle. Food and water were allowed *ad libitum*. The rats were continuously infused with normal saline at a flow rate of 2 μl/min until the time of the experiment, for hydration purposes and to prevent blood clotting in the cannulas. Rats were infused via the jugular vein with either (-)-CBV (135.6 ± 23.0 μg/min/kg, n = 4) for 240 min or (-)-6AC (364.2 ± 44.2 μg/min/kg, n = 5) for 120-150 min. During the infusion ten to eleven blood samples (250μl each) were drawn from the femoral vein followed by replacement with the same volume of heparinized normal saline (10 U/ml). The blood samples were frozen immediately on dry ice to inhibit the potential *in vitro* conversion of (-)-6AC to (-)-CBV. At the end of the infusion the rats were anesthetized with carbon dioxide, decapitated, and the brains were excised. The superficial blood vessels were stripped off. After being rinsed with ice-cold normal saline and blotted dry, the brain was frozen on dry ice. Those rats that received the (-)-6AC infusion were also injected with [^{3}H]inulin (10-15 μCi) via the femoral vein immediately after the last blood sample was drawn. Five minutes after the injection of [^{3}H]inulin, the rats were sacrificed as described above and the blood and brain were collected. The brain and blood samples were stored at -70°C until analysis.

Analytical Methodology. The solid-phase extraction of (-)-CBV and (-)-6AC from blood samples was similar to a previously described method (19). However, since *in vitro* incubation of (-)-6AC with blood showed a minor amount of conversion of (-)-6AC to (-)-CBV at 37°C, with a half-life of a few days (unpublished data), special precautions were taken in handling the blood samples to minimize the conversion during the preparation of samples. After two freeze-thaw cycles used to hemolyze the blood, samples were kept at 0°C until the solid-phase extraction was performed.

The brain was weighed and homogenized with 0.8 μg/ml internal standard solution (2 ml/g brain tissue) and 60% TCA (600 μl/g brain tissue). The homogenates were centrifuged for 10 min at 13,000 × g. The supernatants were then divided into three aliquots, and into each, saturated $NaHCO_3$ was added. The samples were then loaded onto the solid-phase extraction column and handled as previously described (19). Brain tissue standard curves were prepared in a similar way with brains from rats that had received no drug. Control studies with brain homogenate spiked with (-)-6AC indicated a lack of conversion of (-)-6AC to (-)-CBV during the workup of the brain samples.

HPLC separation of the compounds was performed on a 25 cm Spherisorb C_8 cartridge column (0.46 cm i.d., 5 μm particle size, Phase Sep, Norwalk, CT) connected to a 1 cm reversed-phase guard column (0.46 cm i.d., Phase Sep). The solvent delivery system was comprised of a Gilson Model 302 pump (Middleton, WI) with a Gilson Model 231 automatic injector. The compounds were eluted isocratically with a mobile phase consisting of 18% methanol in an ion-pairing buffer. The buffer was composed of 50 mM phosphoric acid, 25 mM HSA, and 10 mM TEA (pH 2.2). A flow rate of 1.5 ml/min was used. The column temperature was kept at 40°C to improve the resolution and to decrease the retention times.

(-)-CBV was monitored as described previously (20) with a Shimadzu RF-530 fluorescence detector (Columbia, MD) at excitation and emission wavelengths of 275 and 345 nm, respectively. (-)-6AC was monitored with the Gilson Model 116 UV detector (Middleton, MI) at 252 nm because of its lack of fluorescence. In some cases, (-)-CBV was monitored by UV detection at 252 nm. Triplicate unweighted standard curves were prepared for each day of analysis. Peak height ratios were used to quantitate the concentration of the compounds of interest. The coefficients of variation were less than 10% in blood for both (-)-CBV and (-)-6AC over the concentration range used (0.156 μg/ml to 5 μg/ml). The coefficients of variation were less than 16% for (-)-6AC and less than 10% for (-)-CBV in brain tissue samples over the concentration range used in the infusion studies (0.125 μg/g to 0.5 μg/g). The coefficients of variation were less than 10% for both compounds over the concentration range used in brain homogenate incubations (0.3125 μg/ml to 10 μg/ml).

Evaluation of the Vascular Space Volume in Brain. The radioactive blood sample (100 μl) was solubilized and decolorized with 200 μl perchloric acid (70%) and 200 μl hydrogen peroxide (30%) in a water bath at 50°C for 3-4 hours. Approximately 0.2 g of the brain sample was treated in a similar manner with 400 μl perchloric acid and 400 μl hydrogen peroxide. Fifteen ml of Eco-lite$^+$ (ICN Biomedicals, Irvine, CA) scintillation liquid were then added to the vials. The vials were left to stand for 24 hr to minimize the effect of chemiluminescence on scintillation counting. The radioactivity was then counted in a Beckman (Fullerton, CA) Model LS 3801 liquid scintillation counter. The counting efficiency was in the range of 10% to 40%.

The blood-brain barrier was assumed to be impermeable to [^{3}H]inulin, and the percent of the brain tissue that was vascular space (V_{brn}) was calculated as follows (21):

$$\%V_{bm}(v/w) = \frac{\text{dpm in brain} \times \text{volume of blood sample (ml)}}{\text{dpm in blood} \times \text{weight of brain sample (g)} \times f} \times 100 \quad (1)$$

Since inulin is a plasma marker (21), and (-)-CBV and (-)-6AC

are evenly distributed throughout the blood (22, unpublished data), the factor f was included to correct for the difference in hematocrit between brain capillaries and the large vessels. An f value of 1.19 was used in this study (21).

Evaluation of the Brain/Blood Concentration Ratios of (-)-CBV and (-)-6AC at Steady-State. The steady-state brain parenchyma/blood concentration ratios were calculated as follows (21):

$$\text{Brain/Blood Ratio} = \frac{C_{ss,brn,ttl} - (V_{brn} \times C_{ss,bld})}{C_{ss,bld}} \quad (2)$$

where $C_{ss,brn,ttl}$ is the total steady-state brain concentration (μg/g) and $C_{ss,bld}$ is the average value of the steady-state blood concentrations. The brain parenchymal concentration was corrected for the drug concentration in the vascular space of the brain. The average value for the vascular space volume in brain determined from the (-)-6AC studies was used for correction in the (-)-CBV studies.

Pharmacokinetic Evaluation. Total body clearance (CL) was calculated as the infusion rate divided by the steady-state concentration of the administered compounds.

The unpaired Student's t-test was used for statistical comparisons. A p value of less than 0.05 was considered to be significant.

RESULTS

Figure 2 shows a typical concentration profile of (-)-6AC and (-)-CBV during 6-hr incubation in brain tissue. While the control incubations showed no (-)-6AC degradation, a slow rate of conversion of (-)-6AC to (-)-CBV was observed in incubations with brain tissue homogenates. The decline of the (-)-6AC concentration-time curve appeared to be mono-exponential. The rate constant for the enzymatic conversion (-)-6AC to (-)-CBV was obtained by linear regression of the log concentration-time data. The rate constant (mean ± s.d.) was 0.074 ± 0.013 hr^{-1} and the half-life (mean ± s.d.) was 9.51 ± 1.77 hr.

Figure 3A shows the blood concentration-time profile of (-)-CBV after iv infusion of (-)-CBV in a representative rat. The concentration of (-)-CBV appeared to reach steady-state within 2 hr. Table I summarizes the parameters obtained in the (-)-CBV infusion studies. Since evaluation of the vascular space volume in brain was not performed in the (-)-CBV infusion studies, the mean value of V_{brn} obtained in (-)-6AC infusion studies was used for the correction. The brain parenchyma/blood concentration ratios were 0.032 ± 0.009, indicating a very poor blood-brain barrier penetration of (-)-CBV.

Figure 3B illustrates the blood concentration-time profile of (-)-CBV and (-)-6AC after (-)-6AC iv infusion in a given rat. The parameters of the (-)-6AC infusion studies are presented in Table I. Among the five rats in this study most of them attained steady-state for both (-)-6AC and (-)-CBV concentrations within 70 to 100 min of infusion. The brain parenchyma/blood concentration ratios for (-)-6AC and for (-)-CBV were 0.076 ± 0.033 and 0.080 ± 0.020, respectively. The (-)-CBV concentration ratio is statistically different from that obtained in the (-)-CBV infusion studies. The (-)-CBV brain parenchyma/blood ratio was improved by approximately 2.5-fold after (-)-6AC infusion.

The total body clearances (CL) for (-)-CBV and (-)-6AC were 55.8 ± 9.0 and 121.5 ± 22.3 ml/min/kg, respectively (Table I). The clearance of (-)-6AC was similar to the value previously reported (15). The clearance of (-)-CBV was significantly lower than the previously reported value (15), suggesting that nonlinear clearance (22,23) was operating at the steady-state concentrations of (-)-CBV achieved in the present study.

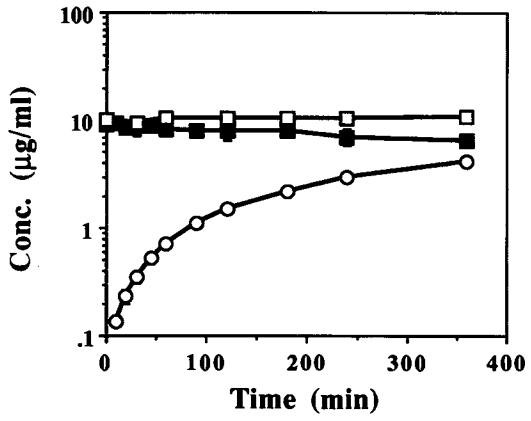

Fig. 2. Representative concentration-time profiles of (-)-CBV (○) and (-)-6AC (■) during (-)-6AC incubation in brain tissue homogenate. Control (□) experiments were incubations of (-)-6AC in PBS. Three rat brains were pooled and triplicate samples were taken for each experiment. The error bars represent one standard deviation from the mean values. Some symbols concealed the error bars.

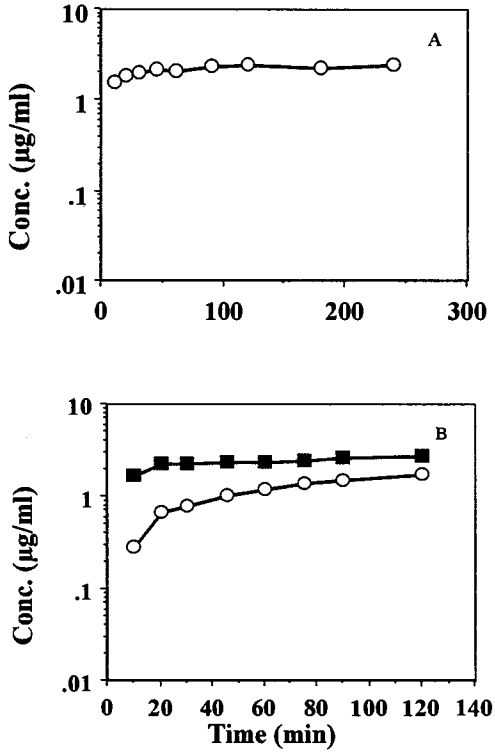

Fig. 3. (A) (-)-CBV (○) concentration-time profile in a representative rat during iv infusion of (-)-CBV (116 μg/min/kg). (B) (-)-CBV (○) and (-)-6AC (■) concentration-time profiles in a representative rat during iv infusion of (-)-6AC (324 μg/min/kg).

Table I. Pharmacokinetics and Brain Exposure to (-)-CBV and (-)-6AC After iv Infusion

	CBV Infusion (n = 4)[a]	6AC Infusion (n = 5)[a]	
	CBV	CBV	6AC
CL (ml/min/kg)	55.8 ± 9.0	—	121.5 ± 22.3
$C_{ss,bld}$ (μg/ml)	2.49 ± 0.61	2.02 ± 0.50	3.07 ± 0.64
$C_{ss,brn,tll}$ (μg/g)	0.16 ± 0.04	0.23 ± 0.07	0.33 ± 0.10
$C_{ss,brn,crtd}$ (μg/g)[b]	0.08 ± 0.03	0.16 ± 0.06	0.23 ± 0.10
V_{brn} (ml/g%)	—	3.19 ± 0.61	3.19 ± 0.61
Brain/Blood Ratio	0.032 ± 0.009	0.080 ± 0.020	0.076 ± 0.033

[a] Data are presented as mean ± standard deviation.
[b] $C_{ss,brn,crtd}$ is the concentration in the brain after correction for the vascular space contribution.

DISCUSSION

(-)-6AC demonstrated a slow rate of conversion to (-)-CBV in the rat brain homogenate. It appeared that (-)-6AC underwent complete conversion to (-)-CBV with no further metabolism of (-)-CBV, because the summation of the amounts of (-)-6AC and (-)-CBV gave mass balance at all times during the incubation. Studies with other tissues have shown that the rank order of the rate of conversion of (-)-6AC was intestine>liver>brain>blood (unpublished data), which is in good agreement with the tissue distribution of adenosine deaminase in rats (24). This enzyme is most likely responsible for the bioactivation of (-)-6AC (18).

(-)-CBV brain uptake was increased by 2.5-fold after (-)-6AC infusion compared to the administration of (-)-CBV itself. (-)-6AC had a higher brain/blood concentration ratio than did (-)-CBV. Although the (-)-6AC ratio is not a true indication of its blood-brain barrier penetration because of the conversion to (-)-CBV, (-)-6AC appeared to penetrate the blood-brain barrier more readily than (-)-CBV and was converted *in vivo* to (-)-CBV in the brain parenchyma. However, the absolute brain/blood concentration ratio of (-)-CBV was only 0.080 ± 0.020, so (-)-6AC may not be a satisfactory candidate for enhancing the brain delivery of (-)-CBV. In comparison, 6-halo-dideoxypurines are activated by adenosine deaminase and have been reported to increase the brain/plasma concentration ratio of ddI by 20 to 34-fold (12,13). 6-Halo-CBV may have potential for penetrating into the CNS, and should be evaluated. Other brain targeting approaches (8-11) for (-)-CBV are worthy of testing as well.

ABBREVIATIONS

AIDS: acquired immunodeficiency syndrome; CNS: central nervous system; AZT: zidovudine, 3'-azido-3'-deoxythymidine; ddC: 2',3'-dideoxycytidine; ddI: 2',3'-dideoxyinosine; (-)-6AC: (-)-6-aminocarbovir, (-)-carbocyclic 2',3'-didehydro-2',3'-dideoxy-6-deoxy-6-amino-guanosine; (-)-CBV: (-)-carbovir, (-)-carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine; HSA: hexanesulfonic acid; TCA: trichloroacetic acid; TEA: triethylamine; CL: total body clearance; f_m: fraction metabolized.

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