

Fatty Acid Derivatives of Clozapine: Prolonged Antidopaminergic Activity of Docosahexaenoylclozapine in the Rat

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Stable amides of clozapine derived from fatty acids prominent in cerebral tissue might enhance the central activity of clozapine and reduce its exposure to peripheral tissues. Such derivatives might enhance the safety of this unique drug, which is the only agent with securely established superior antipsychotic effectiveness, but with a risk of potentially lethal systemic toxicity. Amide derivatives of clozapine were prepared from structurally varied fatty acid chlorides and evaluated for ability to inhibit behavioral arousal in rat induced by dopamine agonist apomorphine and to induce catalepsy. Their duration-of-action and potency were compared to free clozapine, and concentrations of clozapine were assayed in brain and blood. Selected agents were also evaluated for affinity at dopamine receptors and other potential drug-target sites. Clozapine-N-amides of linoleic, myristic, oleic, and palmitic acids had moderate initial central depressant activity but by 6 h, failed to inhibit arousal induced by apomorphine. However, the docosahexaenoic acid (DHA) derivative was orally bioavailable, 10-times more potent (ED_{50} 5.0 $\mu\text{mol/kg}$) than

clozapine itself, and very long-acting (≥ 24 h) against apomorphine, and did not induce catalepsy. DHA itself was inactive behaviorally. Clozapine showed expected dopamine receptor affinities, but DHA-clozapine was inactive at these and other potential target sites. After systemic administration of DHA-clozapine, serum levels of free clozapine were very low, and brain concentrations somewhat lower than after administering clozapine. DHA-clozapine is a long-acting central depressant with powerful and prolonged antidopaminergic activity after oral administration or injection without inducing catalepsy, and it markedly reduced peripheral exposure to free clozapine. It lacked the receptor-affinities shown by clozapine, suggesting that DHA-clozapine may be a precursor of free, pharmacologically active clozapine. Such agents may represent potential antipsychotic drugs with improved central/peripheral distribution, and possibly enhanced safety. [Neuropsychopharmacology 24:55–65, 2001]
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Clozapine is a virtually unique antipsychotic agent in having shown superior effectiveness in a series of randomized comparisons with standard neuroleptics, including for treatment of otherwise treatment-resistant psychotic patients (Kane et al. 1988; Baldessarini and Frankenburg 1991; Lieberman et al. 1994; Bondolfi et al. 1998; Wahlbeck et al. 1999). Clozapine is also one of the

rare psychiatric treatments found to limit suicidal risk (Meltzer 1998; Baldessarini and Jamison 1999). However, its clinical utility is limited by adverse effects that include hypotension and later weight-gain and new-onset insulin-resistant diabetes II, the pathophysiology of which remains incompletely defined, as well as dose-dependent risk of centrally mediated adverse effects, including excessive sedation and epileptic seizures (Baldessarini and Frankenburg 1991; Hägg et al. 1998). Moreover, clozapine is associated with severe bone marrow toxicity, including agranulocytosis that may not be dose-dependent and is hard to predict except by close monitoring of circulating leukocyte counts (Gerlach 1991; Alvir et al. 1993).

Given the unusual benefits and substantial risks of clozapine, we considered that it may be feasible to increase the ratio of utility to risk of adverse effects of this important drug by modifying its access to brain target sites. As a strategy aimed at testing this hypothesis, we followed our previous findings with ester or amide derivatives of fatty acids important in the composition of brain tissue, including some that are effectively transported into the central nervous system (CNS). Such derivatives can markedly enhance entry of some compounds into the brain, including hydrophilic agents such as GABA and dopamine (Jacob et al. 1985; Shashoua and Hesse 1996). Accordingly, we considered preparing derivatives of clozapine and fatty acids that are abundant in brain tissue but must be supplied exogenously and transported through the blood-brain barrier. We hypothesized that such compounds might increase and prolong the uptake of clozapine into the brain, with an enhanced ratio of brain/blood concentrations of clozapine over time, or be neuropsychopharmacologically active in their own right. Such derivatives might yield more clinically favorable risk/benefit relationships (therapeutic index) than are provided by free clozapine itself.

Docosahexaenoic acid (DHA) is a particularly interesting candidate for attachment to clozapine because it is vigorously transported into the CNS and accounts for a high proportion of cerebral fatty acid content (Söderberg et al. 1991; Martin et al. 1994). Moreover, in earlier work, DHA proved particularly effective in facilitating entry of hydrophilic compounds into the brain (Shashoua and Hesse 1996).

DHA is a 22-carbon chain, omega-3, unsaturated, fatty acid containing six *cis*-double bonds. It is present in neuronal tissue, nerve terminals and synapses, predominantly within membrane phospholipid constituents phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (Martin and Bazan 1992; Martin et al. 1994). Of the total fatty acid content in brain regions, DHA constitutes approximately 22% in frontal cerebral cortical gray matter, 16% in hippocampus, and in lower proportions in white matter and in some sub-

cortical regions (Söderberg et al. 1991). Brain is virtually entirely dependent on intake of exogenous, and mainly dietary, DHA (Innis 1991). It and other fatty acids are transported into the CNS by incompletely defined mechanisms that may include receptor-mediation (Bazan et al. 1985; Scott and Bazan 1989; Bazan 1990; Martin et al. 1994).

Based on the preceding considerations, we prepared amide derivatives of clozapine from the acid chlorides of a series of fatty acids of varying chain-length and saturation, and tested them for potency and duration of action in inhibiting behavioral arousal in the rat induced by the centrally-acting direct dopamine receptor agonist R(-)-apomorphine, as well as for simultaneous presence of free clozapine in serum and brain tissue.

METHODS

Materials

Fatty acids used included DHA (obtained by saponification of DHA ethyl ester from Shiseido Corporation, Tokyo, Japan) as well as linoleic, myristic, oleic, and palmitic acids (from Aldrich Chemicals, Milwaukee, WI). The corresponding acid chlorides were prepared at Protarga, Inc. by reacting the fatty acids in methylene chloride (CH_2Cl_2) with oxalyl chloride ($[\text{COCl}]_2$, with DHA) or thionyl chloride (SOCl_2 , with all others) at room temperature. The resulting fatty acid chlorides were then reacted with clozapine (generously provided by Novartis Laboratories, Basel, Switzerland) in the presence of pyridine in dry toluene under argon for 60 min at 120°C.

Resulting crude reaction mixtures were purified by flash chromatography on silica gel (Merck grade 9385, 230–400 mesh, 6.0 nm) with ethyl acetate:methanol (90:10, vol) as the mobile phase. The desired clozapine-5-N-amide products were obtained as viscous oils in yields of 85–95%. Each novel fatty acid amide derivative of clozapine was characterized by ^1H NMR (300 MHz, CDCl_3) and ^{13}C NMR (75 MHz, CDCl_3) spectroscopy. All other chemical and drug materials used were obtained from Sigma Corporation (St. Louis, MO) or its Research Biochemicals International (RBI) division (Natick, MA).

Animal Subjects

All procedures involving laboratory animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of McLean Hospital, and followed pertinent federal and local regulatory guidelines. Young adult, male albino SD rats (200–300 g body wt) were obtained from Charles River Laboratories (Wilmington, MA). Before experimentation, animals were allowed one week to adapt to the laboratory environment, housed under controlled lighting (lights on:

07:00–19:00 h), temperature, and humidity, with free access to tap-water and standard Purina laboratory rat chow. They were tested in groups of 6/condition following injections at a standard test vol of 1 ml/kg, intraperitoneally (i.p.) or by orogastric intubation (p.o.).

Behavioral Procedures

Rats were tested singly in their home cages between 10:00 and 16:00 h to minimize effects of circadian variations in behavioral responses. Locomotion was monitored as an index of behavioral arousal, either as spontaneous behavior or as stimulated by the direct central dopamine mixed D_2 and D_1 agonist $R(-)$ -apomorphine-HCl (RBI). Locomotion was recorded in a 12-channel electronic activity monitor (Stoelting Co., Wood Dale, IL) controlled by a microcomputer, as detailed previously (Campbell et al. 1989, 1990, 1993, 1997). For experiments involving repeated testing after administration of a novel test compound, subjects included randomly constituted samples of rats tested only once or up to three times with a minimum of 24 h between testing sessions. Locomotor activity data were accumulated and recorded every 5 min over 60 min, using MacLab System software (ADInstruments, Castle Hill, NSW, Australia) for the Macintosh microcomputer.

Additional behavioral assessments scored for catalepsy, using methods detailed previously (Campbell et al. 1988). Rats were placed on 45° inclined grids every 10 min for an hour after injections of test agents, and scored for time remaining immobile up to 60 sec/observation, for a maximum score of 360 sec.

All data were then transferred for analysis with Statview-5 biostatistical programs (SAS Research Inc., Cary, NC). Standard analysis-of-variance (ANOVA) methods were used to test for overall effects, followed by Scheffé post-hoc tests of planned comparisons. Differences yielding 2-tailed $p < .05$ were considered statistically significant. Data are presented as means \pm SEM unless stated otherwise.

Solvent Vehicle

Owing to the need to dissolve the large, highly lipophilic novel compounds for testing, preliminary assessments compared their solubility in various vehicles, and tested for effects of the vehicles on behavioral responses in the rat. The solvent system eventually adopted contained α -tocopherol (70 mg) and ascorbic acid (50 mg) as antioxidants, with dilaurylthiopropionic acid (5 mg) in each 100 ml of propyleneglycol. This vehicle mixture was compared with dimethylsulfoxide (DMSO, 100% and 50% in water), and with ethanol (50% and 30% in water). The solvent vehicles all dissolved the test agents, but some limited locomotor behavioral arousal produced by apomorphine.

Compared to saline control injections of physiological saline before apomorphine, pure DMSO reduced behavioral arousal by 51%; diluted DMSO inhibited by 17%, and ethanol reduced motor activity by 25–30% at both concentrations. However, the propyleneglycol mixture did not interfere significantly with either spontaneous motor activity or with arousal induced by apomorphine, and so was adopted as the standard vehicle for subsequent experiments.

Radioaffinity Assays

DHA-clozapine, as well as free DHA and clozapine were evaluated for their interactions with several types of dopamine receptors: D_1 (assayed in competition with $C = 300$ pM [3 H]SCH-23390 with cell membrane-containing homogenates of rat corpus striatum for 30 min at 30°C), D_2 (with 45 pM [3 H]nemonapride and the same tissue for 90 min at 30°C), human D_3 (with 100 pM [3 H]nemonapride and recombinant material in cell membranes from cultured genetically transfected mouse fibroblasts at 30°C for 60 min), and human $D_{4.2}$ (with 400 pM [3 H]nemonapride with transfected CHO-K1 cells at 20°C for 180 min). These receptor assays involved triplicate determinations with at least six concentrations of test agents, following initial screening at 10 μ M. Concentration-inhibition functions were computer-fit to determine values of $IC_{50} \pm SE$, converted to K_i from the relationship $K_i = IC_{50}/[1 + C/K_d]$, where C is the radioligand test concentration, and K_d is the separately determined affinity of the radioligand. These methods have been reported in detail previously (Kula et al. 1997, 1998).

In addition, DHA-clozapine was evaluated by a commercial screening laboratory (Panlabs/Pharma Services, Ltd., Taipei, Republic of China) for potency in the following radioligand competition assays: α_1 -adrenoceptors (vs. 250 pM [3 H]prazosin with rat brain homogenates for 30 min at 25°C), α_2 -adrenoceptors (700 pM [3 H]rauwolscine with rat cerebral cortex for 30 min at 25°C), β -adrenoceptors (250 pM [3 H]dihydroalprenolol with rat brain for 20 min at 25°C), norepinephrine transporter (200 pM [125 I]RTI-55 with human recombinant material expressed in MDCK cells, for 180 min at 4°C), dopamine transporter (150 pM [125 I]RTI-55 with human transporter expressed in transfected CHO cells for 180 min at 4°C), serotonin transporter (150 nM [125 I]RTI-55 with human transporter in HEK-293 cells for 180 min at 4°C), the GABA_A agonist site (1.0 nM [3 H]muscimol with rat brain for 10 min at 4°C), GABA_B receptor (10.0 nM [3 H]GABA with rat cerebellum for 20 min at 25°C), glutamate receptors (1.6 nM [3 H]L-glutamic acid with rat brain for 10 min at 37°C), serotonin 5-HT₁ receptors (2.0 nM [3 H]5-HT with rat cerebral cortex for 10 min at 37°C), and 5-HT₂ receptors (500 pM [3 H]ketanserin with rat brain for 40 min at 25°C).

Tissue Assays of Clozapine

Aqueous homogenates of brain were extracted into organic solvent, and assayed for clozapine and its major metabolites (norclozapine, and clozapine-N-oxide) as reported and extensively characterized previously (Baldessarini et al. 1993; Volpicelli et al. 1993; Centorino et al. 1994). Tissue extracts were assayed for clozapine by highly selective, reversed-phase liquid chromatographic separation, and sensitive detection at either 254 nm or by photodiode-array multi-wavelength (210–340 nm) ultraviolet spectrophotometry, with spectra of peaks in samples compared by microcomputer to those of authentic standards.

For assays of rat brain tissue, ca. 30 mg of striatum was suspended in 1.0 ml of 0.5 M citric acid, sonicated on ice for 5 min, and then centrifuged at 10,000g for 15 min, and held at -70°C until assayed (within two weeks) as with serum samples, adding 300 μl of supernatant to 100 μl of 5 M aqueous NaOH. Serum (600 μl) samples were extracted into ethyl acetate and back into 0.1 N HCl to provide 50 μl aliquots for assay, at recovery averaging 76%. DHA-clozapine was not recovered through this extraction and separation scheme, but recovery of free clozapine (monitored with triprolidine [Sigma] as an internal standard) was 94% at 200 ng/ml. Inter-assay coefficients of variation for independently replicated assays of serum and brain samples averaged 2.0% and 3.2%, respectively. Limits of detection of authentic clozapine were 3.5 ng per ml (or g) serum or 5.5 ng/g brain tissue, and about 550 pg per injected sample.

RESULTS

Preliminary Assessment of Novel Agents

The novel fatty-acid amide derivatives of clozapine (Table 1) were evaluated initially for acute effects on spon-

taneous locomotor activity at 10–70 min after i.p. injection, as well as for ability to inhibit locomotor behavioral arousal induced by apomorphine (1 mg/kg, i.p.) injected at 6 h after the test agents. All fatty acid derivatives showed significant early reduction of spontaneous locomotor activity at 1 h, ranging from 48% reductions for linoleoylclozapine and oleoylclozapine, 52–53% reductions with the myristoyl and palmitoyl derivatives, 70% reduction by free clozapine, and an 85% effect with DHA-clozapine (data not shown). However, by 6 h after injection, only DHA-clozapine showed significant, dose-dependent (1–10 mg/kg, i.p.) reductions of both spontaneous and apomorphine-stimulated locomotor activity.

Based on these observations, several doses of DHA itself were given in control experiments, but the free fatty acid (1–30 mg/kg, i.p.) had no significant behavioral depressant effect on spontaneous locomotion or against arousal induced by apomorphine at 1–2 hours (not shown), nor against apomorphine at 6–7 hours (Table 2). All of the novel test agents were also compared with treatment with free clozapine. Representative results with high doses of all compounds tested are shown (Table 2). Based on these preliminary results, the DHA-clozapine derivative was selected for further testing.

Affinities at Dopamine Receptors and Other Potential Drug Target Sites

Comparisons of clozapine with DHA-clozapine and free clozapine in assays of all four major types of dopamine receptors indicated that clozapine had moderate affinity ($K_i = 132\text{--}158\text{ nM}$) at receptors D_1 , D_2 , and D_3 , and somewhat higher affinity at D_4 receptors in transfected cell lines ($K_i = 43.6\text{ nM}$), consistent with expectations. However, DHA-clozapine and DHA itself showed no detectable potency in any dopamine recep-

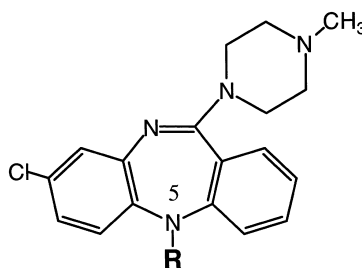


Table 1. Fatty-Acid Amide Derivatives of Clozapine

Compound	Substituent (R)	MW
Clozapine	–H	326.8
N-Myristoylclozapine	–CO(CH ₂) ₁₂ CH ₃	537.2
N-Palmitoylclozapine	–CO(CH ₂) ₁₄ CH ₃	565.2
N-Linoleoylclozapine	–CO(CH ₂) ₇ CH=CHCH ₂ CH=CH(CH ₂) ₄ CH ₃	589.3
N-Oleoylclozapine	–CO(CH ₂) ₇ CH=CH(CH ₂) ₇ CH ₃	591.3
N-Docosahexaenoylclozapine ^a	–COCH ₂ (CH ₂ CH=CH) ₆ CH ₂ CH ₃	637.3

^aDHA-clozapine. Compounds in ascending order of molecular weight (MW).

Table 2. Effects of Clozapine and Fatty-Acid Derivatives Against Apomorphine at Six Hours After Administration

Treatment	Locomotor Activity \pm SEM	% of Apomorphine Response
Vehicle control	878 \pm 130	10.6 \pm 1.6
Apomorphine alone	8,294 \pm 698	100.0 \pm 8.4
Oleoylclozapine	7,545 \pm 490	90.0 \pm 5.9
Linolenoylclozapine	7,416 \pm 598	89.4 \pm 7.2
Myristoylclozapine	7,121 \pm 648	85.8 \pm 7.8
DHA alone	6,793 \pm 781	81.9 \pm 8.4
Palmitoylclozapine	5,940 \pm 1130	71.6 \pm 13.6
Clozapine	5,855 \pm 412	70.6 \pm 5.0 ^a
DHA-clozapine	445 \pm 32.3	5.37 \pm 0.39 ^a

Results for clozapine, its derivatives, and DHA at 10 mg/kg, i.p. (other doses, 1, 3, 5 mg/kg, not shown).

^aTreatment with DHA-clozapine and, weakly, clozapine were significantly and dose-dependently active in reducing acute apomorphine (1 mg/kg, i.p.) induced locomotor activity at 6–7 h after injection of test drugs, based on 1-way ANOVA for dose ($F[4; 25 \text{ df}] \geq 5.34$, for overall $p \leq 0.03$), and post-hoc Scheffé tests ($p < .05$ for dose shown). Agents ranked in ascending order of apparent efficacy as apomorphine antagonists.

tor assay, except for a weak interaction of DHA-clozapine at D_1 sites ($K_i = 800 \text{ nM}$) in rat forebrain (Table 3). Moreover, broader screening of DHA-clozapine for activity in radioligand competition assays for other potential drug target sites also failed to show substantial potency (all $K_i = 10,000 \text{ nM}$) at any site considered, including adrenergic, serotonin, GABA, and glutamate receptors, and monoamine transporters, indicating that DHA-clozapine, in contrast to free clozapine, showed little or no evidence of direct neuropharmacological activity.

Comparison of Duration and Potency of DHA-Clozapine and Clozapine

Representative doses of clozapine and DHA-clozapine were further tested for ability to inhibit behavioral arousal induced by a standard dose of apomorphine (1 mg/kg, i.p.) given just prior to recording locomotor activity, at various times post-injection of the test agents

Table 3. Dopamine Receptor Affinities of Docosahexaenoic Acid, DHA-Clozapine and Clozapine

Receptor	DHA	K_i (nM) by Agents DHA-Clozapine	Clozapine
D_1	>10,000	800 \pm 90	132 \pm 10
D_2	>10,000	>10,000	154 \pm 55
D_3	>10,000	>10,000	158 \pm 35
D_4	>10,000	>10,000	43.6 \pm 8.5

Data derived from radioreceptor affinity assays described in Methods, shown as K_i (nM) \pm SE values determined by computed data-fits. D_1 and D_2 assays vs. [^3H]SCH-23390 or [^3H]nemonapride with rat striatal membrane-containing homogenates; other assays used [^3H]nemonapride with cell membranes from transfected mouse fibroblasts (D_3) or CHO-K1 cells (D_4) expressing human receptor genes.

(Figure 1). Both agents produced dose-dependent inhibition of apomorphine-induced arousal. However, at doses of 1 mg/kg and 3 mg/kg, the DHA derivative was more potent initially, even though its molar doses were 51% of those of free clozapine. Moreover, the behaviorally inhibitory effects of DHA-clozapine were much more prolonged than those of free clozapine. For example after 10 mg/kg doses, the effects of clozapine were largely dissipating by 4–8 h post-injection, whereas DHA-clozapine continued to antagonize acute challenges with apomorphine for at least 24 h, when arousal was still decreased by 36% by the fatty-acid derivative (Figure 1).

Similarly prolonged anti-apomorphine effects of DHA-clozapine (10 mg/kg or 15.7 $\mu\text{mol/kg}$) were found after oral (p.o.) administration as well as systemic injections (i.p.)—again, much longer than with a twice-larger molar dose of clozapine (10 mg/kg or 30.6 $\mu\text{mol/kg}$). The behavioral effects of DHA-clozapine following oral administration remained within 20–30% of those found after injection, indicating substantial oral bioavailability for this compound (Figure 2).

Doses of DHA-clozapine and clozapine were compared by behavioral testing for 1 h after administering apomorphine (1 mg/kg, i.p.) at 3 h after the test agents were injected at doses of 1–30 mg/kg, i.p. (Figure 3). DHA-clozapine inhibited apomorphine-induced behavioral arousal by 50% at a dose of about 3.2 mg/kg, i.p., whereas free clozapine required a dose of about 17 mg/kg. The computed molar-equivalent, half-maximally effective doses ($\text{ED}_{50} \pm \text{SE}$) for DHA-clozapine vs. clozapine were 5.0 \pm 0.7 vs. 52.0 \pm 11.6 $\mu\text{mol/kg}$, indicating that the DHA-derivative was over 10-times more potent on a molar-equivalent basis (Figure 3).

Catalepsy Ratings

Rats (4/group) were tested for catalepsy for 60 min after injections of the following test agents in doses of (10 mg/kg, i.p.): clozapine, DHA-clozapine, DHA, as well as the standard test vehicle (not shown). The control vehicle injections produced a mean \pm SEM score of 2.00 \pm 0.41 units. Clozapine produced a somewhat higher mean score of 7.50 \pm 2.25 ($p = .05$), than an approximately equimolar dose of DHA-clozapine (20 mg/kg) at 6.00 \pm 1.08, and DHA (10 mg/kg, at a score of 2.50 \pm 0.29) was indistinguishable from vehicle controls.

When DHA-clozapine was given at the same dose as that of clozapine, its cataleptic effect was even weaker, at 2.75 \pm 0.48 units, and not significantly different from the vehicle controls. By comparison, we found in separate experiments that haloperidol (RBI) produced a mean score of 180 \pm 40 units at a computed dose of 0.25 mg/kg, indicating that clozapine and DHA-clozapine had very little cataleptic activity.

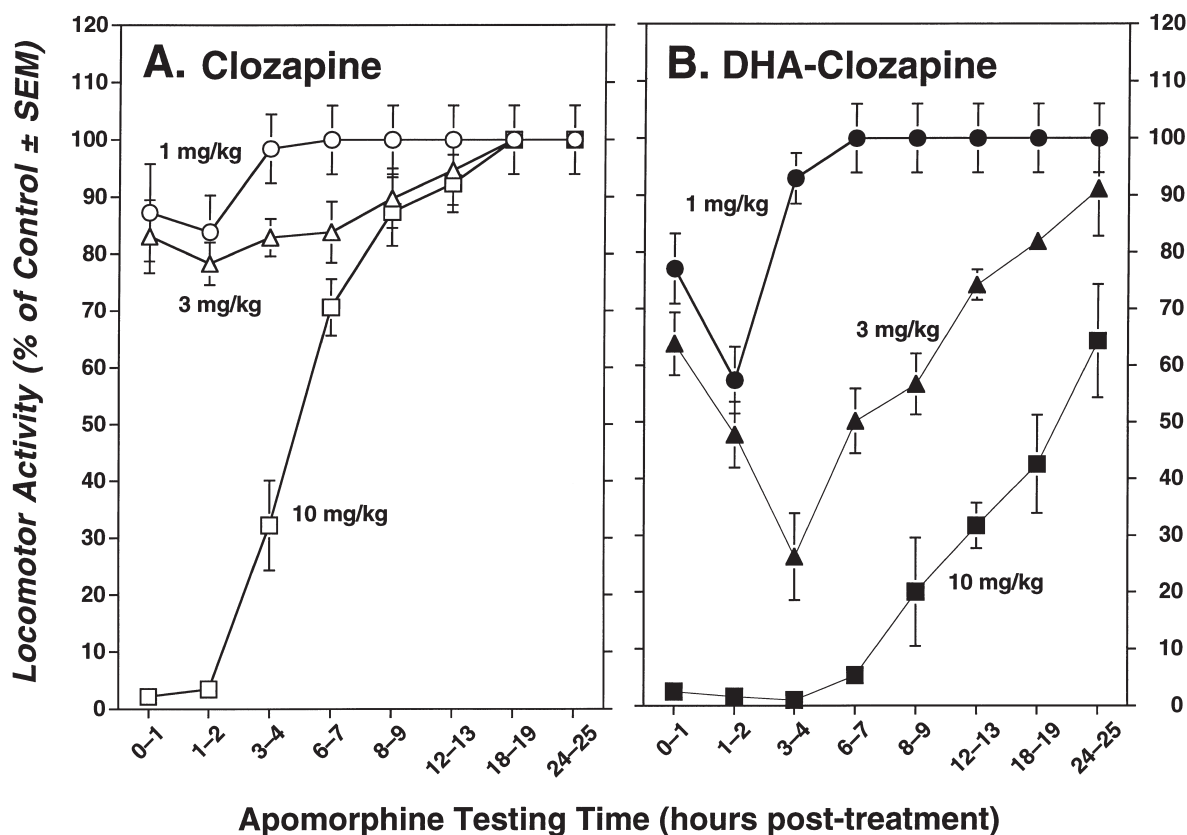


Figure 1. Mean \pm SEM percentage of locomotor activity for $n = 6$ fresh rats per group, induced by challenge doses of apomorphine (1 mg/kg, i.p.) given at indicated times following treatment with a range of doses of clozapine or DHA-clozapine (1–10 mg/kg, i.p.). Control values (100%) are for apomorphine following vehicle injections at the stated times.

Tissue Concentrations of Clozapine

Assays of serum and brain concentrations of clozapine (found in brain and serum) and its metabolites (nor-clozapine and clozapine-N-oxide, found only in serum) were done at 45 min or 8 h after a large dose of clozapine (20 mg/kg, i.p.) or an equimolar dose of DHA-clozapine (61.2 μ mol/kg). Tissue analyte concentrations obtained at 45 min following injection of clozapine were very similar to previously reported findings with rat tissue (Baldessarini et al. 1993). In contrast, DHA-clozapine yielded very low levels of clozapine or its metabolites in serum at 45 min after injection, and undetectable levels at 8 h (Figure 4). In the brain, levels of free clozapine were much lower after treatment with DHA-clozapine than after clozapine (1075 vs. 550 ng/g) at 45 min, and were lower but still detectable (ca. 100 ng/g) at 8 h after giving both agents (Figure 4). Giving DHA-clozapine not only greatly limited serum levels of free clozapine at both sampling times, but also yielded relatively lower brain levels than were found following injections of clozapine. The ratio of brain:serum concentrations of clozapine was much greater following DHA-clozapine, or approximately two at 45 min following a

large dose of clozapine (ca. 1,200/600 ng/g), but >20 following an equimolar dose of DHA-clozapine (ca. 570 vs. 25 ng/g). These findings are consistent with the view that DHA-clozapine favored delivery of clozapine away from blood to brain in comparison to treating with free clozapine itself.

DISCUSSION

The present findings indicate that fatty acid amide derivatives of clozapine can be produced and that at least one of these, DHA-clozapine, shows several pharmacologically interesting characteristics. These include central depressant and anti-apomorphine activity in the rat following oral administration as well as systemic injection, resembling the actions of free clozapine itself, while lacking evidence of direct neuropharmacological activity at dopamine receptors or other potential drug target sites, in contrast to free clozapine. In addition, DHA-clozapine proved to be more than 10-times more potent than clozapine (Figure 3), and much longer-acting, with substantial anti-apomorphine effects found at

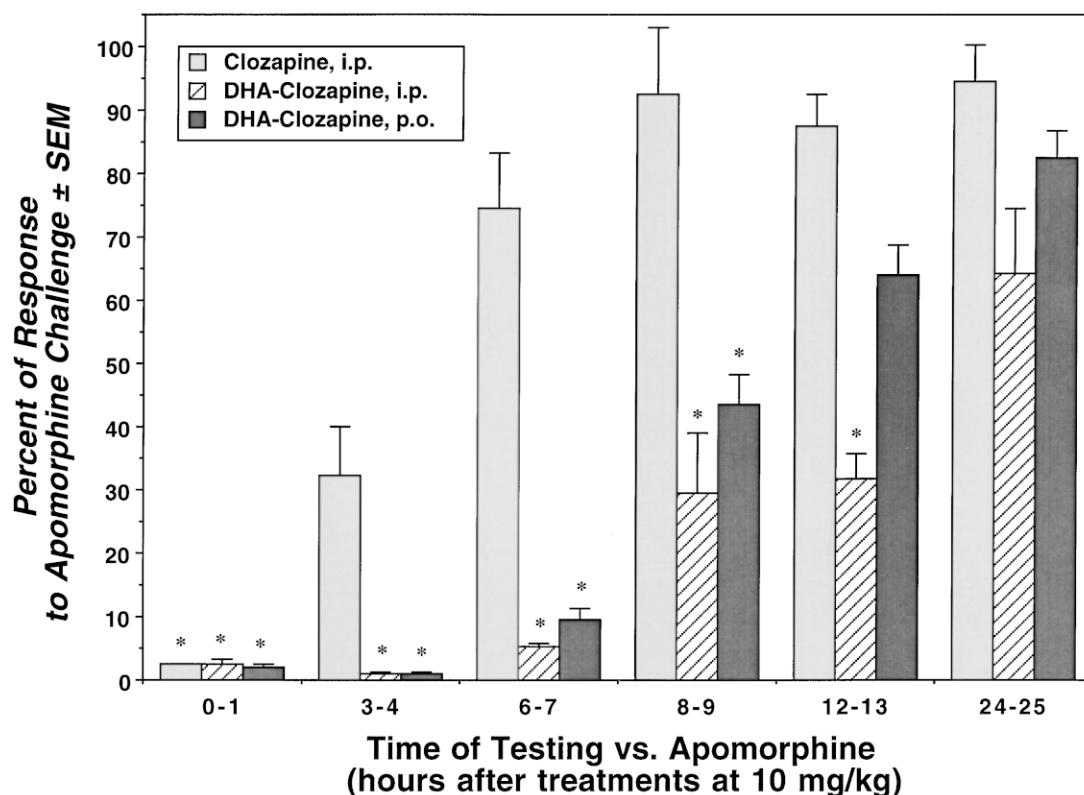


Figure 2. Mean \pm SEM percentage of locomotor activity for $n = 6$ fresh rats per group, induced by challenge doses of apomorphine (1 mg/kg, i.p.) given at indicated times following treatment with a dose of clozapine (10 mg/kg, i.p. [open bars]), DHA-clozapine (10 mg/kg, i.p. [striped bars], or p.o. [dark bars]). Control values (100%) are for apomorphine following vehicle injections at the stated times. * indicates statistically significant differences from controls ($p < .05$) based on 2-way ANOVA (for treatment and time), with post-hoc testing for planned comparisons.

24 h after administration of 10 mg/kg, either intraperitoneally or orally (Figure 2). Moreover, DHA-clozapine produced even weaker cataleptic effects than clozapine, which has very limited extrapyramidal activity, suggesting that it, too, may prove to be an atypical antipsychotic agent with improved pharmacological properties.

In addition, administration of a large dose of DHA-clozapine led to very low circulating concentrations of clozapine and its principal metabolites at 45 min, and undetectable levels at 8 h. In contrast, injections of clozapine itself produced high serum concentrations of clozapine analytes at 45 min, and detectable serum levels were found 8 h later (Figure 4). Some clozapine was detected in brain at both 45 min after an injection of DHA-clozapine, but these concentrations were lower than were produced by clozapine itself. At 8 h, similar low concentrations of free clozapine were detected in brain after treatment with clozapine or DHA-clozapine (Figure 4), at a late time when the behavioral effects of clozapine had virtually disappeared, but DHA-clozapine was still very active (Figures 1 and 2).

These findings based on tissue assays of clozapine and its metabolites are consistent with the hypothesis

that administration of DHA-clozapine might limit peripheral tissue-exposure to potentially toxic free clozapine and its metabolites, while enhancing central clozapine-like neuropsychopharmacological activity. This limitation of exposure to clozapine is particularly significant from the perspective of total exposure of peripheral tissues to free clozapine over the 24-h active time-course of action of DHA-clozapine. If access of clozapine to peripheral sites is important in mediating its systemic adverse effects, this effect may be clinically useful.

The low concentrations of clozapine detected in brain tissue following administration of DHA-clozapine almost certainly represent free clozapine and not the clozapine moiety in DHA-clozapine since DHA-clozapine does not extract or co-elute with clozapine under the reported assay conditions (Flood JG 1999, unpublished observations). The presence of free clozapine in brain may mediate the antidopaminergic behavioral effects reported. It is also possible that free clozapine in brain tissue may contribute to risk of centrally mediated adverse effects including excessive sedation or seizures. However, since seizure-risk is reportedly dose-dependent (Welch et al. 1994), the slow delivery of

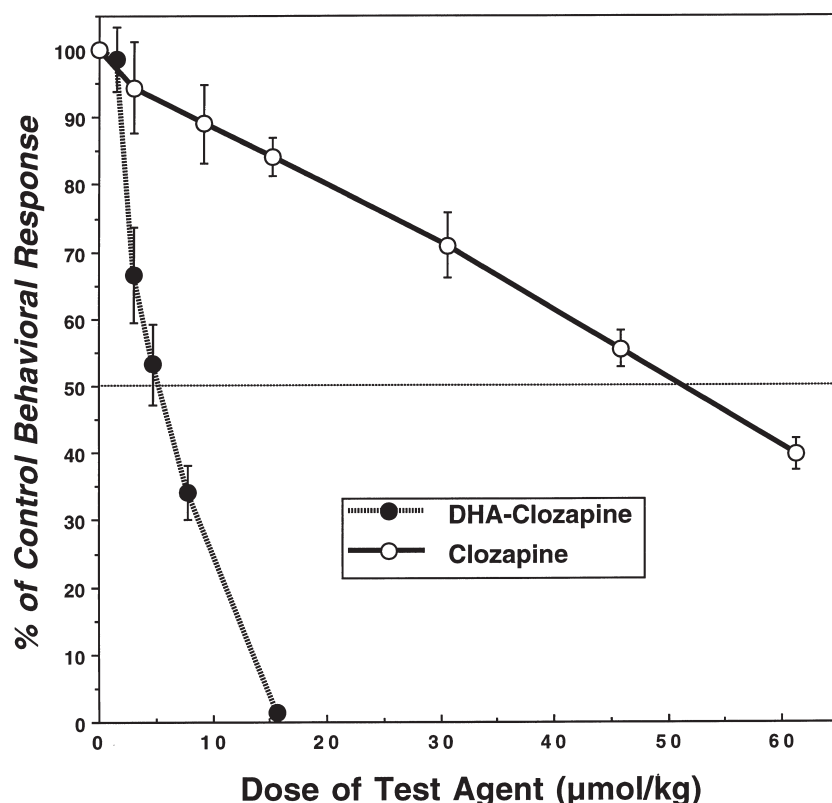


Figure 3. Dose-response comparisons for clozapine (open circles) and DHA-clozapine (filled circles), based on inhibition of locomotor arousal induced by apomorphine challenges (1 mg/kg, i.p.) at 6–7 h after initial treatment with a range of indicated comparable molar doses of test agents ($\mu\text{mol/kg}$, i.p.). Data are mean \pm SEM percentage of control locomotor activity for $n = 6$ fresh rats per group. Dotted horizontal line indicates 50% inhibition (at ED_{50}).

lower levels of clozapine to brain over the 24-h cycle than were found after giving clozapine (Figure 4) may limit seizure risk as well as risk of peripheral toxicity associated with high tissue burdens of free clozapine and its metabolites. Adequate assessment of these predictions will require further direct experimental testing in human subjects.

The lack of evidence of direct neuropharmacological activity of DHA-clozapine in assays of representative receptor and other potential drug target sites, coupled with the presence of detectable levels of free clozapine in brain tissue appear to be consistent with the hypothesis that DHA-clozapine may not be active itself but, instead, a vector and precursor of free clozapine as an active metabolic product, possibly by the action of ubiquitous tissue peptidases. It is also possible that amide substitution at the normally accessible 5-nitrogen of clozapine prevents or delays its oxidative inactivation to clozapine-N-oxide (Baldessarini and Frankenburg 1991; Jann et al. 1993), further contributing to prolonged pharmacological activity.

A related question is whether the prolonged, potent behavioral actions of DHA-clozapine may reflect effects of the fatty acid moiety DHA, rather than the clozapine component of the amide derivative. This question is particularly timely in view of recent evidence that long-chain unsaturated fatty acids in certain natural-product seed or fish oils that include DHA may have antimanic

or antidepressant effects in patients with bipolar disorder, perhaps including those refractory to standard treatments (Stoll et al. 1999). However, control experiments using injections of DHA alone in the present study found no evidence of behavioral depressant effects, nor of significant potency at dopamine receptors (Tables 2 and 3).

A major question arising from the present observations is how to account for the special properties of DHA-clozapine not found with other long-chain, unsaturated (linoleic, oleic) or saturated fatty acids (myristic, palmitic). The brain is highly dependent on external supplies of fatty acids, which readily pass through the blood-brain barrier (Spector 1988). It is not clear that DHA reaches the brain by mechanisms not shared with other unsaturated fatty acids. However, there is evidence that saturated and unsaturated fatty acids may enter by dissimilar mechanisms. Saturated fatty acids, especially those with relatively short carbon-chains, enter by a probenecid-sensitive, low-affinity, high-capacity transport process that may involve protein receptor or carrier molecules, whereas entry of unsaturated fatty acids occurs through a less well understood process that is not inhibited by probenecid (Spector 1988; Avelini et al. 1994). Further complication is introduced by the uncertain role of circulating albumin and proteolipids as avid carriers of fatty acids that reach the brain, possibly through the mediation of low-density proteo-

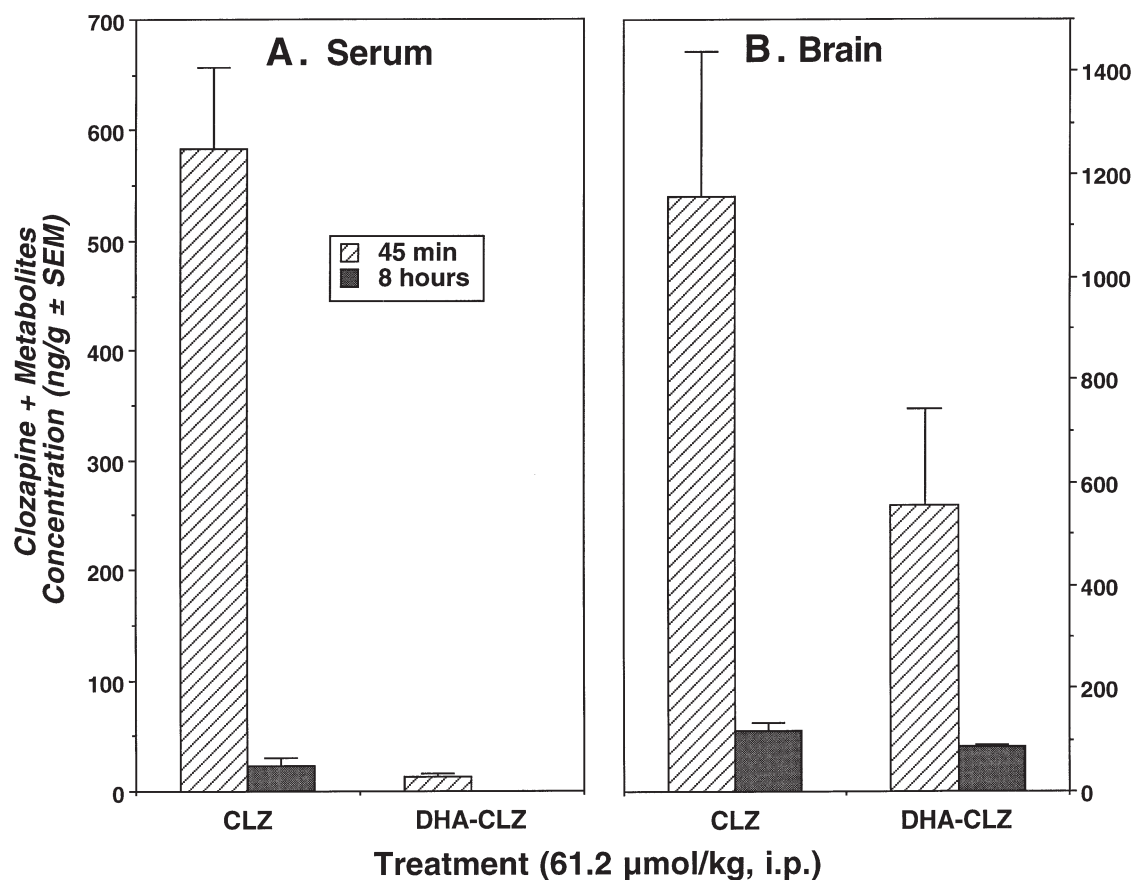


Figure 4. Mean \pm SEM serum concentrations of clozapine and metabolites in serum (A) and whole brain tissue (B) from $n = 6$ rats per condition, at 45 min (striped bars) and 8 h (dark bars) after injections of large, equimolar doses of clozapine and DHA-clozapine (61.2 μ mol/kg, i.p.).

lipid receptor proteins at the blood-brain barrier (Spector 1988; Avellini et al. 1994; Dehouck et al. 1997). The tendency for fatty acids to attach to proteins may also contribute to the prolonged actions of DHA-clozapine. Although a full explanation of the unique properties of the DHA derivative of clozapine found in the present study is not available, it is important to emphasize that the fatty acid amides of clozapine are significantly modified molecules, including occlusion of their usually free carboxylic groups, so that their tissue disposition and brain entry may not necessarily parallel that of the free fatty acids from which they were prepared.

To recapitulate, among amide derivatives of clozapine linked to a series of long-chain, unsaturated or saturated fatty acids that are important components of cerebral lipids, only the DHA-derivative, N-docosahexaenoylclozapine (but not the clozapine-5-N-amides of linoleic, myristic, oleic, or palmitic acids) had potent and long-acting central depressant and anti-apomorphine (antidopaminergic) activity in rat. DHA itself was inactive up to very high doses (30 mg/kg, i.p.). DHA-clozapine was orally bioavailable and more than 10-

times more potent ($ED_{50} = 5.0 \mu$ mol/kg) than clozapine itself. It was also very long-acting against behavioral arousal induced by acute challenge doses of apomorphine, for at least 24 h after a single i.p. dose of DHA-clozapine at 10 mg/kg (16 μ mol/kg), including rats exposed to apomorphine only once. DHA-clozapine produced even less catalepsy than clozapine, suggesting limited extrapyramidal effects. After systemic administration of DHA-clozapine, brain concentrations of free clozapine were much greater than corresponding serum levels, but lower than after injecting clozapine. Taken together, the thrust of the available evidence is that DHA-clozapine is not likely to be directly neuropharmacologically active as an intact molecule, and more likely to serve as a delivery molecule to provide free clozapine selectively to cerebral tissues over prolonged times after systemic or oral administration.

In conclusion, DHA-clozapine is a unique neuropsychopharmacologically active, orally bioavailable and long-acting agent with potent and prolonged antidopaminergic activity after systemic administration, associated with markedly reduced peripheral exposure to

free clozapine over time, but with delivery of free clozapine to brain over times as long as 24 hours. DHA-clozapine was virtually inactive in direct assays of affinity at all dopamine receptor types as well as to a number of other potential drug target sites. Given these pharmacological properties, DHA-clozapine or similar agents should be evaluated as potential antipsychotic drugs with improved central/peripheral distribution, and possibly enhanced safety that requires verification in clinical trials. Moreover, the principle represented in linking a known centrally active drug with a fatty acid carrier molecule such as DHA as a means of enhancing CNS distribution and activity may be applicable to other compounds of neuropsychopharmacological interest, including hydrophilic agents that poorly penetrate the blood-brain barrier (Jacob et al. 1985; Shashoua and Hesse 1996).

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