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Anticonvulsant effects of the 3-hydroxyanthranilic acid dioxygenase inhibitor NCR-631

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Summary. The kynurenine pathway intermediate 3-hydroxyanthranilic acid (3-HANA) is converted by 3-HANA 3,4-dioxygenase (3-HAO) to the pro-convulsive excitotoxin quinolinic acid. In the present study, the anticonvulsant effect of the 3-HAO inhibitor NCR-631 was investigated in models of chemically- and sound-induced seizures. Administration of NCR-631 i.c.v. at a dose of 300 nmol in Sprague-Dawley rats was found to prolong the latency of occurrence of pentylenetetrazole (PTZ)-induced seizures. Also systemic pre-treatment with NCR-631 s.c. in N.M.R.I. mice subjected to PTZ-induced seizures provided an increase in the latency until onset of seizures, concomitant with a reduction in the severity of the seizures. However, the anticonvulsant effect of NCR-631 was short lasting (15–30 min), and only observed at a dose of 250 mg/kg. A similar dose- and time-dependent anticonvulsant effect of NCR-631 was found in seizure-prone DBA/2J mice following sound-induced convulsions. Hence, the findings show that NCR-631 has anticonvulsant properties against generalized tonic-clonic seizures of different origin, suggesting that it may constitute a useful tool to study the role of kynurenines in various convulsive states.

Keywords: Amino acids – Seizures – Epilepsy – Kynurenines – Quinolinic acid

Abbreviations: EAA, excitatory amino acid; 3-HANA, 3-hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilic acid 3,4-dioxygenase; NMDA, N-methyl-D-aspartate; N.M.R.I., Naval Medical Research Institute; PTZ, pentylenetetrazole; QUIN, quinolinic acid; SEM, standard error of the mean.

Introduction

The kynurenine pathway is the principal route of L-tryptophan metabolism in mammals, leading to the formation of the essential products nicotinamide and its nucleotide conjugates. Also certain intermediates of this pathway may have important actions, such as the dicarboxylic acid quinolinic acid (QUIN). QUIN has been shown to interact with excitatory amino acid (EAA)

receptors of the brain, acting as an agonist at N-methyl-D-aspartate (NMDA) receptors (see Stone, 1993). In line with its excitatory properties, QUIN causes convulsions and neurodegeneration when administered into the brain (Lapin, 1978; Schwarcz et al., 1983, 1984).

QUIN is produced from 3-hydroxyanthranilic acid (3-HANA) by the enzyme 3-hydroxyanthranilic acid 3,4-dioxygenase (3-HAO; EC 1.13.11.6) (Okuno et al., 1987; Malherbe et al., 1994), while it is metabolized by quinolinate phosphoribosyltransferase (QPRT). Both 3-HAO and QPRT have been reported to be present in the rat CNS (Köhler et al., 1988; Okuno et al., 1987). Altered kynurenine pathway function, including enhanced cerebral QUIN levels or increased 3-HAO activity, have been found in several human neurodegenerative diseases as well as in animal models of neurodegeneration (e.g. Heyes et al., 1992). QUIN has also been implicated in hepatic encephalopathy (Moroni et al., 1986), conditions characterized by prominent neuroimmunological involvement (Heyes et al., 1992) and in epilepsy (Lloyd et al., 1990). Indeed, decreased activity of QPRT has been described in human epileptic tissues (Feldblum et al., 1988), while at the same time a reduction of QUIN levels in cerebrospinal fluid has been reported in patients with complex partial seizures (Heyes et al., 1990, 1994). 3-HAO activity has been found to be markedly higher in the brains of seizure-prone El and DBA/2 mice than in non-epileptic strains (Eastman et al., 1994; Nakano et al., 1992). Moreover, increased QUIN content and 3-HAO mRNA levels have been found in El mice (Nakagawa et al., 1995; Nakano et al., 1993). There is also a report that electrical kindling in rats causes increased cerebral extracellular levels of kynurenic acid (KYNA); a kynurenine pathway metabolite with antagonistic actions on EAA receptors (Wu et al., 1995).

It is therefore possible that QUIN may play a role as an endogenous excitatory factor involved in lowering the threshold for events leading to neurodegeneration or seizures. To address this hypothesis, a novel 3-HAO inhibitor, 4,6-dibromo-3-hydroxyanthranilic acid (NCR-631; Linderberg et al., 1999), was studied in models of generalized tonic-clonic seizures (Fisher, 1989). Chemically-induced seizures utilizing the $GABA_A$ receptor antagonist pentyleneterazole (PTZ) were first employed in rats, which had been pre-treated with NCR-631 by intracerebroventricular (i.c.v.) injection to avoid potential problems with brain accessibility or metabolic and pharmacokinetic properties. The time- and dose-response relationships after systemic NCR-631 administration were subsequently characterized in N.M.R.I. mice subjected to PTZ-induced seizures as well as in sound-induced seizures in DBA/2J mice (Schlesinger et al., 1965).

Materials and methods

Animals and treatments

Male Sprague-Dawley rats (170–180 g; B&K Universal AB, Sollentuna, Sweden) and male mice of the N.M.R.I. (Naval Medical Research Institute; 20–25 g; B&K Universal AB, Sollentuna, Sweden), or DBA/2J BOM (21 days old at arrival; approx. 9 g, Gl. Bomholtgaard, Denmark) strains were housed under controlled conditions of

temperature (21°C), relative humidity (55–65%) and light-dark cycle (12:12 h, lights on 6 a.m.). Food and tap water were available *ad libitum* in the home cage. The animals arrived from the breeder at least seven days before initiation of the experiments. The animal experimental protocols performed in the present study were approved by the Swedish Committee for Ethical Experiments on Laboratory Animals (S210/92, S211/92, S8/95 and S7/96; South Committee, Stockholm, Sweden).

PTZ (Pentetrazol, Apoteketsbolaget AB, Sweden) was purchased in commercial sterile saline solution (30 mg/ml), which was further diluted in saline for subcutaneous (s.c.) injection of 5 ml/kg. For systemic administration, NCR-631 (Astra Arcus AB, Södertälje, Sweden; batch numbers 100/94 and 205/94; MW: 310.9) was dissolved immediately before use in a few drops of NaOH (1 mM), diluted in physiological saline, and the pH was adjusted to approximately 8 by HCl (1 mM). Systemic administrations were performed by s.c. injections into the neck skin (5 ml/kg). Control animals were given 5 ml/kg of vehicle only with the same pH as the test compound solution. For i.c.v. injections, NCR-631 was dissolved in a few drops NaOH, and then diluted in a 10 mM phosphate buffer (pH 7.4), containing 105 mM NaCl, 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$ and 0.1% ascorbic acid. Control animals were given i.c.v. injections of vehicle only. The prepared solutions were kept away from light, and used within 2 h.

PTZ-induced seizures in rat

Rats were anesthetized in a closed compartment with a 5.0% of enflurane (Efrane; Abbott, Campoverde, Italy) and O_2/N_2O (30/70%) mix. They were thereafter placed in a stereotaxic frame (Stoelting; Wood Dale, IL, USA). A mask was fitted over the nose of the rat, and the anesthesia was maintained by free breathing of 3.5–5.0% enflurane at a flow-rate of 3 l/min of O_2 and 7 l/min of N_2O. The body temperature was kept at 37°C using a heating pad controlled via a rectal thermometer (CMA/12; CMA Medicine AB, Sweden). The skull was oriented with the horizontal plane passing through bregma and lambda, adjusted using a miniature water-level, and through the intra-aural line. The skin over the skull was opened, and a unilateral hole on the right side of the skull bone was drilled with a 1 mm burr. A Hamilton microliter syringe (gauge 22 S, 25 μl) was lowered 3.9 mm vertically from the surface of the brain at AP 0 mm (bregma) and L 1.0 mm. An i.c.v. injection of 10 μl of 300 nmol NCR-631 in buffer, or vehicle only, was performed over 1 min. The syringe was then removed and the skin closed by wound clips. The rats were allowed to recover in separate home cages for 2 h. PTZ was then administered at a dose of 60 mg/kg s.c., followed by an observation period of 30 min and sacrifice by decapitation. The animals were randomly allocated to the different treatments groups and the rating was made blinded to the treatment protocol. During the observation period, the latency until appearance of behavioral seizures and the severity of the seizures were recorded. Behavioral seizures were scored using a modification of the graded scale defined by Racine (1972). Grades were; 0, normal behavior; 1, crawling behavior, forelimb clonus; 2, rearing, falling on forelimbs; 3, falling on side or back, all limbs up; 4, death.

PTZ-induced seizures in N.M.R.I. mice

NCR-631 was given s.c. to N.M.R.I. mice before PTZ-induced convulsions to study its time-response and dose-response effects. After the NCR-631 administration, PTZ was given s.c. in a dose of 80 mg/kg s.c., which was followed by an observation period of 20 min in the home cages. The animals were thereafter immediately sacrificed by cervical dislocation. The animals were randomly allocated to the different treatments groups and the rating was made blinded to the treatment protocol. During the observation period, the time until appearance of behavioral seizures and their severity were recorded. Behavioral seizures were scored using a graded scale; 0, normal behavior; 1, crawling behavior, forelimb clonus; 2, rearing, falling on forelimbs; 3, falling on side or back, all limbs up; 4, death.

Audiogenic seizures in DBA/2J mice

DBA mice (age 24–29 days) were given NCR-631 s.c. at different time points or doses before they were exposed to sound-induced seizures. The mice were placed in a sound insulated test chamber (25 × 25 × 50 cm) equipped with a sound generating system (radio-noise frequence at 130 dB) with a speaker positioned in the ceiling of the chamber. The mice were allowed 1 min habituation before the sound exposure was initiated. They were thereafter exposed to the sound for 2 min, when they were observed through the Plexiglas door of the test chamber. Immediately after termination of the sound exposure the mice were sacrificed by cervical dislocation. The animals were randomly allocated to the different treatments groups and the rating was made blinded to the treatment protocol. Behavioral seizures were scored using a graded scale; 0, normal behavior; 1, jumps; 2, falling on side or back, all limbs up; 3, death.

Data analysis

The most severe score observed for each animal as well as the time until first symptoms appeared (no time data were recorded when score = 0) were used for evaluation of treatment effects. The scoring data were analyzed using either Mann-Whitney U-test or Kruskal-Wallis testing followed by *post hoc* testing with Dunn's test, while the time-data were analyzed using Student's t-test, or by analysis of variance (ANOVA), followed by Dunnett's *post hoc* testing. Statview or SuperAnova; (for Macintosh; Abacus Concepts Inc., Berkeley, CA, USA) were used for statistical analysis as well as the program Prism (Graph Pad Software, San Diego, CA, USA) for statistical analysis and generation of graphs. The data is presented as mean ± standard error of the mean (SEM).

Results

PTZ-induced seizures in rats and mice

The rats given NCR-631 at a dose of 300 nmol i.c.v. before PTZ-induced seizures showed a significant delay in the occurrence of the seizures, when PTZ was administered 2 h later (Fig. 1). In spite of a trend towards a decrease in the scores, there were no significant effects on the severity of the seizures, (Mann-Whitney U-test; $p = 0.20$) in the NCR-631 treated rats (1.65 ± 0.23; $n = 13$) compared to the vehicle group (1.04 ± 0.34; $n = 13$).

N.M.R.I. mice were given NCR-631 at different time points, using a fixed dose of 250 mg/kg s.c., before the induction of PTZ seizures. A reduction was found in the severity of seizures, whereas the latency to seizure onset was prolonged (Fig. 2A,B). However, the effect of NCR-631 pre-treatment was fairly short lasting, with a loss of the effect when the compound was given 30 min or longer before PTZ.

The dose-response effect of NCR-631 was subsequently studied using a fixed pre-administration time (15 min). Only the 250 mg/kg s.c. dose of NCR-631 provided a significant effect on PTZ-induced seizures, assessed by both the severity of the seizures and the latency to seizure onset. The higher dose of NCR-631 (750 mg/kg s.c.), as well as the lower doses studied, were without significant effects (Fig. 3A,B).

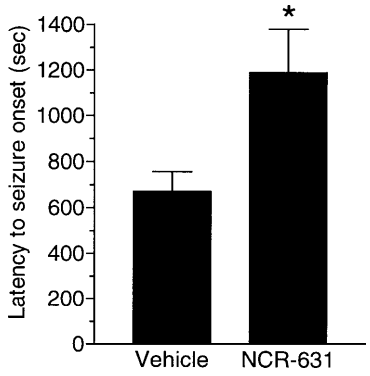

Fig. 1. Effects of NCR-631 given i.c.v. in a dose of 300 nmol on PTZ-induced seizures in rats 2 h later. There was a significant delay in the latency to seizure onset in the NCR-631 treated animals compared to vehicle-injected rats. * = $p < 0.05$ compared to the vehicle-treated group (Student's t-test). The data are presented as mean ± SEM; n = 13 per group

Audiogenic seizures in DBA/2J mice

NCR-631 was given at a dose of 250 mg/kg s.c. at 15 min, 30 min and 60 min before sound-induced seizures in DBA/2J mice (Fig. 4A). A significant effect against the severity of the seizures was found when NCR-631 was given 15 min or 30 min before the induction of the seizures, while the effect had disappeared at the 60 min pre-treatment time. The dose-response effect of NCR-631 was subsequently studied at the 30 min pre-treatment timepoint (Fig. 4B). Only the highest dose of NCR-631 studied, 250 mg/kg s.c., provided a significant anticonvulsant effect.

Discussion

The present results show that NCR-631, a 3-HANA analog inhibitor of 3-HAO, possesses protective effects against PTZ-induced seizures in rats and mice as well as against sound-induced seizures in mice. It was also found that NCR-631 was active against PTZ-induced seizures after both intracerebral and systemic administration. This observation suggests that the anticonvulsant effect of NCR-631 was mediated through a direct action of NCR-631 in the brain. Moreover, since intracerebral administration of NCR-631 was active, it is also less likely that the anticonvulsant action depended on alterations in systemic levels of kynurenines, which subsequently could have affected the seizure threshold. Following systemic administration in mice, a relatively short lasting anticonvulsant effect was obtained with NCR-631, with a loss of its action within one hour. This finding is in line with the short half-life of NCR-631 in plasma and the rapid loss of cerebral inhibition of 3-HAO observed in rats (Luthman et al., submitted), providing further support for a direct action of NCR-631 in the brain. At the same time, it should be noted that there was a 5–15 min latency until the anticonvulsant effects occurred

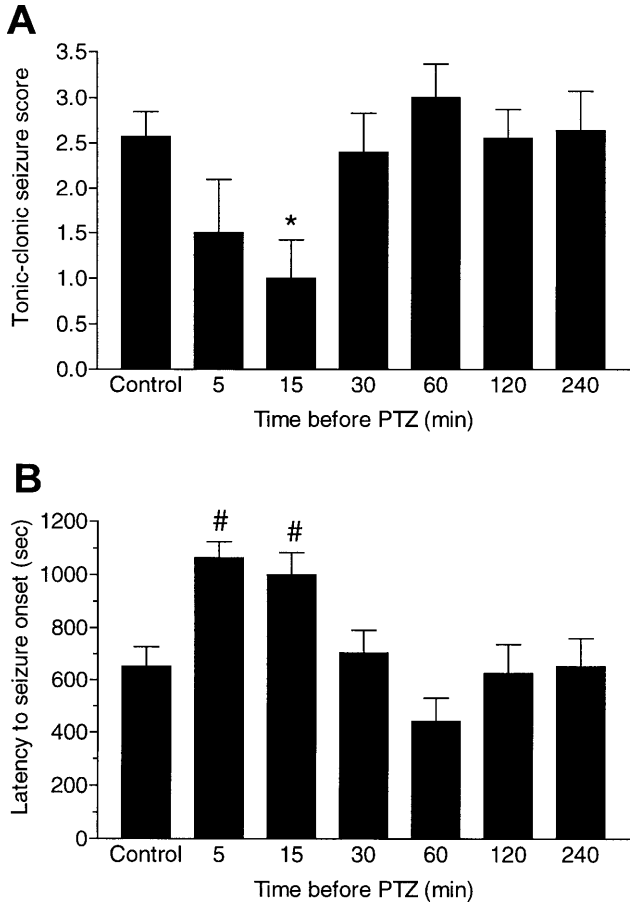

Fig. 2. Effects of NCR-631 given s.c. in a dose of 250 mg/kg at different time points before PTZ-induced seizures in N.M.R.I. mice. There was a significant decrease in the seizure score (**A**) when NCR-631 was given 15 min before PTZ, while there was a significant increase in the latency to seizure onset when NCR-631 was given both 5 min and 15 min before PTZ (**B**). * = $p < 0.05$ compared to the vehicle-treated group (Kruskal-Wallis test, followed by Dunn's *post hoc* test). # = $p < 0.05$ compared to the vehicle-treated group (ANOVA, followed by Dunnett's post *hoc test*). The data are presented as mean ± SEM; n = 10–11 per group

after s.c. administration of NCR-631. This may relate to the distribution time of NCR-631, but it may also reflect that a time delay was needed for the pharmacological action, e.g. requiring the occurrence of alterations in the levels of kynurenine pathway metabolites.

Previous studies have shown that modulation of the kynurenine pathway at other enzymatic sites than 3-HAO may provide anticonvulsant effects. Thus, treatment with the kynurenine hydroxylase and kynureninase inhibitor nicotinylalanine prolonged the time until seizures occurred in mice given leptazol and reduced the severity of electroshock-induced seizures (Connick et al., 1992). Treatment with an analog to nicotinylalanine, meta-nitrobenzoylalanine, has also been shown to provide protection against

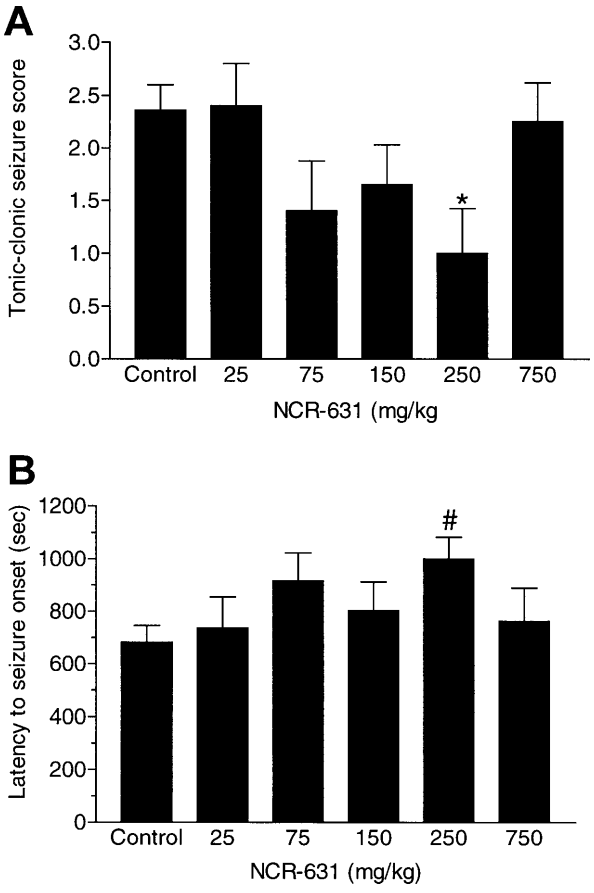

Fig. 3. Effects of NCR-631 given s.c. at different doses at 15 min before PTZ-induced seizures in N.M.R.I. mice. A significant decrease in seizure score (**A**) and a significant prolongation of the latency to seizure onset (**B**) when NCR-631 was given at 250 mg/kg. * = $p < 0.05$ compared to the vehicle-treated group (Kruskal-Wallis test, followed by Dunn's post *hoc test*). # = $p < 0.05$ compared to the vehicle-treated group (ANOVA, followed by Dunnett's *post hoc* test). The data are presented as mean ± SEM; n = 8–10 per group

electroshock-induced seizures in rat and against sound-induced seizures in DBA/2 mice (Carpenedo et al., 1994). While it has been shown that NCR-631 is active *in vivo* as an inhibitor of cerebral 3-HAO, counteracting increases in QUIN levels after substrate loading (Luthman et al., 1996; Linderberg et al., 1999), the effects of the nicotinylalanine compounds are likely to be related to an enhancement of KYNA levels (Connick et al., 1992). It is therefore possible that there are fundamental mechanistic differences between the anticonvulsant effects seen with the nicotinylalanine class of kynurenine pathway inhibitors and NCR-631. In support of this notion is the observation that the nicotinylalanine compounds appear to act as anticonvulsants only in association with the occurrence of sedation (Carpenedo et al., 1994), while NCR-631 does not show any sedative effects (Luthman et al., unpublished).

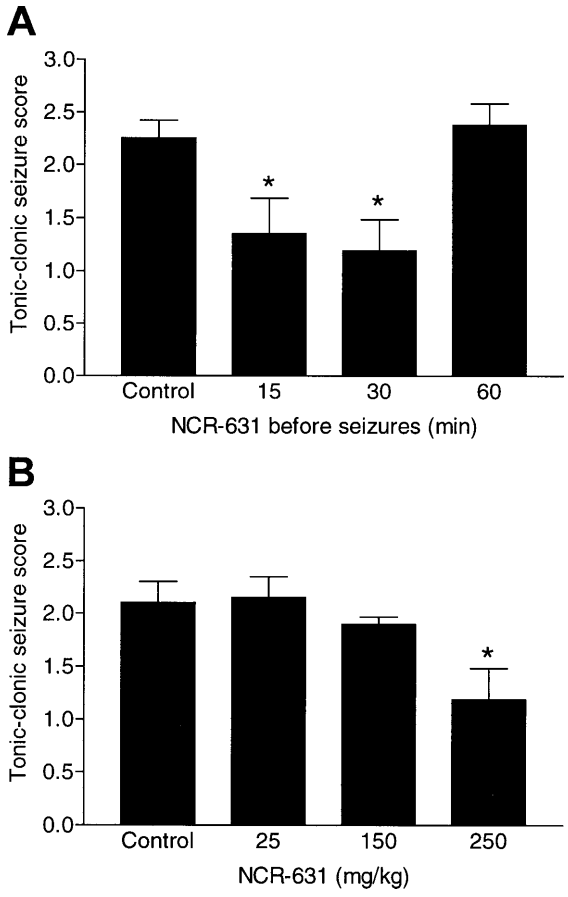

Fig. 4. Effects of NCR-631 given s.c. at different times (**A**) or doses (**B**) before audiogenic seizures in DBA/2J mice. There was a significant decrease in seizure score when 250 mg/kg NCR-631 was given 15 min and 30 min before seizure induction, while only the highest dose provided protection, when given 30 min before the seizures. * = $p < 0.05$ compared to the vehicle-treated group (Kruskal-Wallis test, followed by Dunn's *post hoc* test). The data are presented as mean ± SEM; n = 10–15 per group

Since it has been shown that increased 3-HAO activity and QUIN concentration occur in the brains of seizure-prone mice (Eastman et al., 1994; Nakagawa et al., 1995; Nakano et al., 1992, 1993) one may speculate that the anticonvulsant effect observed after NCR-631 administration is related to the ability of this compound to affect QUIN synthesis. At the same time, in normal rats NCR-631 provides very restricted, if any, effects on endogenous levels of QUIN, while a major accumulation of the substrate 3-HANA occurs in both brain and blood (Fornstedt et al., 1999). The cerebral levels of QUIN, or 3-HANA, were not determined in the present study. Hence, it remains to be established whether the anticonvulsant effect of NCR-631 is related to a reduction of endogenous QUIN in the brain, or whether other mechanisms, such as an accumulation of upstream kynurenines, are implicated.

In a previous study it was shown that NCR-631 provided protection against anoxia-induced loss of hippocampal pyramidal neurons in organotypic cultures, when it was present during the insult (Luthman et al., 1998). NCR-631 also counteracted lipopolysaccaride- and interleaukin-1β-mediated neurotoxicity in the same culture system (Luthman et al., 1998). Furthermore 4-Cl-3-HANA, another 3-HAO inhibitor, has been shown to provide neuroprotection *in vivo* in a spinal cord injury model (Blight et al., 1995). While the pharmacology of NCR-631 has to be further characterized, the present findings therefore suggest that 3-HANA analogs may have anticonvulsant actions in addition to their observed neuroprotective properties, suggesting that the 3-HAO enzyme may be implicated in lowering the threshold for both excitotoxic and pro-convulsive events.

It can be concluded that a protective effect of NCR-631 was found in two different rodent models of generalized tonic-clonic seizures, spurring further studies on the potential of 3-HANA analog 3-HAO inhibitors as anticonvulsant drugs, and on the role of kynurenines in epilepsy.

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