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4-HALO-3-HYDROXYANTHRANILIC ACIDS: POTENT COMPETITIVE INHIBITORS OF 3-HYDROXY-ANTHRANILIC ACID OXYGENASE *IN VITRO*

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Abstract—The mechanism of action of three potent inhibitors of 3-hydroxyanthranilic acid oxygenase (3HAO), the enzyme responsible for the production of the endogenous excitotoxin quinolinic acid, was examined *in vitro*. Using either liver homogenate or purified 3HAO, and following the rapid synthesis of the immediate enzymatic product α-amino-β-carboxymuconic acid ω-semialdehyde spectrophotometrically, 4-halogenated (F, Cl, Br) 3-hydroxyanthranilic acids were found to inhibit enzymatic activity in a reversible fashion. Because of the very tight binding of the drugs to 3HAO, reversibility was detected only after warming the protein–inhibitor complexes at 37°. Further studies showed that enzyme inhibition was competitive in nature (apparent K_i values: 190, 6 and 4 nM for the F-, Cl- and Br-compounds, respectively), and suggested that the drugs are metabolized by the enzyme. Specific, reversible, and tightly binding 3HAO inhibitors can be expected to become valuable tools for the study of quinolinate neurobiology. The drugs could also be of interest for the diagnostics and therapeutics of brain diseases which have been speculatively linked to a pathological overabundance of quinolinic acid.

Quinolinic acid, an established component of the tryptophan–NAD^+ pathway in the periphery, has gained prominence recently for its neuroactive properties [1]. In particular, the compound was shown to activate selectively *N*-methyl-D-aspartate (NMDA‡) receptors, which are likely to mediate most of the "fast" excitatory synaptic transmission in the brain [2]. Since NMDA receptors also have been linked convincingly to the pathogenesis of a wide spectrum of neurological diseases [2], the neurobiology of quinolinic acid has recently become the subject of intensive scrutiny. Abnormalities in cerebral quinolinic acid content or metabolism have been found in temporal lobe epilepsy [3], Huntington's disease [4], olivopontocerebellar atrophy [5], hepatic encephalopathy [6] and, most dramatically, in the AIDS–dementia complex [7].

In the brain as in the periphery of mammals, quinolinic acid appears to be synthesized exclusively by the Fe^{2+}-dependent dioxygenase 3-hydroxyanthranilic acid oxygenase (3HAO). 3HAO actually catalyzes the conversion of 3-hydroxyanthranilic acid (3HANA) to α-amino-β-carboxymuconic acid ω-semialdehyde, which, in turn, transforms spontaneously into quinolinic acid [8–10]. Identical 3HAO proteins are present in the liver and in the brain, where the enzyme is localized in astrocytes [11].

Inhibitors of 3HAO activity, namely 4-halogenated substrate analogs, have been described [12, 13], and are potentially useful tools for the study of quinolinate function [13–15]. They are also of obvious therapeutic interest in diseases which can be traced to an overabundance of quinolinic acid. Therefore, we decided to examine the mechanism of 3HAO inhibition by 4-halo-3HANAs in detail. Some of the results have been published in abstract form [16].

METHODS

Materials. 4-F-, 4-Cl-, and 4-Br-3HANAs were synthesized as described previously [13]. All other reagents were obtained from commercial sources.

Enzyme preparations. Cell-free homogenate was prepared from the liver of male Sprague–Dawley rats (250–400 g). After storage of whole liver at −20° for up to 3 months, the tissue was thawed, minced and homogenized on ice by 3 times 2-sec bursts of sonication (1:4, w/v) in ≤1 mL of 150 mM 2[*N*-morpholino]-ethanesulfonic acid (MES) buffer, pH 6.5, using a cell disruptor (Ultrasonics Inc., Plainview, NJ; maximal setting). After centrifugation (10,000 *g*, 10 min), the supernatant was collected and diluted 1:4 in distilled water.

Purified 3HAO was prepared from rat liver using a slight modification of the procedure of Okuno *et al.* [11]; thus, in the final stages of purification, the CM-cellulose column was ommitted and Sephacryl S-200 was substituted for Sephadex G-150.

Enzyme assay. 3HAO activity was measured according to the method of Bokman and Schweigert

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‡ Abbreviations: NMDA, *N* - methyl - D - aspartate; 3HAO, 3-hydroxyanthranilic acid oxygenase; 3HANA, 3-hydroxy-anthranilic acid; MES, 2[*N*-morpholino]-ethanesulfonic acid; 4-halo-3HANA, 4-halogenated-3-hydroxyanthranilic acid; 4-*F*-HANA, 4-fluoro-3-hydroxyanthranilic acid; 4-Cl-3HANA, 4-chloro-3-hydroxyanthranilic acid; and 4-Br-3HANA, 4-bromo-3-hydroxyanthranilic acid.

[8] by following the conversion of 3HANA to α-amino-β-carboxymuconic acid ω-semialdehyde.* The assay mixture contained (final concentrations) 30 μM 3HANA, 0.3 mM $(NH_4)_2$ $Fe(SO_4)_2$, enzyme inhibitors at various concentrations and cell-free homogenate or purified enzyme and 60 mM MES buffer, pH 6.5, in a total volume of 1 mL.† Routine assays were performed by preincubating the buffer, $(NH_4)_2$ $Fe(SO_4)_2$, inhibitor and enzyme preparation for 1 min at 30°, and initiating the reaction by adding 3HANA. Subsequently, the incubation was continued at 30°, and the production of α-amino-β-carboxymuconic acid ω-semialdehyde over the next 60 sec was measured spectrophotometrically by the increase in absorbance at 360 nm. A unit of enzyme activity was defined as the amount of 3HAO which produces 1 μmol α-amino-β-carboxymuconic acid ω-semialdehyde [10] per min under routine assay conditions.

Dialysis. Enzyme preparations containing 63 units of 3HAO activity in approximately 2 mL in the presence or absence of inhibitors were dialyzed overnight at 4° against 400 mL of 60 mM MES buffer, pH 6.5, containing 10 mM β-mercaptoethanol. Subsequently, the sample was split, and 3HAO activity was determined either immediately or after further incubation for 1 hr at 37°.

Kinetic analyses. Kinetic studies were performed with tissue homogenates by varying the substrate concentration between 0.25 and 1 μM. The concentration of 3HAO used per sample was calculated to be approximately 45 nM [11]. Routinely, buffer, $(NH_4)_2$ $Fe(SO_4)_2$ and enzyme preparation were incubated for 1 min at 30°. The reaction was then initiated with a mixture of 3HANA and inhibitor, the solution was mixed and data collection begun within 3 sec. The increase in absorbance at 360 nm was followed using a Beckman model DU-30 spectrophotometer. Recordings were taken every 0.2 sec and recorded electronically. The first 6 sec of the recorded reaction were chosen for the determination of the reaction rate. K_i values were determined from Lineweaver–Burk plots.

Protein determination. Protein was measured according to the method of Bradford [19] using bovine serum albumin as a standard.

RESULTS

Reversibility of 3HAO inhibition. Initial experiments were designed to assess the reversibility of the inhibition of 3HAO activity by the drugs. To this end, the enzyme reaction mixture without substrate was incubated in the presence or absence of enzyme inhibitors for up to 2 hr at 37°. Subsequently, the mixture was equilibrated at 30° (1 min) and the reaction started by the addition of

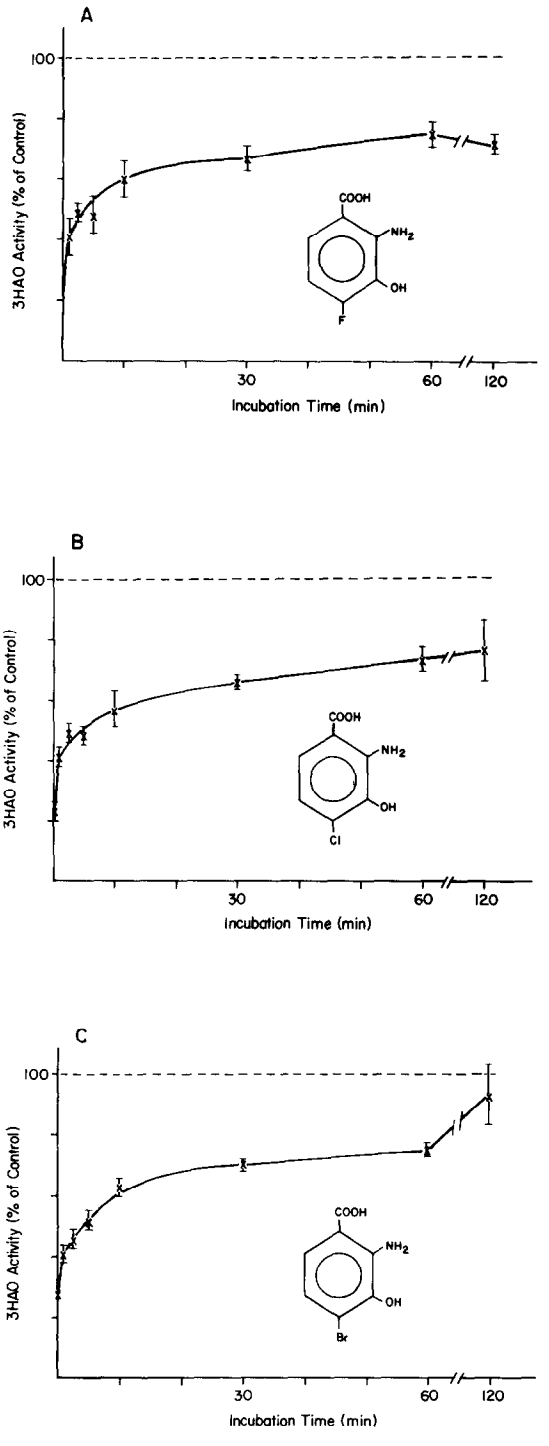

Fig. 1. Reversibility of the association between 4-halo-3-HANAs and 3HAO enzyme. Samples containing buffer, $(NH_4)_2$ $Fe(SO_4)_2$, inhibitor, and 1 unit of enzyme activity were incubated at 37° for the indicated time periods and subsequently assayed for 3HAO activity using the routine assay procedure. The concentrations of 4-halo-3HANAs were 312 nM [4-*F*-3HANA; (A)], 135 nM [4-Cl-3HANA; (B)] and 32 nM [4-Br-3HANA; (C)]. Data are means ± SEM (N = 3 per time point). 3HAO activity is expressed as a percentage of the activity obtained under otherwise identical assay conditions in the absence of inhibitor (between 0.97 and 1.47 units for the different time points).

* The half-life of conversion of α-amino-β-carboxymuconic acid ω-semialdehyde into quinolinic acid is not influenced by pH and is reported to be 43 min at 25° [17]. Therefore, the concentration of the analyte for the assay will show no significant decrease over the time interval of measurement.

† The assay conditions used (pH 6.5, non-chelating buffer) minimize the auto-oxidation of Fe^{2+} (cf. Ref. 18).

Table 1. Effect of overnight dialysis in the presence of 4-halo-3HANAs on 3HAO activity in liver homogenate

	3HAO activity (% of control)		
		Overnight at 4°	
4-halo-3HANA	Initial activity	Dialyzed	Non-dialyzed
4-F-3HANA	25 ± 3	10 ± 3 (100 ± 6)*	31 ± 11
4-Cl-3HANA	27 ± 3	40 ± 8 (108 ± 19)*	47 ± 3*
4-Br-3HANA	33 ± 2	78 ± 6† (114 ± 14)†	67 ± 6*

Samples initially contained 63 units of 3HAO activity and 1.6 μM 4-F-3HANA, 0.68 μM 4-Cl-3HANA or 0.16 μM 4-Br-3HANA, respectively. Samples (2 mL) were either incubated for 16 hr on ice or dialyzed against 400 mL buffer for the same time period. All data are expressed as a percentage of respective control values which were obtained under identical conditions in the absence of drugs (controls lost 74 and 22% of the initial activity after overnight incubation with and without dialysis, respectively). Values are the means ± SEM of four separate experiments. Data in parentheses demonstrate that full recovery of enzyme activity was obtained when aliquots of dialyzed samples were incubated for 1 hr at 37° (N = 4 per group).

* $P < 0.01$ as compared to respective controls (one-tailed Student's *t*-test).

† $P < 0.001$.

3HANA. Drug concentrations (see legend to Fig. 1) were chosen to effect approximately 75% inhibition of enzyme activity under routine incubation conditions.

For all three drugs, prolonged incubation at 37° resulted in an increase in apparent 3HAO activity with time. Reversion of the inhibitory effect was most prominent during the first 2.5 min of incubation and proceeded at a slower pace thereafter (Fig. 1). At 1 hr of incubation, 3HAO activity was inhibited by 25, 27 and 25% for 4-F-, 4-Cl- and 4-Br-3HANA, respectively. Virtually identical data, demonstrating the reversibility of enzyme inhibition, were obtained with purified 3HAO (data not shown). In separate experiments, it was found that incubation of the drugs alone (60 min, 37°) did not significantly ($P > 0.01$) affect their inhibitory potency.

Dialysis. The physical association between enzyme and inhibitors was examined by dialysis. Drug concentrations were chosen to produce 75% inhibition of control enzyme activity prior to dialysis.

As shown in Table 1, dialysis of the enzyme overnight at 4° in the presence of 4-F-3HANA did not result in an increase in apparent 3HAO activity, indicating a lack of dissociation between enzyme and inhibitor under these conditions. Enzyme activity was increased moderately, but not significantly, following dialysis with 4-Cl-3HANA. Dialysis in the presence of 4-Br-3HANA resulted in a substantial restoration of enzyme activity ($P < 0.001$) as compared to control values.

Since these results could be due either to physical removal of 4-Br-3HANA from the enzyme during dialysis or to chemical instability of the compound, separate samples were incubated in 2 mL buffer under otherwise identical conditions. These non-dialyzed samples showed the same selective decrease in enzyme inhibition with 4-Br-3HANA as described above for dialyzed preparations (Table 1). Thus, 4-Br-3HANA appears to be less stable than the other two drugs upon prolonged incubation at 4°, but no indication was obtained for a physical dissociation of the inhibitors from the enzyme under these experimental conditions.

Another series of experiments was designed to evaluate the recovery of enzyme activity during further incubation of the dialyzed samples at 37° for 1 hr. In all cases (i.e. using each of the three drugs), 3HAO activity was restored, thus confirming the reversibility of enzyme inhibition at physiological temperature (Table 1).

Kinetic analysis. After establishing the reversibility of the association of enzyme and inhibitors, the next set of experiments was designed to explore the precise mechanism of 3HAO inhibition by 4-halo-3HANAs. Since the dialysis study had suggested that the drugs were bound quite tightly to the enzyme, kinetic analyses had to be performed by following the initial rate of the reaction after starting the assay with a mixture of substrate and inhibitor. Preliminary experiments using this paradigm showed that the reaction rate remained reproducibly linear for at least 6 sec; therefore, a 6-sec reaction time was chosen for subsequent analyses.

In preliminary experiments, the concentration of 3HANA was varied between 0.03 and 1 mM. Double-reciprocal plots derived from these studies showed curvature (data not shown). The linear range of these plots, representing substrate concentrations of 0.25 to 1 mM, was chosen therefore for kinetic analyses. Lineweaver–Burk representations derived from Michaelis–Menten equations are depicted in Fig. 2. The graphs show apparent competitive inhibition of the enzyme by all three drugs. The K_i values estimated from these plots were 190, 6 and

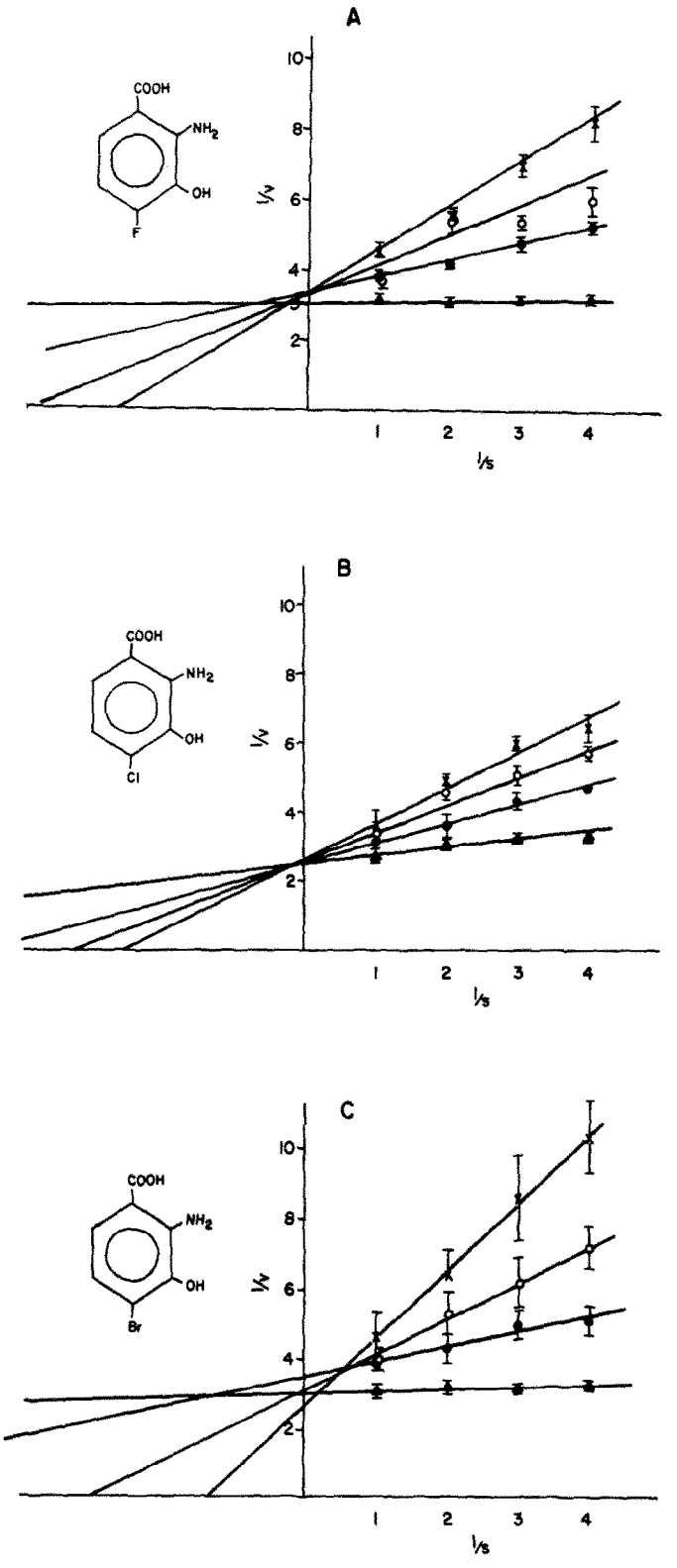

Fig. 2. Kinetic analyses of the initial reaction rate of 3HAO activity in the presence of 4-halo-3HANAs. Liver homogenate 3HAO activity (1 unit) was measured by following ΔA/min at 360 nm for the first 6 sec of the reaction initiated by addition of a mixture of 3HANA and the respective 4-halo-3HANA. The following concentrations of 4-halo-3HANAs were used: A (4-F-3HANA): no drug (▲), 10 μM (●), 15 μM (○), and 20 μM (×); B (4-Cl-3HANA): no drug (▲), 400 nM (●), 600 nM (○), and 800 nM (×); C (4-Br-3HANA): no drug (▲), 200 nM (●), 400 nM (○), and 600 nM (×). Data are means ± SEM (N = 3–6).

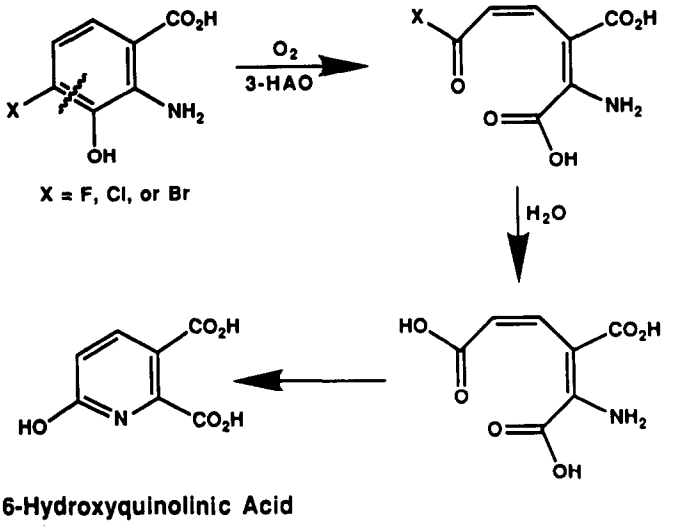

Fig. 3. Hypothetical scheme for the metabolism of 4-halo-3-HANAs by 3HAO.

4 nM for 4-F-3HANA, 4-Cl-3HANA and 4-Br-3HANA, respectively. Thus, K_i values were of the same order of magnitude as the enzyme concentration used in the experiment (cf. Methods), qualifying the inhibitors as "tight binding" drugs [20].

DISCUSSION

In the present study, analysis of 3HAO inhibition was based on the measurement of α-amino-β-carboxymuconic acid ω-semialdehyde, the immediate reaction product of the enzyme-catalyzed process. Using this assay system, both the rank order and the absolute inhibitory potency of the three 4-halo-3HANAs were comparable to those obtained previously with a radiometric assay in which the production of [^{14}C]quinolinic acid from [^{14}C]-3HANA was used as an analytical endpoint. In those studies, conducted with rat and human brain tissue, incubation proceeded for 1 hr to allow for the quantitative (non-enzymatic) formation of quinolinic acid [13].

Previous kinetic studies using either quinolinic acid production after a 30-min incubation [21] or α-amino-β-carboxymuconic acid ω-semialdehyde formation after a 1-min incubation [12] led to the proposal that 4-Cl-3HANA, the only drug tested in those experiments, acted as a non-competitive 3HAO inhibitor. However, it is difficult to distinguish between competitive and non-competitive enzyme inhibition when dealing with drugs which bind very tightly to the protein [22]. Under the experimental conditions used previously, particularly when testing substrate analogs which display very high affinity to the 3HAO protein, it is also virtually impossible to differentiate reversible from irreversible binding. For example, measurement of enzyme activity following dialysis of the enzyme–inhibitor complex at 4° leads to the conclusion that the drug–protein interaction is irreversible [12].

The experimental approaches used here to re-evaluate the mechanism of 3HAO blockade by 4-Cl-3HANA and its congeners confirmed the tight physical association between enzyme and inhibitor(s) but also demonstrated unequivocally the reversibility of their interaction. Irreversible inhibitors decrease enzyme activity with increasing association time [22]. This is clearly in contrast to the present results, which showed a loss of apparent inhibitory potency when protein and inhibitor(s) were incubated together at 37° for up to 1 hr. Notably, reversibility by brief increases in temperature was observed not only after the protein and the inhibitor(s) were mixed acutely, but also after they had been kept together on ice for an extended period of time (data not shown). Moreover, overnight incubation of the enzyme with 4-Br-3HANA at 4° resulted in a loss of enzyme inhibition. Taken together, these data suggest that the inhibitors can serve as substrates for 3HAO. Thus, the apparent preferential loss of inhibition by warming of the drugs in the presence of enzyme—but not in its absence—or in the case of 4-Br-3HANA merely by incubation with the enzyme overnight at 4°, is likely due to enzymatic metabolism of the drugs. The relatively lower concentration of 4-Br-3HANA used in these studies may explain why its metabolism was detected more readily than that of 4-Cl-3HANA and 4-F-3HANA (i.e. even after incubation on ice). Since all three inhibitors are substrate analogs, they may be metabolized in an identical manner as 3HANA itself, yielding 6-hydroxyquinolinic acid (Fig. 3). The production of this hypothetical product is currently under investigation in our laboratories.

Once the reversibility of the inhibition by the drugs was ascertained, the precise nature of drug action could be investigated. Mixing the enzyme with the inhibitor(s) prior to initiation of the reaction with the substrate would have allowed the drug to bind completely to the protein. Since the inhibitors

bind very tightly to 3HAO, Michaelis–Menten analysis would certainly result in the appearance of noncompetitive inhibition [12]. In contrast, initiation of the reaction with a mixture of substrate and inhibitor(s), combined with the assessment of the initial (6 sec) reaction rate, permitted characterization of the drug effect(s) as apparent competitive inhibition. While the present study does not differentiate unequivocally between truly competitive inhibitors and allosteric modulators of enzyme activity [22], the structural resemblance of the drugs to the substrate and their probable metabolism by the enzyme (cf. above) indicate that they act competitively at the metabolic site.

The availability of reversible 3HAO inhibitors could be of value for the development of novel treatment strategies for brain diseases which are caused by a pathological overabundance of quinolinic acid (see beginning of article). Moreover, if proven selective, the tight binding to their target enzyme may make appropriately radiolabeled 4-halo-3HANAs attractive candidates for attempts to positron label and PET scan 3HAO *in vivo*. At present, poor penetration of the compounds through the blood–brain barrier (unpublished observation) appears to constitute the major obstacle to the use of 4-halo-3HANAs both for therapeutic and diagnostic purposes and as research tools for the study of brain quinolinate function. The present data indicate, however, that structurally modified chemical congeners, which can enter the brain after systemic administration, may indeed possess the necessary pharmacological properties to become of value for experimental and clinical applications.

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REFERENCES

1. Stone TW, Burton NR and Smith DAS, Electrophysiology of quinolinic acid, kynurenic acid, and related compounds in the CNS. In: *Quinolinic Acid and the Kynurenines* (Ed. Stone TW), pp. 113–148. CRC Press, Boca Raton, FL, 1989.
2. Watkins JC and Collingridge GL (Eds.), *The NMDA Receptor*. IRL/Oxford University Press, Oxford, 1989.
3. Feldblum S, Rougier A, Loiseau H, Loiseau P, Cohadon F, Morselli PL and Lloyd KG, Quinolinic-phosphoribosyltransferase activity is decreased in epileptic human brain tissue. *Epilepsia* **29**: 523–529, 1988.
4. Schwarcz R, Okuno E, White RJ, Bird ED and Whetsell WO Jr, 3-Hydroxyanthranilate oxygenase activity is increased in the brains of Huntington disease victims. *Proc Natl Acad Sci USA* **85**: 4079–4081, 1988.
5. Kish SJ, Du F, Parks DA, Robitaille Y, Ball MJ, Schut L, Hornykiewicz O and Schwarcz R, Quinolinic acid catabolism is increased in cerebellum of patients with dominantly inherited olivopontocerebellar atrophy. *Ann Neurol* **29**: 100–104, 1991.
6. Moroni F, Lombardi G, Carlà V, Lal S, Etienne P and Nair NP, Increase in the content of quinolinic acid in cerebrospinal fluid and frontal cortex of patients with hepatic failure. *J Neurochem* **47**: 1667–1671, 1986.
7. Heyes MP, Rubinow D, Lane C and Markey SP, Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Ann Neurol* **26**: 275–277, 1989.
8. Bokman AH and Schweigert BS, 3-Hydroxyanthranilic acid metabolism. IV. Spectrophotometric evidence for the formation of an intermediate. *Arch Biochem Biophys* **33**: 270–276, 1951.
9. Long CL, Hill HN, Weinstock IM and Henderson LM, Studies of the enzymatic transformation of 3-hydroxyanthranilate to quinolinate. *J Biol Chem* **221**: 405–417, 1954.
10. Wiss O and Bettendorf G, Über die Umwandlung der 3-Hydroxyanthranilsäure in Chinolinsäure und Nicotinsäure im tierischen Organismus. II. Die Isolierung und vorläufige Charakterisierung des primären Oxydations-produktes der 3-Hydroxyanthranilsäure. *Hoppe Seyler's Z Physiol Chem* **306**:145–153, 1957.
11. Okuno E, Köhler C and Schwarcz R, Rat 3-hydroxyanthranilic acid oxygenase: Purification from the liver and immunocytochemical localization in the brain. *J Neurochem* **49**: 771–780, 1987.
12. Parli CJ, Krieter P and Schmidt B, Metabolism of 6-chlorotryptophan to 4-chloro-3-hydroxyanthranilic acid: A potent inhibitor of 3-hydroxyanthranilic acid oxidase. *Arch Biochem Biophys* **203**: 161–166, 1980.
13. Todd WP, Carpenter BK and Schwarcz R, Preparation of 4-halo-3-hydroxyanthranilates and demonstration of their inhibition of 3-hydroxyanthranilate oxygenase activity in rat and human brain tissue. *Prep Biochem* **19**: 155–165, 1989.
14. Heyes MP, Hutto B and Markey SP, 4-Chloro-3-hydroxyanthranilate inhibits brain 3-hydroxyanthranilate oxidase. *Neurochem Int* **13**: 405–408, 1988.
15. Cook JS and Pogson CI, Tryptophan and glucose metabolism in rat liver cells. The effects of DL-6-chlorotryptophan, 4-chloro-3-hydroxyanthranilate and pyrazinamide. *Biochem J* **214**: 511–516, 1983.
16. Walsh JL, Todd WP, Carpenter BK and Schwarcz R, 4-Chloro-, 4-fluoro- and 4-bromo-3-hydroxyanthranilic acids are potent competitive inhibitors of 3-hydroxyanthranilic acid oxygenase. *Soc Neurosci Abstr* **15**: 816, 1989.
17. Mehler AH, Formation of picolinic and quinolinic acids following enzymatic oxidation of 3-hydroxyanthranilic acid. *J Biol Chem* **218**: 241–254, 1956.
18. Lambeth DO, Ericson GR, Yorek MA and Ray PD, Implications for *in vitro* studies of the autoxidation of ferrous ion and the iron-catalyzed autoxidation of dithiothreitol. *Biochim Biophys Acta* **719**: 501–508, 1982.
19. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
20. Morrison JF, Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochim Biophys Acta* **185**: 269–286, 1969.
21. Ji X-D, Nishimura M and Heyes MP, Non-competitive inhibition of 3-hydroxyanthranilate-3,4-dioxygenase by 4-chloro-3-hydroxyanthranilic acid in whole brain of rat. In: *Kynurenine and Serotonin Pathways: Progress in Tryptophan Research* (Eds. Schwarcz R, Young SN and Brown RR), pp. 563–565. 1991 Plenum Press, New York, NY.
22. Dixon M and Webb EC, *Enzymes*, 3rd Edn. Academic Press, New York, 1979.