7-CHLOROKYNURENIC acid (7-Cl-KYNA) and 5,7-dichlorokynurenic acid (5,7-Cl$_2$-KYNA) are of therapeutic interest as potent glycine/N-methyl-D-aspartate (NMDA) receptor antagonists, but are excluded from brain by the blood–brain barrier. We examined whether these compounds could be delivered to brain through their respective precursors, L-4-chlorokynurenine (4-Cl-KYN) and L-4,6-dichlorokynurenine (4,6-Cl$_2$-KYN), which are amino acids. 4-Cl-KYN was shown to be rapidly shuttled into the brain by the large neutral amino acid transporter of the blood–brain barrier (K_m = 105 ± 14 μM, V_{max} = 16.9 ± 2.3 nmol min^{-1} g^{-1}) and to be converted intracerebrally to 7-Cl-KYNA. 4,6-Cl$_2$-KYN also expressed affinity for the transporter, but four-fold less than that of 4-Cl-KYN. In summary, the results show that because of their facilitated uptake 4-Cl-KYN and 4,6-Cl$_2$KYN might be useful prodrugs for brain delivery of glycine-NMDA receptor antagonists.

Key words: Blood–brain barrier; 4-Chlorokynurenine; Excitotoxicity; Glycine; Kynurenic acid; Neurodegenerative disorders; NMDA; Transport

Facilitated brain uptake of 4-chlorokynurenine and conversion to 7-chlorokynurenic acid

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Introduction

N-Methyl-D-aspartate (NMDA) receptors play important roles in neuronal damage induced by excitatory amino acids following stroke, head injuries and seizures, and have been suggested to contribute to cell death in several neurodegenerative diseases.[1,2] This has stimulated development of a wide range of NMDA receptor antagonists as possible neuroprotective agents.[3] One such group of agents includes kynurenic acid (KYNA) and its derivatives that act as competitive antagonists of the glycine co-agonist site of the NMDA receptor. Within this group, 7-chlorokynurenic acid (7-Cl-KYNA) and 5,7-dichlorokynurenic acid (5,7-Cl$_2$-KYNA) are the prototypic agents and show superb potency and selectivity *in vitro*.[4] As a result of their low lipid solubility, these halogenated KYNA derivatives cross the blood–brain barrier poorly and show minimal penetration into the brain following systemic administration.[3]

An alternative means of delivering 7-Cl-KYNA or 5,7-Cl$_2$KYNA to the brain might be through their precursors, L-4-chlorokynurenine (4-Cl-KYN) and L-4,6-dichlorokynurenine (4,6-Cl$_2$-KYN). As neutral amino acids, both compounds would be expected to be taken up into the brain at the blood–brain barrier and at brain cell membranes by the large neutral amino acid transporter (System L).[5–8] Once within brain, 4-Cl-KYN and 4,6-Cl$_2$-KYN are enzymatically converted to 7-Cl-KYNA and 5,7-Cl$_2$-KYNA, respectively, through the action of kynurenine aminotransferase.[9]

In the present study, we examined the brain uptake of 4-Cl-KYN and 4,6-Cl$_2$-KYN using an *in situ* rat brain perfusion technique that allows ready control of perfusate composition for brain delivery. The results demonstrate that both compounds are taken up into the brain via the large neutral amino acid transporter. Moreover, the study shows that 4-Cl-KYN is readily converted to 7-Cl-KYNA *in vivo*.

Materials and Methods

Transport into the brain was examined using the *in situ* brain perfusion technique described by Takasato *et al*[10] and Smith.[11] Male Sprague–Dawley rats (250–350 g) were anesthetized with sodium pentobarbital (45 mg kg^{-1}, i.p.). A polyethylene catheter (PE-60) filled with heparinized saline (100 IU ml^{-1}) was placed in the right common carotid artery, and

the external carotid artery was ligated to limit flow to extracerebral tissues. The perfusion was begun by rapidly severing the cardiac ventricles and infusing bicarbonate-buffered Krebs–Henseleit saline[11] into the carotid artery at a rate of 20 ml min^{-1} to obtain a stable vascular perfusion pressure of ~100 mmHg. To the saline perfusate was added 0–500 μM L-4-Cl-KYN or L-4,6-Cl$_2$-KYN, with or without 1 mM L-leucine, to examine the rate and mechanism of chlorokynurenine uptake into brain. In addition, the perfusate contained L-[^{14}C]leucine (0.3 μCi ml^{-1}) and [^{3}H]diazepam (1 μCi ml^{-1}) to assess chlorokynurenine inhibition of leucine transport[5,6] and to measure cerebral perfusion flow.[10]

Twenty seconds after the start of perfusion, the animal was decapitated and the pump was turned off. The brain was removed from the skull and placed on ice. Samples were collected from the cortex, hippocampus and striatum of the perfused hemisphere, as well as duplicate specimens of perfusion fluid. Specimens were placed in tared plastic vials and re-weighed. Tracer activity was determined following digestion by liquid scintillation counting.[5,6] Brain levels of 4-Cl-KYN and KYN were determined using the method of Holmes,[12] with retention times for 4-Cl-KYN and KYN of 9 and 3 min, respectively. 7-Cl-KYNA and KYNA were determined by HPLC with fluorescence detection, as described previously,[9] with retention times for 7-Cl-KYNA and KYNA of 16 and 5 min, respectively.

All amino acids were L-isomers. L-Leucine, L-KYN, 7-Cl-KYNA and 5,7-Cl$_2$-KYNA were from Sigma (St. Louis, MO). L-4-Cl-KYN and L-4,6-Cl$_2$KYN were kindly provided by Drs F. Salituro and R. Tomlinson (Marion Merrell Dow, Cincinnati, OH). Radiotracers were from DuPont NEN (Boston, MA).

Transfer constants (K_{in}; ml s^{-1} g^{-1}) for brain uptake were calculated as previously described[5,6,10,11] from the quantity of solute taken up into the brain by the end of perfusion (Q_{br}; d.p.m. or nmol g^{-1} brain)

divided by the product of perfusate concentration (C_{pf}) and time (T):

$$K_{in} = [Q_{br} - V_v C_{pf}]/(C_{pf}T) \qquad (1)$$

Q_{br} was corrected for trapped intravascular solute by subtracting the product of perfusate concentration and brain intravascular volume (V_v; 0.008 ml g^{-1}).[6,10] Perfusion time was limited to 20 s to ensure unidirectional uptake.[6,11] Transfer constants were corrected for flow (F) using the Crone–Renkin equation[6,10,11] and converted to cerebrovascular permeability–surface area products (PA) as:

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

where F was measured using [^{3}H]diazepam[10] and averaged $11.7 \pm 1.5 \times 10^{-2}$ ml s^{-1} g^{-1} ($n = 18$). PA products were converted to influx rates as: Influx = PA*C_{pf}, and estimates of maximal transport capacity (V_{max}) and half saturation constant (K_m) were calculated by fitting the data to the Michaelis–Menten equation [Influx = ($V_{max} C_{pf}$)/($K_m + C_{pf}$)] using nonlinear regression.[5,6] Fifty percent inhibition constants (K_i) were calculated from the reduction in L-[^{14}C]leucine uptake as:

$$\% \text{ Inhibition} = 100 \times [C_i/(C_i + K_i)] \qquad (3)$$

where C_i = perfusate 4-Cl-KYN, 4,6-Cl$_2$-KYN or KYN concentration.[5,6] The inhibition constant provides an index of the ability of the compound to inhibit blood–brain barrier L system transport.

Values are presented as mean ± s.e.m. Statistical significance ($p < 0.05$) was assessed by analysis of variance with the Tukey correction for multiple comparisons (InStat, GraphPad Software, San Diego, CA).

Results

Perfusion with physiologic saline containing 100–500 μM 4-Cl-KYN for 20 s demonstrated significant brain uptake of 4-Cl-KYN and metabolic conversion to 7-Cl-KYNA (Table 1). Measured brain

Table 1. Brain concentrations of 4-Cl-KYN, 7Cl-KYNA, KYN and KYNA after 20 s of brain perfusion with physiologic saline containing 100 or 500 μM 4-Cl KYN

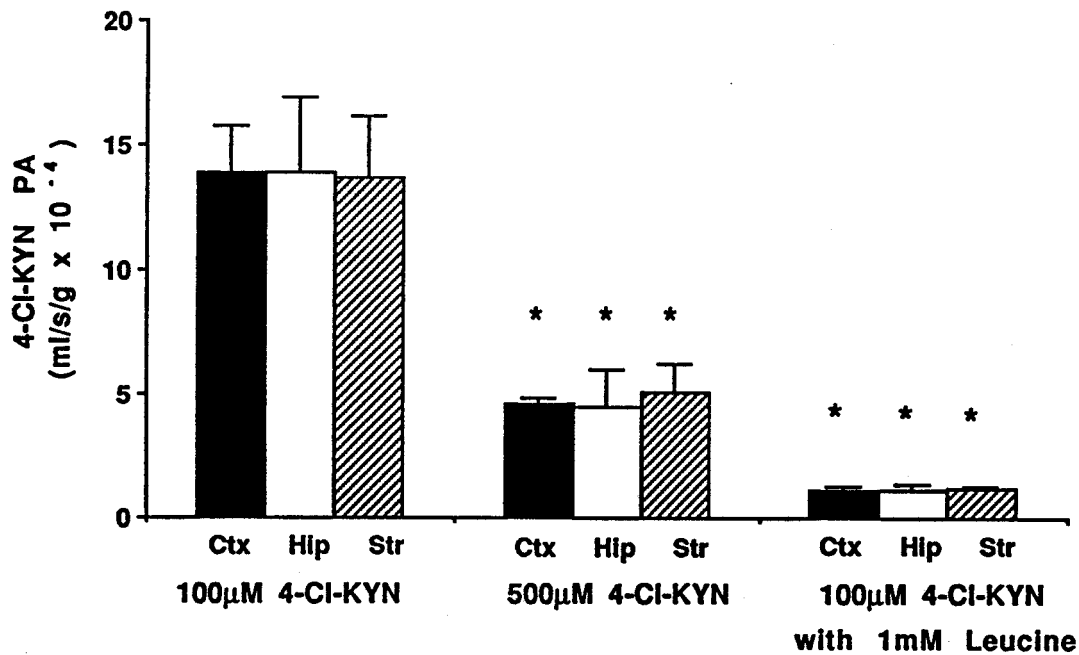| Perfusate Conc. of 4-Cl-KYN		4-Cl-KYN (nmol/g)	KYN (nmol/g)	7Cl-KYNA (pmol/g)	KYNA (pmol/g)
100μM					
	Cortex	2.66 ± 0.19	0.91 ± 0.09	11.07 ± 1.09	10.28 ± 1.01
	Hippocampus	2.70 ± 0.17	0.81 ± 0.06	13.62 ± 1.75	9.23 ± 0.70
	Striatum	2.70 ± 0.27	1.29 ± 0.11	14.76 ± 1.24	17.16 ± 1.31
	Average	2.69 ± 0.21	1.00 ± 0.09	13.15 ± 1.35	12.22 ± 1.00
500μM					
	Cortex	4.87 ± 0.51	0.91 ± 0.15	22.45 ± 1.32	12.81 ± 1.09
	Hippocampus	4.44 ± 0.07	0.62 ± 0.08	23.73 ± 2.69	14.50 ± 1.23
	Striatum	4.29 ± 0.67	0.84 ± 0.04	32.06 ± 1.63	18.46 ± 3.82
	Average	4.53 ± 0.42	0.79 ± 0.09	26.08 ± 1.88	15.26 ± 2.05

Values represent means ± s.e.m. for five animals

FIG. 1. Permeability–surface area (PA) product for 4-Cl-KYN uptake into the brain during perfusion with physiologic saline containing 100 or 500 μM 4-Cl-KYN. Values are mean ± s.d. for four or five animals per group. *Statistically significant difference from corresponding value with 100 μM 4-Cl-KYN alone ($p < 0.05$). The PA product is a measure of the relative ability of the compound to cross the blood–brain barrier. Abbreviations: Ctx, cerebral cortex; Hip, hippocampus; Str, striatum.
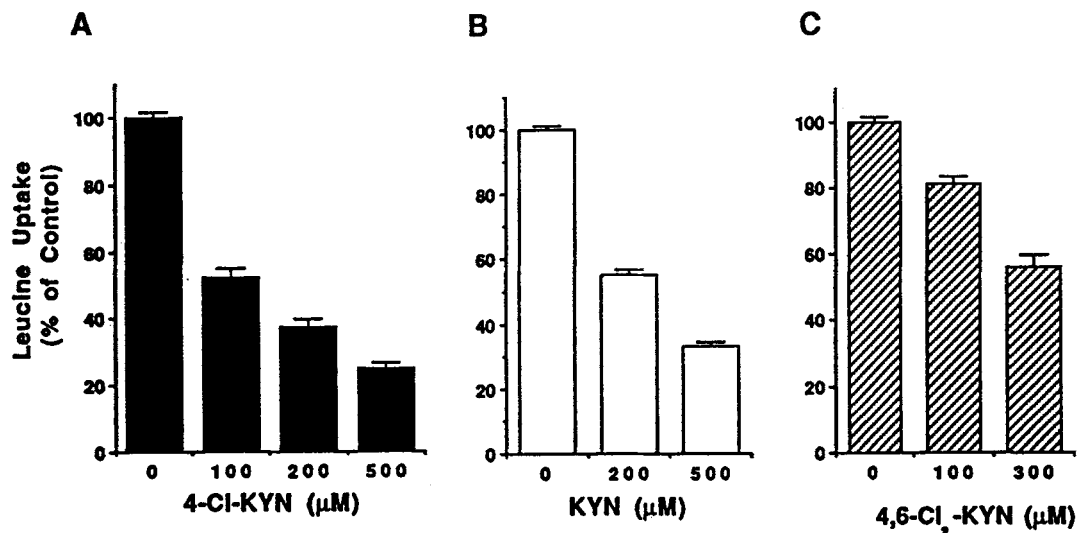

FIG 2 Inhibition of leucine uptake into brain by 4-Cl-KYN (**A**), KYN (**B**) and 4,6-Cl$_2$-KYN (**C**). Values represent mean ± s.d. for three to five animals per group.

concentrations of 4-Cl-KYN and 7-Cl-KYNA in rat cortex, hippocampus and striatum equaled or exceeded respective concentrations of endogenous L-KYN and KYNA. Assuming that conversion to other metabolites was negligible, PA products for 4-Cl-KYN were calculated (Eqns. 1,2) based on the summed cerebral 4-Cl-KYN and 7-Cl-KYNA concentrations in each animal to obtain a relative measure of the ability of 4-Cl-KYN to cross the blood–brain barrier. The PA to 4-Cl-KYN decreased with increasing perfusate 4-Cl-KYN concentration,

as expected for saturation of a transport system (Fig. 1). Influx was mediated by the cerebrovascular large neutral amino acid transporter (L system), as addition of 1 mM L-leucine, a typical L-system substrate,[5-7] to the perfusate almost completely abolished 4-Cl-KYN uptake (Fig. 1). Non-linear regression provided best fit estimates of transport V_{max} and K_m of 16.9 ± 2.3 nmol min^{-1} g^{-1} and 105 ± 14 μM, respectively. These values are of the same order as those reported previously for the brain uptake of L-KYN, which is also mediated by the L system.[6]

Affinity for the blood–brain barrier L-system was confirmed by analysis of L-[^{14}C]leucine uptake (Fig. 2A). 4-Cl-KYN reduced L-[^{14}C]leucine transport into brain in a concentration-dependent manner, with a calculated K_i (116 ± 10 μM; $n = 8$) close to the estimated K_m. L-KYN and 4,6-Cl$_2$-KYN also exhibited affinity for the transporter (Fig. 2B,C). The respective K_i values (255 ± 9 μM and 410 ± 18 μM; $n = 6$) however, were two and four times greater than that for 4-Cl-KYN. Brain uptake of 4,6-Cl$_2$-KYN was not demonstrated because of the poor sensitivity of the HPLC assay for the dichlorinated substrate.

Discussion

7-Cl-KYNA is the prototypic antagonist that helped define the role of the glycine co-agonist site of the NMDA receptor in neurobiology and disease. The poor bioavailability of this compound, and of its more potent congeners, such as 5,7-Cl$_2$-KYNA, however has stimulated attempts to identify new agents with greater CNS access and therapeutic action.[3]

The potential for a prodrug approach to address this problem is based on the knowledge that, first, L-KYN, the precursor of KYNA, can gain ready access to brain via the large neutral amino acid transporter of the blood–brain barrier[6] and brain cell membranes[13] and, second, that the brain has the metabolic machinery to convert L-KYN as well as its halogenated analogs to KYNA derivatives via the enzyme kynurenine aminotransferase.[9] The L system transporter is fairly loose in its requirements for substrate binding and transfer,[5,7] and it is not surprising that both 4-Cl-KYN and 4,6-Cl$_2$-KYN show some affinity for the transport carrier. The affinity for 4-Cl-KYN actually exceeds that for the endogenous L-KYN substrate, by ~ two fold, whereas the addition of the second chlorine reduces affinity by four fold. The transport K_m for 4-Cl-KYN is within the range of that of other large neutral amino acids (10–200 μM),[5] including L-leucine, which has a K_m of 29 μM.[5]

The capacity of the brain to take up 4-Cl-KYN and convert it to 7-Cl-KYNA is demonstrated by the fact that brain concentrations of both 4-Cl-KYN and 7-Cl-KYNA equaled or exceeded corresponding concentrations of L-KYN and KYNA after only 20 s of brain perfusion with 100 μM 4-Cl-KYN (Table 1). Brain concentrations of 4-Cl-KYN and 7-KYNA were higher after perfusion with 500 μM 4-Cl-KYN. As the affinity of 7-Cl-KYNA for the glycine/NMDA receptor is ~70 times greater than that of endogenous KYNA (IC$_{50}$ *in vitro* 0.5 μM)[3] our results suggest that it is possible to achieve therapeutic concentrations of 7-Cl-KYNA following peripheral 4-Cl-KYN administration. Recent preliminary experiments in mice have demonstrated that micromolar concentrations of extracellular 7-Cl-KYNA can be maintained for several hours following the systemic application of 4-Cl-KYN.[14]

Conclusion

This study demonstrates that 4-Cl-KYN is readily taken up into brain by the large neutral amino acid transporter (System L) of the blood–brain barrier and converted *in situ* to 7-Cl-KYNA, a potent selective antagonist of the glycine co-agonist site of the NMDA receptor. The results support the possible use of systemically administered 4-Cl-KYN as a prodrug of 7-Cl-KYNA for the treatment of brain injury in acute and chronic neurodegenerative diseases.[9,14]

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General Summary
The *N*-methyl-D-aspartate (NMDA) receptor plays a central role in neuroexcitatory cell death in stroke and neurodegenerative diseases. This has stimulated development of NMDA receptor blockers as neuroprotective agents. 7-Chlorokynurenic acid and 5,7-dichlorokynurenic acid are the prototypical blockers of the glycine site of the NMDA receptor complex, but are polar and are not taken up into brain after peripheral administration. In the present study, we show that it is possible to achieve good brain delivery of 7-chlorokynurenic acid through its precursor, 4-chlorokynurenine, which is taken up into brain by the large neutral amino acid transport system and converted to 7-chlorokynurenic acid *in situ*. The results present a new means of brain delivery of NMDA receptor blockers which might provide greater specificity and less toxicity than other methods.