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3-Hydroxyanthranilic acid accumulation following administration of the 3-hydroxyanthranilic acid 3,4-dioxygenase inhibitor NCR-631

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Abstract

In the kynurenine pathway of tryptophan metabolism, 3-hydroxyanthranilic acid is the substrate for formation of the excitotoxin quinolinic acid by 3-hydroxyanthranilic acid 3,4-dioxygenase. This study was designed to characterize the effects on 3-hydroxyanthranilic acid after treatment with the 3-hydroxyanthranilic acid 3,4-dioxygenase inhibitor 4,6-di-bromo-3-hydroxyanthranilic acid (NCR-631) in Sprague–Dawley rats. The blood plasma and brain concentrations of 3-hydroxyanthranilic acid were found to increase rapidly in a dose-dependent manner after gavage administration of NCR-631. However, the effect was relatively transient, with a decline in 3-hydroxyanthranilic acid levels already at 1h after NCR-631 treatment. Similar increases in plasma levels of 3-hydroxyanthranilic acid were observed following either gavage or parenteral (i.v. or s.c.) administration of NCR-631 (25 mg/kg). Only a minor enhancement of the NCR-631-induced increase in plasma 3-hydroxyanthranilic acid levels was found after sub-chronic treatment (25 mg/kg by gavage; 7 days, b.i.d.), suggesting a low propensity for altered 3-hydroxyanthranilic acid 3,4-dioxygenase activity following repeated inhibition. Administration of [^{14}C]NCR-631 suggested 20 min initial plasma half life and an oral absorption around 50%. A dose of 250 mg/kg [^{14}C]NCR-631 given by gavage provided plasma levels of almost 2 μmol/ml and a brain concentration of approximately 16 nmol/g, when analyzed 15 min after administration. Neither acute nor sub-chronic administration of NCR-631 caused any substantial effects on quinolinic acid levels in plasma or brain. Also, the plasma levels of kynurenic acid, another neuroactive kynurenine pathway metabolite, were unaffected by acute NCR-631 treatment. Moreover, the brain levels of the major cerebral tryptophan metabolites 5-hydroxytryptamine and 5-hydroxyindoleacetic acid remained unchanged following administration of NCR-631. Although reversible inhibition of 3-hydroxyanthranilic acid 3,4-dioxygenase with NCR-631 in normal rats is insufficient to cause substantial changes in the levels of quinolinic acid or other important tryptophan metabolites, it causes a major accumulation of the substrate 3-hydroxyanthranilic acid.

Keywords: Kynurenine; 3-Hydroxyanthranilic acid; Inhibitor; (Rat); Plasma; Brain

1. Introduction

The opening of the tryptophan indole ring by tryptophan 2,3-dioxygenase in the liver, or by indoleamine 2,3-dioxygenase elsewhere in the body, initiates the formation of a series of compound collectively called kynurenines (Stone, 1993). One important product of this metabolic pathway is nicotinamide adenine dinucleotide (NAD^+), but several other kynurenines have also been found to possess significant biological effects. For example, the dicarboxylic acid quinolinic acid acts as a modestly potent, but specific, agonist at the *N*-methyl-D-aspartate (NMDA) receptor (Stone and Perkins, 1981). Quinolinic acid is formed from 3-hydroxyanthranilic acid through an initial step involving the Fe^{2+}-dependent enzyme 3-hydroxyanthranilic acid 3,4-dioxygenase (EC 1.13.11.6; Malherbe et al., 1994), and a second step in which the enzymatic product, α-amino-β-carboxymuconic-acid ω-semialdehyde, is non-enzymatically transformed to quinolinic acid (Bokman and Schweigert, 1951; Long et al., 1954).

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Quinolinic acid is subsequently metabolized by quinolinate phosphoribosyltransferase (see Fig. 1).

It has been shown that quinolinic acid acts as a convulsant agent (Lapin, 1978) and causes excitotoxic lesions (Foster et al., 1983; Schwarcz et al., 1983) when administered directly into the brain. Moreover, increased levels of quinolinic acid in cerebral tissue or in cerebrospinal fluid (CSF), as well as increases of normal activities of quinolinic acid's metabolic enzymes, have been found in neurological diseases in humans as well as in various animal models of neurological conditions (e.g., Moroni et al., 1986; Schwarcz et al., 1988; Heyes and Nowak, 1990; Heyes et al., 1991; Lloyd et al., 1990; Kish et al., 1991). This has led to the suggestion that quinolinic acid, under certain circumstances, may act as an endogenous neurotoxin that plays an active role in various pathophysiological processes. In order to test this hypothesis, it is useful to employ pharmacological tools which counteract the actions of quinolinic acid, e.g. by inhibiting 3-hydroxyanthranilic acid 3,4-dioxygenase activity. Indeed, it has been found that various 4-halogenated 3-hydroxyanthranilic acids are potent inhibitors of 3-hydroxyanthranilic acid 3,4-dioxygenase in vitro and in vivo (Parli et al., 1980; Heyes et al., 1988; Todd et al. 1989; Walsh et al. 1991; Saito et al., 1994; Walsh et al., 1994; Naritsin et al., 1995).

In spite of showing characteristics of irreversible inhibitors (Parli et al., 1980) due to their tight binding to 3-hydroxyanthranilic acid 3,4-dioxygenase, the 4-halogenated substrate analogues have been shown to act as competitive, reversible enzyme inhibitors (Walsh et al. 1991). 4,6-Di-bromo-3-hydroxyanthranilic acid (NCR-631) is a recently developed reversible 3-hydroxyanthranilic acid 3,4-dioxygenase inhibitor with improved chemical stability and high potency, which has been shown to be active both in vitro (Linderberg et al., 1999; Luthman et al., in preparation) and in vivo (Luthman et al., 1996) against the formation of the product quinolinic acid. In the present study, the in vivo effects of NCR-631 on the 3-hydroxyanthranilic acid 3,4-dioxygenase substrate 3-hydroxyanthranilic acid was examined in blood plasma and brain. A characterization of the pharmacokinetics of NCR-631 was also performed using [^{14}C]labeled compound. Moreover, the 3-hydroxyanthranilic acid measurements in plasma were complemented by the determination of two other important kynurenines, quinolinic acid and kynurenic acid, and by studies on the major cerebral tryptophan metabolites 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA).

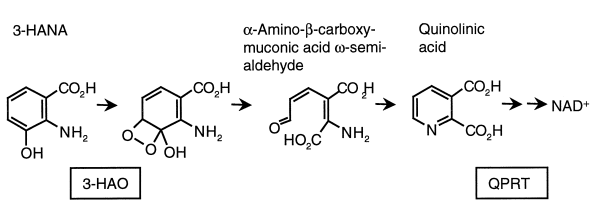

Fig. 1. In the kynurenine pathway, 3-hydroxyanthranilic acid is enzymatically converted by 3-hydroxyanthranilic acid dioxygenase to α-amino-β-carboxymuconic-acid ω-semialdehyde, which is spontaneously transformed to quinolinic acid. Quinolinic acid is subsequently metabolized by quinolinate phosphoribosyltransferase (QPRT), which further downstream leads to the formation of nicotinamide adenine dinucleotide (NAD^+).

2. Materials and methods

2.1. Test compounds

NCR-631 (Astra Arcus; Batch 205/93; MW: 310.9) was dissolved in a small volume of NaOH, further diluted with physiological saline, and the pH was adjusted to approximately 8. The drug solution was kept at +4°C, protected from light and administered within 2 h after preparation. The specific radioactivity of [^{14}C]NCR-631 (Astra Arcus; Batch no. OB 040/21) was 54 μCi/mmol. Radiochemical purity was > 95% as assayed by thin layer chromatography (Silica gel; CH_2Cl_2–EtOAc–HoAc 20:10:1).

2.2. Animals and treatment procedures

Male Sprague–Dawley rats (225–275 g) were used (B&K Universal, Sollentuna, Sweden). The rats were housed five animals per cage under controlled conditions of temperature (21°C), relative humidity (55%–65%) and light–dark cycle (12:12 h, lights on 0600 h). Food and tap water were available ad libitum in the home cage. The animals were acclimatized to the animal quarters for at least 7 days before initiation of the experiments. Systemic NCR-631 treatments were performed by gavage (5 ml/kg), subcutaneous (s.c.; 5 ml/kg) or intravenous (i.v.; 400 μl/animal; tail vein) administrations. Controls were administered with equal volumes of physiological saline. Sub-chronic administration was performed by twice daily administrations (bis in die; b.i.d.) of NCR-631 by gavage in a dose of 25 mg/kg for 7 days. A challenge dose of NCR-631 (25 mg/kg) was given by gavage following 15–16 h wash-out.

To obtain plasma samples, the rats were anesthetized by free breathing of 3.5%–5.0% enflurane (Efrane; Abbot, Campoverde, Italy) in a mix of 30% O_2 and 70% N_2O. The thorax was opened, and a cut was made in the right ventricle of the heart. Blood was collected in heparinized tubes, transferred to 1.5 ml Eppendorf tubes and centrifuged at $2600 \times g$ for 10 min. The supernatants were transferred to new Eppendorf tubes and stored at −70°C until analysis. To clear the blood vessels before brain tissue sampling, the rats were transcardially perfused through the left ventricle with heparinized saline (0.2 μl/ml of 5000 IE/ml; 37°C, followed by ice cold solution) until clear liquid was obtained through a cut in the right side ventricle. The brains were thereafter rapidly

removed, inspected that they did not show any remaining blood, and placed on an ice-chilled Petri dish. The hippocampus and striatum were dissected out and stored at −70°C. For measurement of plasma radioactivity after [^{14}C]NCR-631 administration, serial blood samples were withdrawn via the orbital venous plexus during light enflurane anesthesia.

The animal experimental protocols for the study were approved by the Swedish Committee for Ethical Experiments on Laboratory Animals (S 220/92, and S5/95; South Committee, Stockholm, Sweden).

2.3. Analysis of 3-hydroxyanthranilic acid

Measurements of 3-hydroxyanthranilic acid in plasma and brain were performed using high pressure liquid chromatography (HPLC) coupled to fluorescence detection. Blood plasma analysis was performed by adding 500 μl plasma to 370 μl of an ice-chilled perchloric acid solution, consisting of 300 μl 4 M perchloric acid, 50 μl 10% EDTA (Merck, Darmstadt, Germany) and 20 μl 15% sodium bisulfite (Fisher Scientific, NJ, USA). Following centrifugation (10,000 × *g*, 10 min), the supernatant was separated and kept frozen at −20°C until analysis. Standards were prepared by adding 500 μl of a 3-hydroxyanthranilic acid solution (150–225 pmol/ml) to the perchloric acid solution. The samples were diluted 4–40 times with distilled water immediately before analysis.

Samples from brain tissue, i.e., striatal and hippocampal tissue weighing 35–70 mg, were added to 420 μl of a cold perchloric acid solution, consisting of 350 μl 0.1 M perchloric acid, 50 μl 10% EDTA and 20 μl 15% sodium bisulfite. The samples were ultrasonicated for 10 s, centrifuged (10,000 × *g*, 10 min), and the supernatant was separated and kept at −20°C until analysis. Standards were prepared by adding of 50 μl of a 3-hydroxyanthranilic acid solution (100–150 pmol/ml) to the perchloric acid solution. All preparative work was performed at +4°C.

The analytical system used for measurement of 3-hydroxyanthranilic acid in plasma consisted of a pump (Model LC-10AD; Shimadzu) working at a speed of 0.8 ml/min, an injection valve (Model C6W; Valco Instuments, USA), and a reversed-phase stainless steel column (Supelcosil, 150 × 4.6 mm, 3 μm; Supelco, Bellefonte, PA, USA). The detection was carried out by a fluorescense detector (Model RF-551; Shimadzu, Kyoto, Japan) with a 12-μl cell and a band-width of 18 nm. The wavelengths for excitation and emission were 311 and 414 nm, respectively. An integrator (Model SP 4270; Spectra-Physics, San Jose, CA, USA) was used to monitor the signal. The mobile phase (pH 4.65) consisted of 2.0 mM hexyl sodium sulfate (Research Plus, Bayonne, NJ, USA), 1 mM EDTA, 8% Uvasol® methanol (Merck, Darmstadt, Germany), 48 mM citric acid and 73 mM sodium hydroxide. The analytical method used to analyze 3-hydroxyanthranilic acid in brain tissue was similar, except for the pH of the mobile phase (4.45), and the methanol concentration (1.5%).

In blood plasma samples, the identity of the presumed 3-hydroxyanthranilic acid signal was confirmed by analyzing eluates from the HPLC separation by mass -spectrometry. The HPLC peak for 3-hydroxyanthranilic acid was also examined by varying the excitation wavelength (296–340 nm) and emission wavelength (399–444 nm), and by comparing standard solutions of 3-hydroxyanthranilic acid with the 3-hydroxyanthranilic acid signals obtained from plasma or brain samples. Standards and test samples were affected identically by wavelength modifications. The quantitative loss of sample volume during ultrasonication was 1.8 ± 0.2%, while the coefficient of variation in the HPLC analysis was less than 4.0%. 3-Hydroxyanthranilic acid detection was linear up to 500 pmol with a detection limit of 270 fmol.

2.4. Measurement of radioactivity

Radioactivity after [^{14}C]NCR-631 administration was measured in plasma by liquid scintillation spectroscopy using a Tricarb Model 460 instrument (Pachard Instrument, Downers Grove, IL, USA) with facilities for computing quench corrected disintegrations per min. Samples of plasma were mixed with 10 ml of Biofluor (NEN, Boston, MA, USA) before counting. Pieces of brain tissue (100–150 mg) were combusted in a sample oxidizer (Pachard, Oxidizer Model 306) and carbon dioxide ($^{14}CO_2$) was trapped in 9 ml of Carbosorb (Pachard) before counting in 15 ml of Permafluor V (Pachard). Radioactivity in amounts less than twice the background (40 disintegrations per min) was considered to be below the limit of accurate determination.

2.5. Analysis of quinolinic acid, kynurenic acid and monoamines

Plasma and brain levels of quinolinic acid were determined using gas chromatography coupled to mass spectrometry, as described previously (Luthman et al., 1996).

The plasma levels of kynurenic acid were determined by HPLC with spectrophotometric detection (Turski et al., 1988).

5-HT and 5-HIAA were measured by HPLC with electrochemical detection according to Mohringe et al. (1986).

2.6. Statistics

Statistical calculations were performed by either one- or two-factor analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) post hoc comparison (SuperAnova; Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Effects of NCR-631 on 3-hydroxyanthranilic acid: dose dependency

Control levels of 3-hydroxyanthranilic acid in blood plasma were 18 ± 3 pmol/ml. A dose-dependent increase in the 3-hydroxyanthranilic acid plasma levels was found when studied at 30 min after gavage administration of NCR-631 (Fig. 2A). A slight elevation of plasma 3-hydroxyanthranilic acid was found already following a 2.5 mg/kg dose of NCR-631, while a 25-fold increase was observed after 25 mg/kg and almost a 100-fold increase after 250 mg/kg NCR-631 (ANOVA: $F(4,14) = 20.6$; $P < 0.001$, Fisher's PLSD post hoc test: $P < 0.001$). In the hippocampus (Fig. 2B), 3-hydroxyanthranilic acid levels increased from 1.5 ± 1.0 in controls to 10.8 ± 5.7 pmol/g following administration of 25 mg/kg NCR-631 by gavage. 250 mg/kg NCR-631 raised hippocampal 3-hydroxyanthranilic acid levels to 25 ± 8 pmol/g (ANOVA: $F(4,13) = 5.2$; $P < 0.01$, Fisher's PLSD post hoc test: $P < 0.01$). A similar dose-dependent increase of 3-hydroxyanthranilic acid was observed in the striatum (Fig. 2C). Thus, the highest dose of NCR-631 caused a rise from control levels of 2.7 ± 2.3 pmol/g to 20.2 ± 5.5 pmol/g (ANOVA: $F(4,15) = 8.6$; $P < 0.001$, Fisher's PLSD post hoc test: $P < 0.001$).

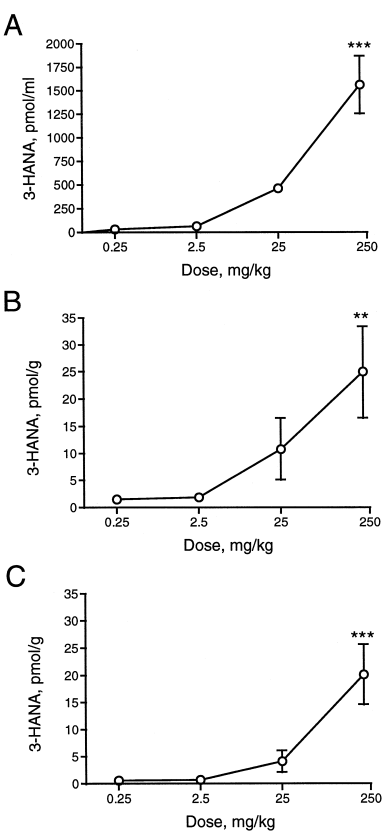

Fig. 2. 3-Hydroxyanthranilic acid concentration in rat blood plasma (A), hippocampus (B) or striatum (C) at 30 min following 0.25, 2.5, 25 or 250 mg/kg NCR-631 administered by gavage. The data are presented as means ± S.E.M ($n = 4$ per dose). Statistical analysis by ANOVA followed by Fisher's PLSD post hoc testing: ** = $P < 0.01$, *** = $P < 0.001$, as compared to controls.

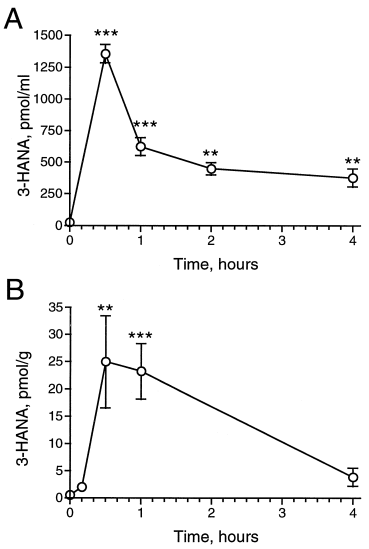

Fig. 3. 3-Hydroxyanthranilic acid concentration in rat blood plasma (A) or hippocampus (B) at different time points after administration of 250 mg/kg NCR-631 by gavage. The data are presented as means ± S.E.M. ($n = 4$–5 per time point). Statistical analysis by ANOVA followed by Fisher's PLSD post hoc testing: ** = $P < 0.01$, *** = $P < 0.001$, as compared to controls.

3.2. Effects of NCR-631 on 3-hydroxyanthranilic acid: time course

In an initial time-course study on plasma levels of 3-hydroxyanthranilic acid, rats were sacrificed at 30 min, 1, 2 and 4 h after the administration of 250 mg/kg NCR-631 by gavage (Fig. 3A). 3-Hydroxyanthranilic acid levels in plasma were enhanced at all time points studied. The largest increase was found at 30 min following NCR-631 (1360 ± 72 pmol/ml; vs. control 26 ± 5 pmol/ml).

The hippocampal levels of 3-hydroxyanthranilic acid were determined in separate animals following administration of the same dose of NCR-631 (250 mg/kg). Ten minutes after NCR-631, 3-hydroxyanthranilic acid levels increased moderately, from 0.6 ± 0.1 (controls) to 2.0 ± 0.7 pmol/g. The highest levels, 25.0 ± 8.4 pmol 3-hydroxyanthranilic acid/g, were seen at 30 min after NCR-631 administration. At 1 h, hippocampal 3-hydroxyanthranilic acid levels were 23.2 ± 5.1 pmol/g, while at 4 h the levels had fallen to 3.9 ± 1.7 pmol/g (Fig. 3B).

The time-dependent effects of NCR-631 on plasma 3-hydroxyanthranilic acid levels were also examined using a lower dose of the drug (25 mg/kg by gavage). The animals were killed at 10 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h and at 24 h following treatment (Fig. 4). Plasma 3-hydroxyanthranilic acid levels reached a maximum as early as 10 min after NCR-631 administration (ANOVA:

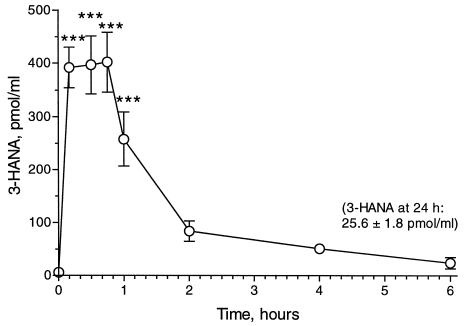

Fig. 4. 3-Hydroxyanthranilic acid concentration in rat blood plasma at different time points after administration of 25 mg/kg NCR-631 by gavage. The data are presented as means ± S.E.M. ($n = 4$ per time point). Statistical analysis by ANOVA followed by Fisher's PLSD post hoc testing: *** = $P < 0.001$, as compared to controls.

$F(8,26) = 25.5$; $P < 0.001$, Fisher's PLSD post hoc test: $P < 0.001$) and remained at peak levels at the 45-min time point. Plasma 3-hydroxyanthranilic acid levels began to decline rapidly 1 h after NCR-631, though they were still elevated as compared to control levels ($P < 0.001$). A marked decrease towards baseline levels was seen at 2 and 4 h. At 6 and 24 h after NCR-631 (24 ± 11 pmol/ml) no effect was seen as compared to vehicle.

3.3. Effects of NCR-631 on 3-hydroxyanthranilic acid: different routes of administration

The effects on plasma 3-hydroxyanthranilic acid following different routes of administration were studied using i.v. and s.c., injections of 25 mg/kg of NCR-631. In an initial experiment, the animals were sacrificed at 10 min, 30 min, 1 h, 2 h and 6 h after treatment (Fig. 5A). For comparison, NCR-631 was administered by gavage, and the 3-hydroxyanthranilic acid accumulation in plasma was determined at 30 min. The plasma levels of 3-hydroxyanthranilic acid were found to peak as early as 10 min following both routes of NCR-631 administration (s.c. administration: 429 ± 101 pmol/ml; i.v. administration: 355 ± 93 pmol/ml). There were both time-dependent (two-factor ANOVA: $F(5,27) = 24.7$; $P < 0.001$) and treatment-dependent effects ($F(1,27) = 4.2$; $P < 0.05$). When separate time points were analyzed, a significantly more rapid decline in the effects on 3-hydroxyanthranilic acid was seen at 1 h following i.v. administration of NCR-631 (Fisher's PLSD post hoc test: $P < 0.01$).

In a follow-up experiment, NCR-631 (25 mg/kg) was administered s.c., i.v., or by gavage, and the rats were killed at 10 min or at 1 h after treatment (Fig. 5B). Maximal increases in 3-hydroxyanthranilic acid levels were found at 10 min after NCR-631 administration (two-factor ANOVA: time effect $F(2,20) = 47.6$; $P < 0.001$). During the time period studied, 3-hydroxyanthranilic acid levels appeared to decline more rapidly following the i.v. treatment, but no significant differences were found between the different routes of administration.

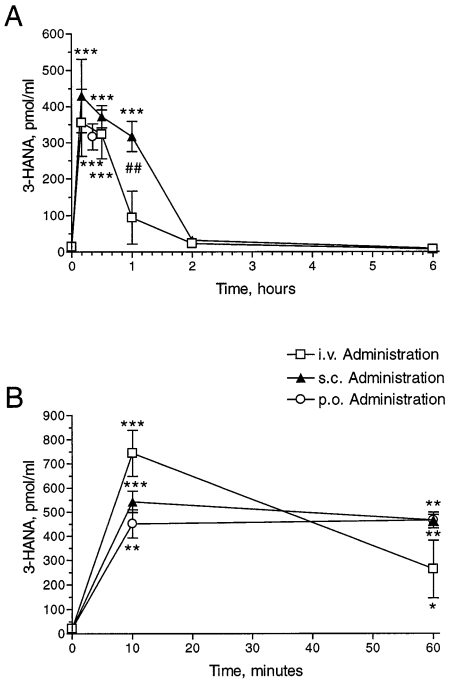

Fig. 5. 3-Hydroxyanthranilic acid concentration in rat blood plasma at different time points after NCR-631 (25 mg/kg) given by various routes of administration. (A) NCR-631 administered i.v. (open squares) or s.c. (solid triangles). For comparison, 3-hydroxyanthranilic acid was also determined at 30 min after administration of NCR-631 by gavage (open circle; slightly moved relative to the *x*-axis). (B) Comparison between administration of NCR-631 (25 mg/kg) i.v. (open squares), s.c. (solid triangles) or by gavage (open circles). The data are presented as means ± S.E.M. ($n = 3$–4 per group). Statistical analysis by two-factor ANOVA followed by Fisher's PLSD post hoc testing: ** = $P < 0.01$, *** = $P < 0.001$, time-dependent effects as compared to controls. ## = $P < 0.01$ treatment-dependent effects; i.v.-treated animals compared to animals given NCR-631 s.c.

3.4. Plasma and brain radioactivity following administration of [^{14}C]NCR-631

After the i.v. injection of [^{14}C]labeled NCR-631 (250 mg/kg), plasma radioactivity declined in a biphasic fashion (Fig. 6). The half-life of the initial phase was approxi-

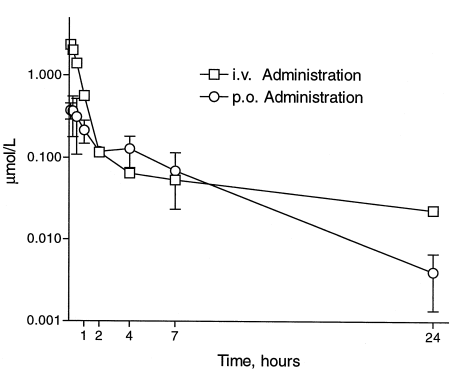

Fig. 6. Concentration of radioactivity in plasma (in µmol equivalents of NCR-631) following i.v. or gavage administration of [^{14}C]NCR-631 (250 mg/kg) in male rats. The data are presented as mean ± S.D. ($n = 3$, serial samples taken from each animal).

mately 20 min and that of the later phase 14 h. Most probably the later phase of plasma decay represented presence of metabolites rather than unchanged NCR-631. Following gavage administration, plasma concentrations showed that [^{14}C]labeled NCR-631 was fairly rapidly absorbed in the gastrointestinal tract of the rat. However, plasma levels were lower than after i.v. administration and comparison of the area under the curve after i.v. and p.o. administrations of [^{14}C]labeled NCR-631 suggested that the degree of oral absorption of the compound was approximately 50%.

Separate experiments showed that concentration–time profiles in major tissues and in plasma were similar, albeit levels of radioactivity in tissues were lower than in plasma (data not shown). At 15 min after i.v. injection of 250 mg/kg of [^{14}C]NCR-631, plasma levels of radioactivity were 1.8 ± 0.2 μmol/ml ($n = 3$). The corresponding concentrations in perfused brain tissue were 16.0 ± 0.9 nmol/g ($n = 3$). Semiquantitative measurement of the unchanged compound in plasma by thin layer chromatography, suggested that approximately 75% the radioactivity represented the unchanged compound at this time point.

3.5. Effects of NCR-631 on 3-hydroxyanthranilic acid: sub-chronic administration

In order to examine whether repeated treatment would alter the NCR-631-induced increase in plasma 3-hydroxyanthranilic acid concentrations, NCR-631 was given b.i.d. by gavage for 7 days at a dose of 25 mg/kg. On the morning of treatment day 8, the levels of 3-hydroxyanthranilic acid were determined in plasma before and at 30 min, 2 and 4 h after a challenge administration of 25 mg/kg NCR-631 (Fig. 7). The rats treated sub-chronically with NCR-631 showed the same time-course pattern of NCR-631-induced increases in plasma 3-hydroxyanthranilic acid content as the animals treated sub-chronically with vehicle (two-factor ANOVA: time effect $F(3,31) = 40.9$; $P < 0.001$). However, a significant difference was found between the rats treated sub-chronically with NCR-631 as compared to the vehicle-treated animals ($F(1,31) = 6.1$; $P < 0.05$). Thus, at 2 h after administration of the challenge dose of NCR-631, a significantly higher elevation in plasma 3-hydroxyanthranilic acid levels was found in the sub-chronically NCR-631-treated animals (Fisher's PLSD post hoc test $P < 0.05$). When studied before administration of the challenge dose, no differences in 3-hydroxyanthranilic acid plasma levels were observed between the animals treated sub-chronically with NCR-631 or vehicle, or compared to a group of treatment naïve rats (sub-chronic NCR-631 group: 12 ± 2 pmol/ml, sub-chronic vehicle group: 12 ± 2 pmol/ml, treatment naïve group: 12 ± 3 pmol/ml).

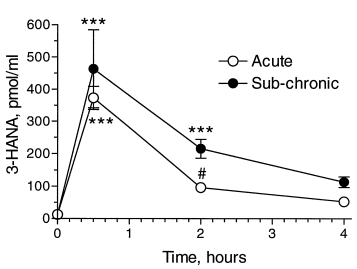

Fig. 7. 3-Hydroxyanthranilic acid concentration in rat blood plasma at 30 min, 2 h and 4 h after administration of a challenge dose of NCR-631 (25 mg/kg by gavage) to sub-chronically NCR-631-treated animals (solid circles; 25 mg/kg, p.o., 7 days b.i.d.) or to vehicle-treated animals (open circles). The data are presented as means ± S.E.M. ($n = 5$ per group). Statistical analysis by two-factor ANOVA followed by Fisher's PLSD post hoc testing: *** = $P < 0.001$; time-dependent effects as compared to pre-treatment levels. # = $P < 0.05$; effect of the sub-chronic treatment; vehicle- vs. NCR-631-treated animals.

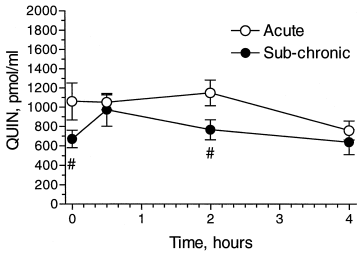

Fig. 8. Quinolinic acid concentration in rat blood plasma at 30 min, 2 h and 4 h after administration of a challenge dose of NCR-631 (25 mg/kg by gavage) to sub-chronically NCR-631-treated animals (solid circles; 25 mg/kg by gavage, 7 days b.i.d.) or to vehicle-treated animals (open circles). The data are presented as means ± S.E.M. ($n = 5$ per group). Statistical analysis by two-factor ANOVA followed by Fisher's PLSD post hoc testing: # = $P < 0.05$; effect of the sub-chronic treatment; vehicle- vs. NCR-631-treated animals.

3.6. Effects of NCR-631 on plasma and brain quinolinic acid content

No significant changes in plasma quinolinic acid levels were found at 2 h after gavage, s.c. or intraperitoneal (i.p.) administration of 250 mg/kg of NCR-631 (control group: 1060 ± 193 pmol/ml, $n = 6$; NCR-631 gavage: 1149 ± 133 pmol/ml, $n = 5$; NCR-631 s.c.: 891 ± 104 pmol/ml, $n = 5$; NCR-631 i.p. 790 ± 237 pmol/ml, $n = 3$; ANOVA $P > 0.05$). Moreover, there was no apparent effect on hippocampal quinolinic acid levels at 2 h after the s.c. administration of NCR-631 (control group: 29 ± 10 fmol/mg, $n = 5$; NCR-631: 41 ± 12 fmol/mg, $n = 5$; $P > 0.05$).

Quinolinic acid levels in plasma were also measured following a challenge administration of NCR-631 in rats treated sub-chronically with NCR-631 (25 mg/kg by gavage, 7 days b.i.d.) or vehicle (Fig. 8). Although there was no significant time-dependent effect, a significant effect of the sub-chronic NCR-631 treatment was found ($F(3,33) = 4.0$; $P = 0.05$). Subsequent analysis revealed significantly lower quinolinic acid levels in plasma in the rats treated sub-chronically with NCR-631 as compared to vehicle-

Table 1
Concentration of kynurenic acid in blood plasma at different time points after administration of NCR-631 (250 mg/kg by gavage). The data are expressed as means ± S.E.M. There are no statistically significant differences

Treatment	Kynurenic acid (pmol/ml)
Control	64.3 ± 10.8
10 min	60.9 ± 12.4
1 h	63.4 ± 9.5
4 h	70.0 ± 8.1

treated animals both before administration of the challenge dose of NCR-631 ($P < 0.05$; i.e., 15–16 h after the previous NCR-631 administration) and at 2 h after the challenge dose of NCR-631 ($P < 0.05$). Also in comparison to treatment naïve rats (1060 ± 193 pmol/ml, $n = 6$) significantly lower plasma levels of quinolinic acid were observed in the rats treated sub-chronically with NCR-631 ($P < 0.05$), when studied before administration of the challenge dose of NCR-631 (Fig. 8).

3.7. Effects of NCR-631 on other tryptophan metabolites

Acute administration of NCR-631 (250 mg/kg by gavage), failed to affect the levels of kynurenic acid in plasma at 10 min, 1 h and 4 h (Table 1). In addition, the same dose of NCR-631 had no significant effect at 30 min, 1 h or 4 h following acute administration on the hippocampal levels of 5-HT (control group: 1.50 ± 0.09 nmol/g, $n = 6$; 10 min: 1.70 ± 0.10 nmol/g, $n = 6$; 1 h: 1.66 ± 0.12 nmol/g, $n = 6$; 4 h 1.67 ± 0.08 nmol/g, $n = 6$; ANOVA $P > 0.05$) or 5-HIAA (control group: 1.39 ± 0.06 nmol/g, $n = 6$; 10 min: 1.41 ± 0.08 nmol/g, $n = 6$; 1 h: 1.57 ± 0.09 nmol/g, $n = 6$; 4 h 1.38 ± 0.06 nmol/g, $n = 6$; ANOVA $P > 0.05$).

The levels of 5-HT and 5-HIAA were also determined in the striatum after the acute administration of NCR-631 in doses up to 250 mg/kg, given by gavage. Measured at 30 min following the NCR-631 treatment, there was no effect on the tissue content of the two indoleamines, as compared to controls (5-HT control group: 3.33 ± 0.23 nmol/g; 5-HIAA control group: 1.98 ± 0.11 nmol/g; treatment data not shown; ANOVA $P > 0.05$).

4. Discussion

The present study was designed to characterize the in vivo effects of 3-hydroxyanthranilic acid 3,4-dioxygenase inhibition using the 3-hydroxyanthranilic acid analogue NCR-631 in rats. Although the effects of NCR-631 on other biologically active tryptophan metabolites was assessed as well, particular attention was paid to the fate of 3-hydroxyanthranilic acid, the substrate of 3-hydroxyanthranilic acid 3,4-dioxygenase. The analytical methods described here made it possible to measure readily the 3-hydroxyanthranilic acid content in both plasma and brain, and to follow NCR-631 treatment-related changes of 3-hydroxyanthranilic acid in both compartments.

NCR-631 caused a marked, dose-dependent elevation in 3-hydroxyanthranilic acid concentrations in both blood and brain after gavage administration, a finding that substantiates an early report of increases in 3-hydroxyanthranilic acid after 3-hydroxyanthranilic acid 3,4-dioxygenase inhibition in rats (Parli et al., 1980). Thus, it is apparent that measurement of 3-hydroxyanthranilic acid levels may constitute a useful and sensitive marker to characterize in vivo efficacy of 3-hydroxyanthranilic acid 3,4-dioxygenase inhibitors. Presumably due to a major first pass effect in the liver, which contains large quantities of 3-hydroxyanthranilic acid 3,4-dioxygenase (Okuno et al., 1987), a peak in plasma 3-hydroxyanthranilic acid accumulation was observed as early as 10 min following gavage administration of NCR-631. The maximal increase in the brain occurred slightly later, which may indicate a delayed uptake of NCR-631 into the brain. However, it is more likely that an enhanced brain entry of 3-hydroxyanthranilic acid occurred subsequent to the surge in circulating 3-hydroxyanthranilic acid since it was found that [^{14}C]NCR-631 entered the brain, albeit in low concentrations, with a similar kinetics as compared to plasma. This interpretation of the data appears most feasible, since 3-hydroxyanthranilic acid is capable of entering the brain from the periphery (Fukui et al., 1991), while 3-hydroxyanthranilic acid 3,4-dioxygenase inhibitors, including NCR-631, have been shown to interfere with cerebral enzymatic cleavage of 3-hydroxyanthranilic acid (Heyes et al., 1988; Walsh et al., 1994; Luthman et al., 1996). Furthermore, the systemic administration of NCR-631 has been found to inhibit cerebral production of quinolinic acid (Luthman et al., in preparation), indicating that NCR-631 may pass into the brain in sufficient concentrations to exert pharmacological effects on 3-hydroxyanthranilic acid 3,4-dioxygenase. It was also found that the effects on plasma 3-hydroxyanthranilic acid levels were reasonably similar after gavage, s.c. and i.v. administration of NCR-631, indicating that the inhibitor is efficiently absorbed in the gastrointestinal tract. This is also supported by the pharmacokinetic studies with [^{14}C]NCR-631, which suggested a degree of absorption of approximately 50%.

3-Hydroxyanthranilic acid 3,4-dioxygenase has been shown to have a large spare capacity, and consequently it does not seem to constitute a rate-limiting step in the kynurenine pathway (Stone, 1993; Reinhard et al., 1994). The finding that NCR-631 caused an extensive and rapid surge of 3-hydroxyanthranilic acid therefore suggests that it is as a very potent inhibitor of 3-hydroxyanthranilic acid 3,4-dioxygenase, even following systemic administration. On the other hand, the transient nature of the induced elevations of 3-hydroxyanthranilic acid in both blood and brain provide evidence for the contention that NCR-631 acts as a reversible enzyme inhibitor with a short half-life.

Indeed, in vitro studies on NCR-631 have shown that it acts as a tight-binding selective inhibitor of 3-hydroxyanthranilic acid 3,4-dioxygenase with reversible competitive characteristics (Luthman et al., in preparation). This contrasts with an early report claiming that the inhibition of 3-hydroxyanthranilic acid 3,4-dioxygenase by a structurally very similar enzyme inhibitor, 4-chloro-3-hydroxyanthranilic acid, is irreversible and non-competitive in nature (Parli et al., 1980), but agrees with the conclusions reached from an in vitro study using several 4-halogenated 3-hydroxyanthranilic acids (Walsh et al., 1991). Thus, while the inhibition kinetics of NCR-631 in vivo still remain to be explored in detail, it appears that irreversible inhibition, or long acting reversible inhibitors, will be required to achieve more sustained effects on 3-hydroxyanthranilic acid 3,4-dioxygenase. This contention is further substantiated by the present finding that sub-chronic treatment with NCR-631 did not lead to increases in basal plasma levels of 3-hydroxyanthranilic acid, and only minor effects on quinolinic acid. Moreover, only a slightly larger 3-hydroxyanthranilic acid increase was observed after the challenge dose of NCR-631, implying that the sub-chronic NCR-631 treatment did not lead to a long-lasting inhibition of the enzyme.

The merely temporary inhibition of 3-hydroxyanthranilic acid 3,4-dioxygenase probably also accounts for the restricted effect of NCR-631 on ambient levels of quinolinic acid following either acute or sub-chronic administration. As shown here and elsewhere (see Stone, 1993), plasma levels of quinolinic acid are 40–60 times higher than those of free 3-hydroxyanthranilic acid, in line with the well-established notion that the catabolic enzyme quinolinate phosphoribosyltransferase, but not 3-hydroxyanthranilic acid 3,4-dioxygenase, plays a rate-limiting role in the kynurenine pathway (cf. Fig. 1; Satyanarayana and Narasinga Rao, 1977; Stone, 1993, Reinhard et al., 1994). It therefore appears that the peripheral pool of quinolinic acid is relatively unresponsive under physiological conditions to transient fluctuations in the concentration of 3-hydroxyanthranilic acid.

Although the levels of both 3-hydroxyanthranilic acid and quinolinic acid are substantially lower in the brain than in the periphery (Baran and Schwarcz, 1990; Reinhard et al., 1994), the ratio between the two metabolites in the brain of normal animals resembles that observed in blood. Since the relative activities of 3-hydroxyanthranilic acid 3,4-dioxygenase and quinolinate phosphoribosyltransferase in the brain, too, duplicate the situation in the periphery (Schwarcz and Du, 1991), it seems reasonable to assume that relatively modest, transient changes in cerebral 3-hydroxyanthranilic acid levels are insufficient to influence brain quinolinic acid levels for the same reasons as in the blood (cf. above). On the other hand, experimentally induced massive increases in cerebral 3-hydroxyanthranilic acid concentrations (e.g. Heyes et al., 1988; Speciale et al., 1989; Walsh et al., 1994), or a prolonged stimulation of the flux through the kynurenine pathway under pathological conditions (e.g., Heyes and Nowak, 1990; Saito et al., 1994; Blight et al., 1995), effectively augment the brain content of quinolinic acid. Under those conditions, substrate analogue inhibitors of 3-hydroxyanthranilic acid 3,4-dioxygenase, such as 4-chloro-3-hydroxyanthranilic acid and NCR-631, have been found to interfere effectively with the de novo production of quinolinic acid in the brain (Heyes et al., 1988; Saito et al., 1994; Walsh et al., 1994; Blight et al., 1995; Naritsin et al., 1995; Luthman et al., 1996; Luthman et al., in preparation). This is in line with the present observation that neither plasma nor cerebral quinolinic acid levels were enhanced after NCR-631-induced surges in 3-hydroxyanthranilic acid.

No effect on the plasma levels of kynurenic acid, an important neuroprotective kynurenine pathway metabolite (Foster et al., 1984), was found following acute NCR-631 treatment. This finding is in contrast with a previous study, which reported an increase in CSF kynurenic acid following intracisternal administration of 4-chloro-3-hydroxyanthranilic acid in macaques (Naritsin et al., 1995). Hence, it cannot be ruled out that NCR-631 may, at least in certain species and following cerebral administration, influence metabolism further upstream in the kynurenine pathway. Nonetheless, the unaffected plasma levels of kynurenic acid as well as the lack of any effects on brain levels of 5-HT or 5-HIAA suggest that in rats systemic administration of NCR-631 exerts a selective action on 3-hydroxyanthranilic acid 3,4-dioxygenase, without affecting other important tryptophan metabolites.

The present findings suggest that 3-hydroxyanthranilic acid 3,4-dioxygenase inhibition with substrate analogue inhibitors, such as NCR-631, could be of therapeutic value in clinical situations which may benefit from a decreased formation of the endogenous excitotoxin quinolinic acid. This concept was first introduced a decade ago after the discovery of increased 3-hydroxyanthranilic acid 3,4-dioxygenase activity in the brain of Huntington's Disease patients (Schwarcz et al., 1988), but did not gain momentum because of the perception that such an intervention would critically interfere with normal kynurenine pathway function (i.e., NAD^+ neo-synthesis; Fig. 1). However, the risks of pharmacological 3-hydroxyanthranilic acid 3,4-dioxygenase blockade, particularly when effected by substrate analogue inhibitors, might not be as grave as previously assumed. In other words, it is conceivable that reversible inhibitors of 3-hydroxyanthranilic acid will have limited action-related side effects in healthy subjects, but will be effective in situations with pathological increases in quinolinic acid production (Moroni et al., 1986; Schwarcz et al., 1988; Heyes and Nowak, 1990; Heyes et al., 1991; Lloyd et al., 1990).

Taken together, it seems timely to expedite the evaluation of the therapeutic potential of this compound class in appropriate experimental models of conditions that may be related to alterations in the kynurenine pathway activity.

Studies in models of neuroinflammation mediated neurotoxicity (Luthman et al., 1998), human immunodeficiency virus (HIV)-1 infection (Kerr et al., 1997) and spinal cord injury (Blight et al., 1995) have indeed yielded promising results concerning the potential of substrate analogue inhibitors of 3-hydroxyanthranilic acid 3,4-dioxygenase as neuroprotective agents.

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