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CARRIER-MEDIATED TRANSPORT OF THE ANTITUMOR AGENT ACIVICIN ACROSS THE BLOOD–BRAIN BARRIER

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Abstract—The cytotoxic agent acivicin has been shown to be effective against several types of tumors. However, the clinical utilization of acivicin has been prohibited because of its dose-limiting neurotoxicity. Acivicin is believed to be transported into the brain by the large neutral amino acid (LNAA) carrier, which is expressed at the blood–brain barrier (BBB). In this study, we used an *in situ* rat brain perfusion technique to determine the kinetics of the LNAA carrier-mediated transport of acivicin across the BBB. We found that the V_{max} of acivicin (1.05 nmol/sec/g) obtained in this study was comparable to the V_{max} of L-leucine (1.07 nmol/sec/g) and other LNAAs as determined by other investigators. The K_m was high compared with other LNAAs, but this could be explained by the low lipophilicity of acivicin. Acivicin transport across the BBB was inhibited by other LNAAs but not by acivicin derivatives with structural modifications at the amino or carboxyl group. The ASC (alanine, serine, cysteine) carrier system did not influence the transport of acivicin across the BBB. These results suggest that the CNS toxicity of acivicin might be reduced by coadministration of other LNAAs. Acivicin derivatives with structural modifications at the amino or carboxyl group of acivicin lack affinity for the LNAA carrier at the BBB and, therefore, will exhibit less CNS toxicity than acivicin.

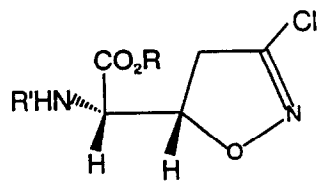
Key words: acivicin; antitumor agent; blood–brain barrier; large neutral amino acid; carrier-mediated transport; octanol–water partition coefficient

Acivicin ((αS,5S)-α-amino-3-chloro-2-isoxazoline-5-acetic acid, Fig. 1), a fermentation-derived amino acid, has been shown to be an effective cytotoxic agent against a variety of tumors [1]. Acivicin is a glutamine antagonist and exhibits cytotoxicity through inhibition of several glutamine-dependent amidotransferases [2–4].

The major reason for the lack of clinical utilization of acivicin is the fact that it causes reversible, dose-limiting neurotoxicities consisting of somnolence, anxiety, lethargy, hallucinations, nightmares, confusion and psychoses [5–7]. The CNS toxicity of acivicin has been attributed to its high BBB† permeability, which results in significant accumulation of the drug within the brain [8].

Evidence from both *in vivo* and *in vitro* experiments suggests that acivicin is transported across the BBB by the LNAA carrier system [8–10]. This evidence includes the observation that acivicin inhibits the transport of [^{3}H]L-leucine across monolayers of BBMECs and that the transport of acivicin itself across BBMEC monolayers is time-, temperature-, and concentration-dependent (K_m = 0.24 mM; V_{max} = 73.5 nmol/mg protein/min) [9]. In addition, Takada *et al.* [10] demonstrated that acivicin could inhibit the transport of [^{14}C]L-leucine by the LNAA carrier at the BBB using an *in situ* rat brain perfusion technique. Williams *et al.* [8] have also shown that the neurotoxicity of acivicin in cats can be reduced by co-injection with a mixture of LNAAs. However, the *in vivo* kinetics of acivicin transport across the BBB have not been reported in the literature.

Therefore, in this manuscript we report the kinetics of LNAA carrier-mediated transport of acivicin across the BBB using an *in situ* rat brain perfusion technique developed by Takasato *et al.*



	R'	R
Acivicin	H	H
Acivicin methyl ester	H	CH_3
t-Boc Acivicin	$(CH_3)_3CCO$	H

Fig. 1. Structures of acivicin and its derivatives.

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† Abbreviations: BBB, blood–brain barrier; BBMEC, bovine brain microvessel endothelial cell; LNAA, large neutral amino acid; log PC(o/w), logarithm of the 1-octanol/water partition coefficient; PA, permeability-surface area product; PC, partition coefficient; MS, mass spectrometry; CI, chemical ionization; and FAB, fast atom bombardment.

[11]. In addition, we have shown that the BBB permeability of this cytotoxic amino acid can be altered by derivatization of the amino or carboxyl group (Fig. 1) or by coadministration of LNAAs that can compete for the LNAA carrier at the BBB, but it is not influenced by the ASC (alanine, serine, cysteine) carrier system at the BBB.

MATERIALS AND METHODS

Materials. Ten- to twelve-week-old Sprague–Dawley male rats (Sasco, Omaha, NE) weighing between 300 and 400 g were used in the *in situ* rat brain perfusion experiments. [^{14}C]L-Leucine (419.2 mCi/g) was purchased from ICN Biochemicals, Inc. (Irvine, CA), and [^{3}H]methoxyinulin (187 mCi/g) was purchased from NEN (DuPont Co., Wilmington, DE). Acivicin and [^{14}C]acivicin (280 mCi/g) were gifts from The Upjohn Co. (Kalamazoo, MI). All other reagents were of the highest grade commercially available.

Synthesis of acivicin derivatives. Acivicin derivatives, acivicin methyl ester and *tert*-butyloxycarbonyl (t-Boc) acivicin, were synthesized using minor modifications of methods previously employed to derivatize other amino acids [12, 13]. The reactions were carried out under ice-cold conditions to avoid racemization of acivicin. Acivicin methyl ester was obtained as yellowish-white crystals. Yield 44 mg; 65%. ^{1}H-NMR (CD_3OD, δ ppm): isoxazoline CH_2 (3.5–3.6, m, 2H); methyl (3.8–3.9, s, 3H); N—CH—C (4.5, d, 1H); isoxazoline CH (5.2–5.3, m, 1H). MS (CI, FAB, Exact Mass): 193 $(M + H)^+$. C,H,N analysis was calculated for $C_6H_9N_2O_3Cl$. The t-Boc acivicin was obtained as a white solid. Yield 70 mg; 75%. ^{1}H-NMR (CD_3OD, δ ppm): t-butyl (1.6–1.7, s, 9H); isoxazoline CH_2 (3.4–3.6, m, 2H); N—CH—C (4.5–4.6, bs, 1H); isoxazoline CH (5.2–5.3, m, 1H). MS (CI, FAB, Exact Mass): 279 $(M + H)^+$. C,H,N analysis was calculated for $C_{10}H_{15}N_2O_5Cl$.

HPLC analysis of acivicin and [^{14}C]acivicin. Reverse-phase HPLC was used to purify [^{14}C]-acivicin and determine its 1-octanol/water partition coefficient. The HPLC system consisted of a Beckman model pump (112 Solvent Delivery Module) and a Perkin–Elmer LCI-100 integrator. A Zorbax C-8 column (9.4 mm × 25 cm; DuPont Co.) and a Brownlee Spheri-5 RP-8 (5 μm, 4.6 × 30 mm; Applied Biosystems, San Jose, CA) guard column were used. The mobile phase consisted of 6% methanol in water adjusted to pH 3.4 with glacial acetic acid. The flow rate was 1.5 mL/min at room temperature. Samples (50 μL) were injected into the HPLC system and detected by a Kratos Spectroflow 783 absorbance detector set at 214 nm. Using this method, the retention time for acivicin was 10 min. For the purification of [^{14}C]acivicin, the effluent fractions were collected at 0.2-min intervals using a Bio-Rad (model 2110) fraction collector; 3 mL of scintillation fluid (3a70BTM from RPI Corp., Mount Prospect, IL) was then added to 150 μL of the collected fractions. The ^{14}C-radioactivity in each fraction was determined by liquid scintillation counting (Beckman LS 6000 IC).

1-Octanol/water partition coefficient determination. The 1-octanol/water partition coefficient of acivicin was determined after an 18-hr equilibration time at room temperature according to standard procedures. Aqueous samples were collected from different 1-octanol:water systems (25:1, 35:1, and 45:1) and analyzed in triplicate using the HPLC system described above. The PC for acivicin was obtained by dividing the concentration of acivicin in the 1-octanol phase by the concentration in the water phase after reaching equilibrium.

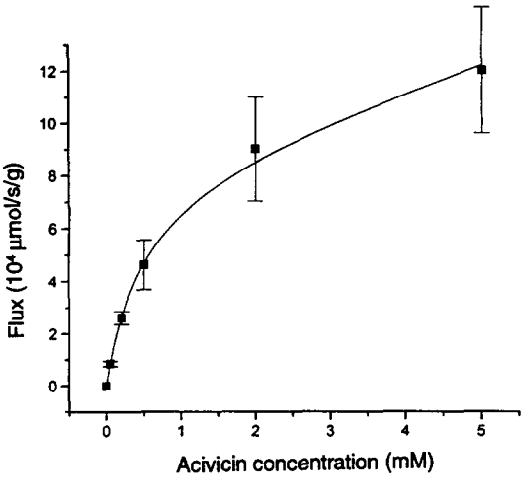

Fig. 2. Relationship between the unidirectional acivicin influx into the rat parietal cortex and acivicin concentration in the perfusate, as determined using the *in situ* rat brain perfusion technique. Each point is the mean ± SD for three animals. The curve represents total (saturable and non-saturable) acivicin influxes calculated using Equation 3. Curve fitting, using MicroCal Origin®, resulted in the following values for the Michaelis–Menten parameters: K_m = 0.667 mM, V_{max} = 1.05 nmol/sec/g and K_d = 5.55 × 10^{-5} mL/sec/g.

In situ *rat brain perfusion.* The transport of [^{14}C]-L-leucine and [^{14}C]acivicin across the BBB was determined in pentobarbital-anesthetized rats (50 mg/kg, i.p.) using the *in situ* rat brain perfusion technique developed by Takasato *et al.* [11] and described in more detail by Chikhale *et al.* [14]. Briefly, the right pterygopalatine, occipital, and superior thyroid arteries were coagulated, and the right external carotid artery was catheterized for retrograde perfusion. The perfusate consisted of a pH 7.4 bicarbonate-buffered physiological saline (128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 9 mM D-glucose) containing [^{3}H]methoxyinulin, a vascular marker, and [^{14}C]L-leucine or [^{14}C]acivicin in a ratio between 3:1 and 10:1 (plus a 2–10 mM concentration of competitor in the competition studies). The perfusate was perfused into the right hemisphere of the rat brain at a flow rate of 4.5 mL/min in the right external carotid artery. After a perfusion period of 30 sec, the perfusion was terminated by decapitation of the animal. A brain tissue sample (20–30 mg) from the parietal cortex of the right cerebral hemisphere was weighed and digested overnight at 50° in 1 mL of water containing 10% piperidine. After overnight digestion, the

sample was counted for radioactivity using a dual-label scintillation spectrometer (Beckman LS 6000 IC). The original radioactivity levels of the radiolabeled tracers in 30-μL samples of the perfusion fluid were also determined.

Calculations of the parietal BBB permeability. The *in situ* rat brain perfusion technique performed in our laboratory for measuring cerebrovascular permeability was validated using [^{14}C]L-leucine to assess the expression of LNAA carrier at the BBB. The Michaelis–Menten equation was used to described transport consisting of a saturable and a non-saturable component [15]. Least-square curve fitting was performed using MicroCal Origin®.

The transport of [^{14}C]L-leucine and [^{14}C]acivicin across the BBB was expressed in terms of three parameters: the parietal unidirectional blood-to-brain transfer constant (K_{in}), the parietal cerebrovascular permeability-surface area product (PA), and the parietal cerebrovascular influx (J_{in}).

The unidirectional blood-to-brain transfer constant was calculated from the quantity of radiolabeled solute taken up into the parietal cortex using the following equation:

$$K_{in} = (q_{tot} - V_v C_{pf})/TC_{pf} \quad (1)$$

where q_{tot} is the total measured ^{14}C-radioactivity in the parietal sample (dpm/g), V_v is the regional intravascular volume (mL/g), C_{pf} is the solute concentration in the perfusion fluid (dpm/mL), and T is the net perfusion time (sec). V_v was determined in each animal from the brain-to-perfusion fluid ratio of [^{3}H]methoxyinulin, a vascular marker [16].

The K_{in} values were converted to PA products using the Crone–Renkin model of capillary transfer [17]:

$$PA = -F \ln(1 - K_{in}/F) \quad (2)$$

where F is the regional perfusion fluid flow (mL/sec/g). F was measured using [^{3}H]diazepam [18] and was determined in a separate set of experiments to be $4.91 \pm 0.54 \times 10^{-2}$ mL/sec/g in the parietal cortex (N = 3).

The parietal cerebrovascular influx (J_{in}) was calculated using the following equation:

$$J_{in} = PA \cdot C \quad (3)$$

where C is the solute concentration in the perfusate (mM).

Statistical analysis. Student's *t*-test was used to compare individual means. In all cases, the criterion for statistical significance was $P < 0.05$.

RESULTS AND DISCUSSION

To validate the *in situ* rat brain perfusion technique, we determined the kinetics of [^{14}C]L-leucine transport across the BBB. The kinetic parameters V_{max} and K_m, were calculated to be $9.81 \pm 1.7 \times 10^{-4}$ μmol/sec/g and 0.041 ± 0.020 mM, respectively (data not shown). These values are in good agreement (not significantly different) with the previously reported V_{max} and K_m values for L-leucine transport across the BBB *in situ* (1.07×10^{-3} μmol/sec/g and 0.026 mM, respectively [16].

Similarly, the kinetics of the carrier-mediated transport of [^{14}C]acivicin across the BBB *in situ* were determined. The concentration dependency of [^{14}C]-acivicin transport across the BBB *in situ* is shown in Fig. 2, and these data were used to calculate $V_{max} = 1.05 \pm 10^{-3}$ μmol/sec/g and $K_m = 0.667 \pm 0.079$ mM. The non-saturable component for acivicin transport, K_d, was determined to be $5.55 \pm 0.12 \times 10^{-5}$ mL/sec/g. The K_m (0.667 mM) for acivicin transport across the rat BBB *in situ* is about two and a half times larger than the K_m (0.24 mM) reported for acivicin transport using an *in vitro* BBB model consisting of BBMECs [9]. Whether this difference in K_m for the carrier-mediated transport of acivicin across the BBB is due to different species (rat and bovine) is not known. However, the rank order of affinities for the LNAA carrier (phenylalanine > leucine > acivicin) is the same in both models of the BBB [9, 16, 18, 19]. The V_{max} values for acivicin, determined in these two models of the BBB, cannot be compared because the V_{max} obtained in the *in situ* model is expressed as μmol/sec/g brain tissue, whereas the V_{max} obtained in the *in vitro* model is expressed as μmol/sec/g protein. The *in situ* V_{max} of acivicin ($V_{max} = 1.05$ nmol/sec/g) is comparable to the *in situ* V_{max} of L-leucine ($V_{max} = 1.07$ nmol/sec/g) and other LNAAs [18]. The *in situ* K_m (0.667 mM) of [^{14}C]acivicin determined in this study was substantially larger than the K_m (0.29 mM) previously estimated from its *in situ* K_i value (acivicin concentration producing 50% inhibition of [^{14}C]L-leucine transport across the BBB *in situ*) [10].

The logarithm of the partition coefficient of acivicin in the 1-octanol/water system, log PC(o/w), was determined to be −2.2. When compared with the log PC(o/w) values determined for other LNAAs, the log PC(o/w) and K_m values determined for acivicin (data not shown) were consistent with the hypothesis that lipophilicity is the major determinant of the amino acid affinity to the LNAA carrier at the BBB [18]. Therefore, the relatively high K_m value of acivicin (0.667 mM) in comparison with that of L-leucine ($K_m = 0.026$ mM) and other LNAAs [18] can be explained by the low 1-octanol/water partition coefficient of acivicin [log PV(o/w) = −2.2].

The effects of 2–10 mM concentrations of various LNAAs and acivicin derivatives on the parietal PA of [^{14}C]acivicin are shown in Table 1. Glycine, which is not an LNAA, did not inhibit significantly the transport of [^{14}C]acivicin across the BBB (control, $PA = 1.62 \times 10^{-3}$ mL/sec/g). In contrast, acivicin itself and the LNAAs L-tyrosine, L-leucine, L-tryptophan, and L-phenylalanine significantly decreased the PA of [^{14}C]acivicin ($P < 0.05$). However, when the amino group (t-Boc acivicin) or the carboxyl group (acivicin methyl ester) were derivatized, these analogs did not inhibit significantly the carrier-mediated transport of acivicin across the BBB (Table 1; $P > 0.05$). Thus, acivicin-methyl ester and t-Boc-acivicin demonstrated no significant affinity for the LNAA carrier, as indicated by their inability to prevent the carrier-mediated transport of [^{14}C]acivicin across the BBB (Table 1). This confirms previous observations that both a free carboxyl and a free amino group are required in the LNAA for affinity to the LNAA carrier [20].

Table 1. Influence of the presence of 2–10 mM concentrations of various LNAAs and acivicin derivatives on the parietal permeability-surface area product (PA) of tracer concentration (0.0015 mM) of [^{14}C]acivicin as determined by the *in situ* rat brain perfusion technique

Addition (2 mM)	PA × 10^4 (mL/sec/g)	% of control PA
None (control)	16.23 ± 0.47	100 ± 2.9
Acivicin-methyl ester	15.80 ± 2.08	97.4 ± 12.8
Glycine	15.51 ± 0.25	95.6 ± 1.5
t-Boc-acivicin	14.40 ± 1.47	88.7 ± 9.1
BCH**	4.91 ± 0.94†	30.3 ± 5.8†
L-Tryptophan	4.63 ± 0.25†	28.5 ± 1.5†
Acivicin	4.51 ± 0.99†	27.8 ± 6.1†
L-Phenylalanine	4.16 ± 0.53†	25.6 ± 3.3†
BCH + L-alanine**	3.92 ± 0.68†	24.2 ± 4.2†
L-Tyrosine	3.35 ± 0.07†	20.6 ± 0.4†
L-Leucine	1.96 ± 0.92†	12.1 ± 5.7†

Each value is the mean ± SD for three animals.

** BCH (2-amino-2-norbornane-carboxylic acid) and L-alanine were used at a 10 mM concentration each.

† Significantly different from control ($P < 0.05$).

An interesting observation was that L-leucine was a better inhibitor of acivicin transport than L-phenylalanine, despite the fact that L-leucine had a lower affinity for the LNAA carrier at the BBB than L-phenylalanine. This was also observed in competition studies between L-threonine and L-leucine or L-phenylalanine at the BBB [21]. L-Leucine was capable of almost completely inhibiting acivicin transport to the non-saturable K_d value of 0.555×10^{-4} mL/sec/g. The lack of significant further inhibition of acivicin transport by the addition of 10 mM L-alanine to a perfusate containing 10 mM BCH (Table 1) indicates that the ASC carrier system [21] does not contribute significantly to the mechanism of acivicin transport across the BBB.

The results of this study provide for the first time direct evidence that [^{14}C]acivicin is transported into the brain predominantly by the LNAA carrier. The affinity of acivicin for the LNAA carrier at the BBB can be correlated with its 1-octanol/water partition coefficient similar to other LNAAs. Our results also demonstrated the feasibility of coadministering LNAAs with acivicin as a strategy to reduce the CNS permeability of acivicin by competition, thus possibly reducing its toxicity. In addition, acivicin derivatives with structural modifications at the amino or carboxyl group of acivicin lack affinity for the LNAA carrier at the BBB. Therefore, transient and bioreversible analogs (prodrugs) of acivicin obtained by derivatization of the N- and(or) the C-terminal end are expected to exhibit less CNS toxicity than acivicin.

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