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European Journal of Pharmacology 323 (1997) 181–192

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Pharmacological characterization of MDL 105,519, an NMDA receptor glycine site antagonist

Bruce M. Baron [a,*], Boyd L. Harrison [a], John H. Kehne [a], Christopher J. Schmidt [a], Paul L.M. Van Giersbergen [b], H. Steven White [c], Barry W. Siegel [a], Yaw Senyah [a], Timothy C. McCloskey [a], Gina M. Fadayel [a], Vicki L. Taylor [a], Michael K. Murawsky [a], Phillip Nyce [a,1], Francesco G. Salituro [a,1]

[a] *Hoechst Marion Roussel, 2110 E. Galbraith Rd., Cincinnati, OH 45215, USA*
[b] *Marion Merrell, 16 Rue d'Ankara, Strasbourg, France*
[c] *University of Utah, 408 Biopolymers Research Building, Salt Lake City, UT 84112, USA*

Received 13 December 1996; accepted 10 January 1997

Abstract

MDL 105,519, (*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid, is a potent and selective inhibitor of [^{3}H]glycine binding to the NMDA receptor. MDL 105,519 inhibits NMDA (*N*-methyl-D-aspartate)-dependent responses including elevations of [^{3}H]*N*-[1,(2-thienyl)cyclohexyl]-piperidine ([^{3}H]TCP) binding in brain membranes, cyclic GMP accumulation in brain slices, and alterations in cytosolic Ca^{2+} and Na^+-Ca^{2+} currents in cultured neurons. Inhibition was non-competitive with respect to NMDA and could be nullified with D-serine. Intravenously administered MDL 105,519 prevented harmaline-stimulated increases in cerebellar cyclic GMP content, providing biochemical evidence of NMDA receptor antagonism in vivo. This antagonism was associated with anticonvulsant activity in genetically based, chemically induced, and electrically mediated seizure models. Anxiolytic activity was observed in the rat separation-induced vocalization model, but muscle-relaxant activity was apparent at lower doses. Higher doses impair rotorod performance, but were without effect on mesolimbic dopamine turnover or prepulse inhibition of the startle reflex. This pattern of activities differentiates this compound from (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801) and indicates a lower psychotomimetic risk.

Keywords: Excitatory amino acid; Anticonvulsant; Anxiolytic; Dopamine; Psychosis; Sensory processing

1. Introduction

The *N*-methyl-D-aspartate (NMDA)-preferring glutamate receptor is the most extensively characterized of excitatory ionotropic receptors due, in part, to the availability of numerous pharmacological tools. This receptor possesses a number of distinct recognition sites capable of regulating the ion flux occurring through the Ca^{2+}-permeable pore (Palfreyman, 1994). Of particular interest is this receptor's co-dependence upon two agonist species for activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Seeburg et al., 1995). Thus, channel opening requires the simultaneous presence of both glutamate and glycine (for review, see Palfreyman and Baron, 1991).

Glycine site antagonists have been shown to provide complete inhibition of NMDA-mediated responses in a variety of tissue preparations supporting the role of glycine as an obligatory co-agonist (Baron et al., 1992). The inhibition observed in the presence of these compounds is non-competitive and therefore may represent a useful mechanism in pathophysiological conditions involving elevated glutamate (Baron et al., 1994; Leeson and Iversen, 1994). NMDA receptor glycine antagonists have shown a pattern of in vivo activity which is similar, but not identical to that exhibited by other types of NMDA receptor antagonists. Of particular interest is the difference in behavioral and neurochemical effects as they may relate to undesired pharmacology such as motoric, sensory, and cognitive deficits (Baron et al., 1994; Kehne et al., 1995; Wiley and Balster, 1994; Muir and Lees, 1995). Since the appearance of distinct pharmacological profiles among agents believed to act at the same receptor target, albeit at

* Corresponding author. Tel.: (1-513) 948-6351; Fax: (1-513) 948-6439; e-mail: brucebaron@hmri.com

[1] Current address: Vertex Pharmaceuticals, Cambridge, MA, USA.

0014-2999/97/$17.00
PII S0014-2999(97)00045-9

different sites on the target, was at first surprising, it prompted us to characterize and contrast the properties of structurally dissimilar molecules acting at the NMDA receptor.

We report here on the properties of MDL 105,519, (*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid, which exemplifies a new class of molecules acting at the glycine recognition site on the NMDA receptor. This series of compounds produces potent antagonism in vitro and exhibits sufficient systemic potency to permit a characterization of their behavioral and neurochemical effects. MDL 105,519 can be recognized as a hybrid structure combining structural features of two previously described chemical series. This molecule incorporates optimal side-chain substituents from MDL 100,748 (4-carboxymethylamino-5,7-dichloroquinoline-2-carboxylic acid (Harrison et al., 1990; Baron et al., 1992)) and MDL 100,458 (3-(benzoylmethylamino)6-chloro-1*H*-indole-2-carboxylic acid (Salituro et al., 1991)) oriented on a common indole-2-carboxylic acid scaffold. This combination of ligand-receptor attachment points results in a multiplicative gain in receptor affinity and in the appearance of prominent in vivo activity.

2. Materials and methods

2.1. Sources of NMDA antagonists

MDL 105,519, MDL 100,748, MDL 104,653 (7-chloro-4-hydroxy-3-phenylquinolin-2(1*H*)-one; Leeson et al., 1993), MDL 29,951 (3-(4,6-dichloro-2-carboxyindol-3-yl)propionic acid), MDL 100,458, ACEA 1021 (5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione; Woodward et al., 1995) were synthesized at Hoechst Marion Roussel and were $>98\%$ pure as assessed by chromatographic, spectroscopic and elemental analyses. MK-801 (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) was purchased from Research Biochemicals International (Natick, MA, USA). Membranes containing cloned human dopamine receptors were obtained from Biosignal (Montreal, Canada). Chinese hamster ovary cells expressing muscarinic cholinergic receptors were obtained from the National Institute of Health (Bethesda, MD, USA). Radioligands were obtained from New England Nuclear Dupont or Amersham.

2.2. Animals

Adult male mice (Charles River CD-1), adult male rats (Charles River CD, Wistar or CDF) or 18-day-old, male audiogenic seizure-susceptible mice (DBA/2J Jackson Laboratories) were used for the present in vivo studies. Neonatal (Charles River CD) rats were used for cell culture and brain slice experiments. Animals were housed in animal rooms on a 14:10 h light/dark cycle, and allowed free access to food and water. Neonatal rats were housed with the mother in a breeding box with sawdust bedding. All experiments were performed during the light phase of the light/dark cycle. Drugs were dissolved in distilled water, saline, or 50 mM Tris base (ACEA 1021) and injected at a volume of 1 ml/kg (rats) or 10 ml/kg (mice).

2.3. Radioligand binding

Methods for [^{3}H]glycine, [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ([^{3}H]CPP) and [^{3}H]*N*-[1-(2-thienyl)cyclohexyl]-piperidine ([^{3}H]TCP) binding to the rat brain NMDA receptor complex are described in Baron et al. (1988). Binding to rat brain non-NMDA glutamate receptors using [^{3}H]kainate and [^{3}H]α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ([^{3}H]AMPA) was measured as described by Baron et al. (1990). Binding to α_1-adrenoceptors ([^{3}H]prazosin, rat cortex), α_2-adrenoceptors ([^{3}H]rauwolscine, rat cortex), β-adrenoceptors ([^{3}H]dihydroalprenolol, rat cortex), L-type Ca^{2+} channels ([^{3}H]nitrendipine, rat heart), dopamine D_1 ([^{3}H]SCH23390, cloned human receptor), dopamine D_2 receptors ([^{3}H]spiperone, cloned human receptors), histamine H_1 receptors ([^{3}H]pyrilamine, rat cortex), muscarinic M_2, M_3 receptors ([^{3}H]*N*-methylscopolamine, cloned human receptors), opiate receptors ([^{3}H]naloxone, rat cortex), 5-HT_{1A} receptors ([^{3}H]8-OH-DPAT, [^{3}H]2-(*N*,*N*-dipropylamine)-8-hydroxy-1,2,3,4-tetrahydronaphthalene, rat cortex), 5-HT_{2A} receptors ([^{3}H]ketanserin, rat cortex), 5-HT_3 receptors ([^{3}H]GR65630, NG108-15 cell membranes) and central benzodiazepine receptors ([^{3}H]flunitrazepam, bovine cortex) was performed as described by Kehne et al. (1996).

2.4. In vitro functional assays

NMDA-elicited cyclic GMP (cGMP) was measured in 8-day-old CD rat cerebellar slices as described by Baron et al. (1992). Cytosolic free Ca^{2+} concentrations were measured in fura-2 AM-loaded cultured rat cerebellar granule cells (8 days in vitro) using microspectrofluorimetry (as described by Baron et al., 1992).

Na^+ and Ca^{2+} currents were measured by the whole-cell configuration of the patch-clamp technique. Rat brain neurons (CD rat, 4–18 days in vitro) were continually perfused at 0.5 ml/min at 22°C in a HEPES-buffered extracellular saline solution consisting of (in mM): 142 NaCl, 1 $CaCl_2$, 8 KCl, 10 glucose, 10 HEPES pH 7.4. Patch pipettes were fabricated from Fisher brand microhematocrit capillary tubes and filled with an intracellular solution consisting of (in mM): 153 $CsCl_2$, 10 HEPES, 5 EGTA pH 7.38. Test compounds were prepared as 10 mM stocks in dimethylsulfoxide. Immediately prior to use, appropriate concentrations were prepared by dilution with external solution (dimethyl sulfoxide final concentrations ranged from 0.001–1%). NMDA 100 μM/glycine 2 μM

and/or test compounds were applied via a U-tube (rapid solution exchanger) positioned within 200 μm of the neuron of interest. With this system, a complete solution change in the vicinity of a cultured neuron was achieved well within 500 ms (Bormann, 1992). The relative potencies of test compounds were assessed by comparison of the response to NMDA (100 μM)/glycine (2 μM) obtained in the presence of the antagonist relative to that elicited by NMDA/glycine alone. Recordings from 3–8 cells were collected for each compound examined. All cells successfully patched responded to NMDA/glycine.

2.5. In vivo neurochemical assays

Inhibition of harmaline-elevated cyclic GMP accumulation in the cerebellum of CD-1 mice was measured as described by Reynolds et al. (1991). Cyclic GMP content was normalized to the amount of membrane protein. Rat brain (CD rat) dihydroxyphenylalanine accumulation and extracellular dopamine levels were assayed as in Schmidt et al. (1992) and Schmidt and Fadayel (1995, 1996), respectively.

2.6. Behavioral paradigms

Antagonism of sound- and i.c.v. quinolinic acid-induced seizures was measured as described by Baron et al. (1992) using DBA/2J and CD-1 mice, respectively. Maximal electroshock-induced seizure testing in CDF rats was performed as described by Kehne et al. (1992). Assessment of anxiolytic potential was performed using the separation-induced vocalization test in CD rat pups described by Kehne et al. (1991, 1995). Assessment of potential ataxia was measured by disruption of rotorod performance in CD-1 mice (Kehne et al., 1992) or by the ability of a compound to decrease the amount of time that an 8-day-old CD rat pup was able to retain its grasp on the lip of an ice bucket (termed 'time on an inclined plane' and described in detail by Kehne et al., 1991). Effects of compounds on sensory gating were assessed by their effect on prepulse inhibition of the startle reflex in Wistar rats and were measured as described by Kehne et al. (1992).

2.7. Data analysis

Concentration-response data were fit to a logistic function using non-linear regression analysis. Agonist-induced responses (e.g., NMDA-stimulated cGMP formation) used a function of the form: Response $= B + MX^n/(K^n + X^n)$ where B = observed basal response, M = calculated maximal response, X = concentration of agonist, n = slope factor, and K = calculated EC_{50} of the agonist. Antagonist-induced inhibition of responses was fit using a similar model of the form: Inhibition $= 100X^n/(K^n + X^n)$ where X = concentration of antagonist, n = slope factor, and K = IC_{50} of the antagonist. Dose-response data was fit using quantal analysis (anticonvulsant and rotorod experiments) or linear regression of log-transformed values (continuous variables from anxiolytic and muscle-relaxant tests).

2.8. Statistical analysis

Harmaline-stimulated cGMP accumulation data was analyzed using one-way ANOVA followed by Dunnett's multiple comparison test (StatView 4.01 software, Abacus Concepts). Dopamine turnover experiments were analyzed by one-way ANOVA followed by post-hoc comparisons with the least-significant difference test (Microstat-II software, Ecosoft). Microdialysis experiments were analyzed using two-way repeated-measures ANOVA followed by post-hoc comparisons using Tukey's test (Sigmastat software, Jandel Scientific). Prepulse inhibition experiments were analyzed using two-way ANOVA followed by Dunnett's test (RS/1 software, BBN Software Products). An adjusted P-value less than 0.05 was used as a criterion for statistical significance throughout.

3. Results

3.1. MDL 105,519 is a selective ligand for the glycine recognition site

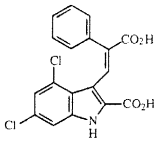
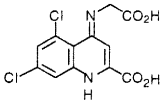
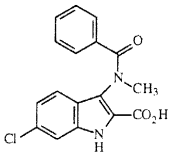
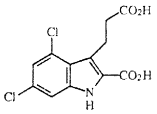
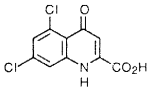
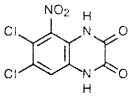
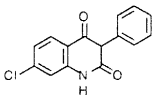
MDL 105,519 was identified as a potent NMDA receptor glycine site ligand using radioligand binding assays. MDL 105,519 could completely inhibit the binding of [^{3}H]glycine to rat brain membranes with a K_i value of 10.9 ± 1.4 nM ($n = 6$). The binding isotherms exhibited a Hill slope near unity (0.97 ± 0.12) consistent with the measured binding occurring to a population of non-interacting binding sites with uniform affinity for the unlabeled ligand. Table 1 reports the affinity and structures of MDL 105,519 and selected reference glycine site antagonists in the glycine binding assay. MDL 105,519 was also examined for its affinity at the glutamate recognition site using [^{3}H]CPP as the radioligand. The K_i value was greater than 100 μM, indicating a high degree of selectivity for the glycine recognition site.

MDL 105,519 was profiled for binding affinity vs. a panel of other 16 receptor species including G-protein-coupled and ionotropic receptors. The results are shown in Table 2 and indicate that MDL 105,519 is approximately 10 000-fold selective for the glycine recognition site relative to the other receptor types investigated.

3.2. MDL 105,519 modulates the binding of the open channel blocker [^{3}H]TCP

The non-equilibrium binding of radiolabeled use-dependent blockers of the NMDA receptor-associated ion channel has been used extensively to localize the site of action of agents active at the NMDA receptor and to provide

Table 1
Binding affinity at the NMDA receptor-associated glycine recognition site for MDL 105,519 and reference glycine site antagonists

Compound	Structure	IC_{50} vs. [^{3}H]glycine, nM
MDL 105,519		10.9 ± 1.4
MDL 100,748		70 ± 20 [a]
MDL 100,458		136 ± 25 [b]
MDL 29,951		140 ± 20 [a]
5,7-DCKA		80 ± 20 [a]
ACEA 1021		23.5 ± 1.5
MDL 104,653		273 ± 46 [c]

Values are means ± S.E.M. of three or more experiments.
[a] Baron et al. (1992).
[b] Kehne et al. (1995).
[c] Chapman et al. (1995).

preliminary information about intrinsic efficacy. We have characterized the effect of a fixed concentration (2 μM) of MDL 105,519 on the allosteric modulation of [^{3}H]TCP binding by exogenous amino acids (Fig. 1). Both glycine and NMDA produced a concentration-dependent elevation in the level of specific binding which at a 1 mM concentration of the amino acids was approximately 8- to 10-fold that observed in their absence. In the presence of 2 μM MDL 105,519, specific binding was reduced by 67%, indicating that the compound functioned as an antagonist in this assay. Moreover, added glutamate site agonist (NMDA) was unable to stimulate channel opening as indicated by the limited increase in specific binding observed in its presence. However, glycine was able to restore normal stimulation of binding although higher concentrations of the co-agonist were required to accomplish this effect in the presence of MDL 105,519 than were needed in its absence. These results suggest that MDL 105,519 inhibits receptor activation via antagonism at the glycine recognition site.

3.3. MDL 105,519 inhibits NMDA-elicited biochemical changes

The antagonist nature of MDL 105,519 was characterized by measuring its ability to inhibit NMDA-elicited biochemical responses in vitro. NMDA elicits a rapid rise in the cGMP content of neonatal cerebellar slices. Under our assay conditions, the cGMP response occurs in the absence of added glycine. However, this response is readily inhibited by glycine site antagonists, indicating that it is glycine-dependent but presumably sufficient glycine was present in the slice to saturate the binding site. MDL 105,519 completely inhibited the response to 15 μM NMDA with an EC_{50} value of 0.282 ± 0.109 μM ($n = 3$). As shown in Fig. 2A, NMDA elicits a concentration-dependent increase in cGMP content. The EC_{50} value for the agonist was 9.5 μM and the maximal response was 102.7 pmol cGMP/mg protein. In the presence of 0.5 μM MDL 105,519, a rightward shift in the NMDA concentration-response curve (EC_{50} 62.2 μM) was observed which was accompanied by a 16% reduction in the maximal response (86.6 pmol/mg protein). At 3 μM MDL 105,519, there was no detectable response to NMDA even at an agonist concentration 2 orders of magnitude higher than the agonist EC_{50} value determined in control tissues. These results indicate that MDL 105,519 acts as a non-competitive inhibitor of the NMDA response.

The mechanism of inhibition of the cGMP response was

Table 2
Binding selectivity of MDL 105,519

Receptor	Inhibition by MDL 105,519		
	1 μM	10 μM	100 μM
α_1-Adrenenoceptor	3	3	14
α_2-Adrenenoceptor	11	10	38
β-Adrenenoceptor	0	4	1
Benzodiazepine	0	10	56
L-type Ca channel	9	8	7
Dopamine D_1	38	15	29
Dopamine D_2	10	1	11
Histamine H_1	4	13	32
Muscarinic M_2	20	2	8
Muscarinic M_3	14	1	20
Opiate	0	16	14
5-HT_{1A}	6	0	13
5-HT_{2A}	8	16	18
5-HT_3	2	1	13
Glutamate AMPA	−8	−7	35
Glutamate kainate	16	20	35
Glutamate NMDA	8	12	22

Results are expressed as percent inhibition of specific radioligand binding at the indicated concentrations of MDL 105,519. Values are the means of two independent experiments conducted in triplicate.

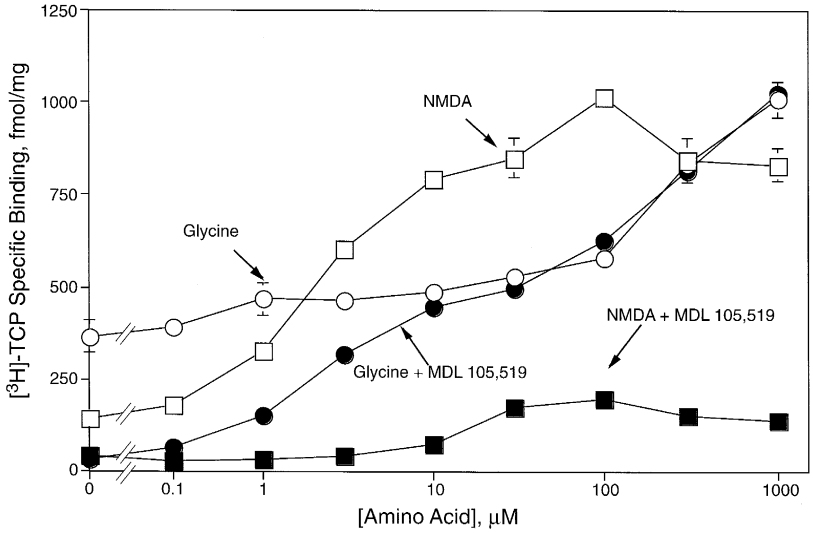


Fig. 1. MDL 105,519 prevents channel opening as shown by inhibition of open-channel blocker binding. The effect of exogenous amino acids on the specific binding of [^{3}H]TCP was measured in the presence (closed symbols) or absence (open symbols) of MDL 105,519 (2 μM). The concentration of NMDA (squares) and glycine (circles) is shown on the abscissa whereas the specific binding (fmol/mg protein) of the radioligand is shown on the ordinate. Results are means ± S.E.M. of triplicate determinations taken from a representative experiment.

evaluated by measuring the effect of inclusion of a high concentration of the glycine-mimetic D-serine (Fig. 2B). The glycine site agonist had a slight potentiating effect on the NMDA response, increasing the maximal response by almost 20% to 121.4 pmol/mg protein and slightly decreasing the EC_{50} value of NMDA to 8.0 μM. Inclusion of D-serine completely nullified the ability of the antagonist to inhibit the response. Under these conditions, curves constructed with 0.5 μM and 3.0 μM MDL 105,519 gave respective maximal responses of 145.2 and 151.4 pmol/mg protein and EC_{50} values of 10.3 and 8.3 μM.

The primary biochemical event occurring after NMDA receptor activation is an influx of Ca^{2+} ions through the receptor-associated pore. Large elevations in intracellular free Ca^{2+} can be elicited in fura-2-loaded cerebellar granule cells using 100 μM NMDA in combination with 10 μM glycine. As shown in Fig. 3, MDL 105,519 (1 μM) completely inhibited the NMDA-induced response. The effect of the antagonist was reversible by superfusion with antagonist-free buffer with a $t_{1/2}$ of approximately 8 min (Fig. 3, inset).

Whole-cell patch-clamp studies confirmed the antagonist properties of MDL 105,519- vs. NMDA-mediated responses. Na^+ and Ca^{2+} currents were elicited in cortical neurons by superfusion with 100 μM NMDA + 2 μM glycine. MDL 105,519 (1 μM) inhibited the current magnitude by 80–90% (Fig. 4). Maximal block occurred within 2–3 s after antagonist application and was slowly reversible, recovery requiring approximately 5 min of superfusion with antagonist-free media. The recovery kinetics were slower than those observed with other glycine site antagonists (MDL 104,653 and 5,7-dichlorokynurenic acid)

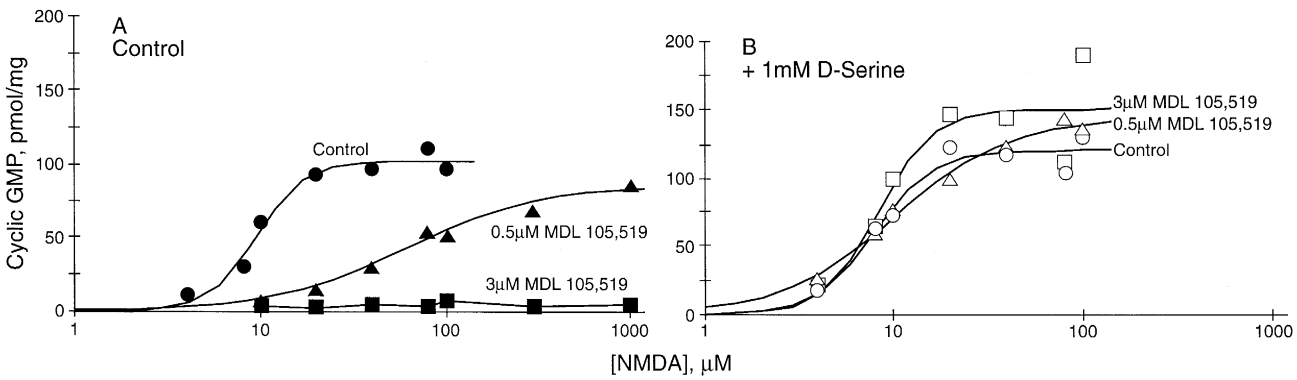


Fig. 2. MDL 105,519 is a noncompetitive inhibitor of NMDA-elicited cGMP accumulation in cerebellar slices. Reversal by D-serine. The concentration-response relationship for NMDA-induced elevation of cGMP content was determined in a neonatal rat cerebellar slice preparation. Control slices are designated by circles. The effect of MDL 105,519 on the NMDA response was investigated by inclusion of 0.5 μM (triangles) or 3 μM (squares) antagonist in the assay tubes. MDL 105,519 led to a flattening of the concentration-response curve (panel A) which was prevented by inclusion of the glycine-mimetic D-serine (1 mM, panel B). Results are from a representative experiment conducted in duplicate.

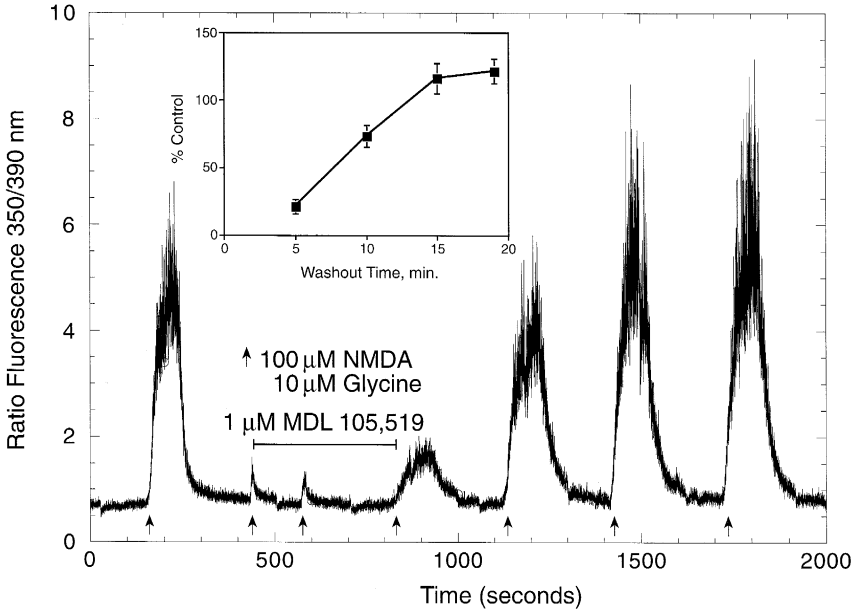


Fig. 3. MDL 105,519 reversibly inhibits the NMDA-induced rise in cytosolic free Ca^{2+} in cultured neurons. The effect of MDL 105,519 (1 μM) on the NMDA (100 μM)/glycine (10 μM) elicited Ca^{2+} rise was assessed in superfused fura 2-loaded cerebellar granule cells. Cytosolic free Ca^{2+} concentration is indicated as the fluorescence ratio (350/390 nm) on the ordinate and time (s) on the abscissa. The periods of compound application are indicated by vertical arrows (NMDA/glycine) or the horizontal bar (MDL 105,519) adjacent to the fluorimeter trace. Data shown are from a representative cell. Inset: Time-course of recovery from MDL 105,519 inhibition. Following antagonist removal, the measured response amplitude is plotted as percent control (NMDA/glycine response prior to MDL 105,519) vs. time using data collected from three cells.

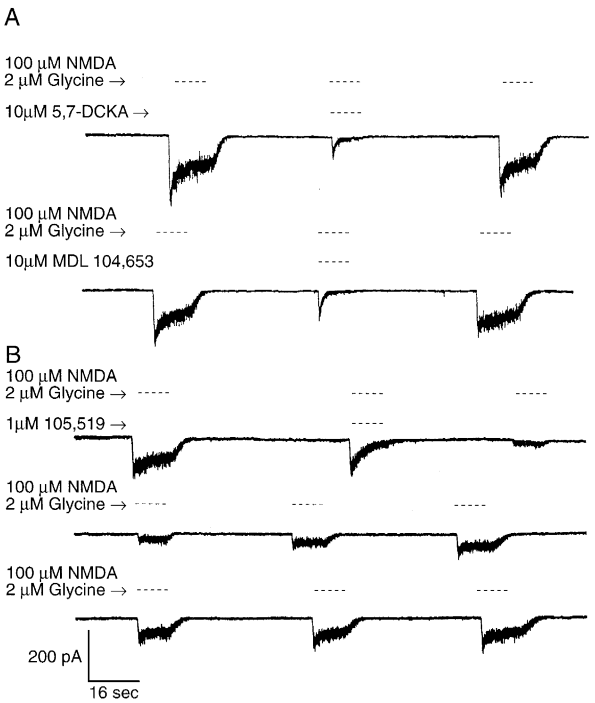


Fig. 4. Effect of MDL 105,519 and reference NMDA receptor glycine antagonists on NMDA-induced Na^{+} and Ca^{2+} currents in cultured neurons. NMDA (100 μM)/glycine (2 μM) was applied to cultured rat cortical neurons and ionic currents were monitored using whole-cell patch-clamp electrophysiology. The period of compound application is indicated by the notations above the recorder traces. The onset and recovery of block after a brief application of antagonist is shown in panel A for 5,7-dichlorokynurenic acid (5,7-DCKA; 10 μM) and MDL 104,653 (10 μM) and in panel B for MDL 105,519 (1 μM). The results shown are for representative recordings.

at biologically equivalent concentrations (Fig. 4). Antagonism by MDL 105,519 was not significantly affected by applied membrane potential over the range of −75 to +40 mV (data not shown).

3.4. MDL 105,519 is an NMDA receptor antagonist in *vivo*

Systemic administration of MDL 105,519 to rodents resulted in central antagonism of NMDA-mediated biochemical events. The alkaloid harmaline is known to activate the climbing fiber pathway to release glutamate at its

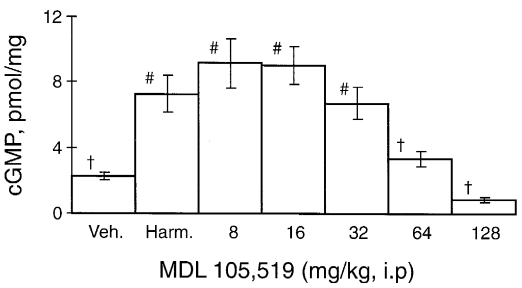


Fig. 5. MDL 105,519 is an NMDA receptor antagonist in vivo. NMDA receptor antagonism was measured by monitoring the ability of i.p. administered MDL 105,519 to inhibit the ability of harmaline to elevate cerebellar cGMP content. Mice (adult male CD-1) were injected with various doses of MDL 105,519 intraperitoneally and 30 min later were administered harmaline (50 mg/kg s.c.). Sixty minutes after the first injection, the mice were killed and cerebellar cGMP content was measured by radioimmunoassay. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test. Symbols above the bars indicate $P < 0.05$ versus vehicle (#) and versus harmaline (+) treatment.

Table 3
Relative potency of MDL 105,519, ACEA 1021, and MK-801 in tests of anticonvulsant and motor impairment activity in CD-1 mice

Compound	Quinolinic acid, ED_{50} (95% C.L.) mg/kg [a]	Rotorod, ED_{50} (95% C.L.) mg/kg [a]	Selectivity ratio [b]
MDL 105,519	36.3 (25.3–58.7)	72.9 (52.0–107.0)	1.9
ACEA 1021	14.7 (9.2–22.3)	3.9 (2.4–5.7)	0.3
MK-801	0.08 (0.06–0.13)	0.18 (0.15–0.21)	2.3

[a] Potency determinations were made 5 min after intravenous administration. The 95% confidence limits (C.L.) are shown in parentheses.
[b] Selectivity ratio was calculated as the ED_{50} in the rotorod test divided by the ED_{50} in the quinolinic acid seizure model.

synapses on Purkinje cell dendrites in the cerebellum. As in the cerebellar slice, the consequent NMDA receptor activation elevates the tissue content of cGMP. This functional assay provides a biochemical index of NMDA receptor antagonism in vivo and enables definition of a dose range for test compounds producing these desired pharmacological effects. In CD-1 mice, MDL 105,519 inhibited the cerebellar response to subcutaneously administered harmaline in a dose-dependent fashion (Fig. 5). The ED_{50} value using a 1 h pretreatment paradigm was 56 mg/kg by the i.v. route and 59 mg/kg by the i.p. route. For comparison, the reference NMDA receptor glycine antagonist ACEA 1021 demonstrated an ED_{50} value of 3.5 mg/kg in this assay by the i.v. route.

3.5. Anticonvulsant effects of MDL 105,519

NMDA receptor antagonists have shown efficacy vs. chemically, electrically, and genetically induced seizures suggesting a potential use for this mechanistic approach in treating epilepsy. MDL 105,519 was profiled in each of these test systems. Following i.p. administration to audiogenic seizure-susceptible DBA/2J mice, MDL 105,519 displayed a rapid onset of anticonvulsant activity. The time of peak effect was approximately 15 min and the ED_{50} value was 13.4 mg/kg (95% confidence limits: 8.2–19.9). Similarly, ACEA 1021 displayed a time of peak effect of 30 min and an ED_{50} value of 3.7 mg/kg (3.2–4.2). Intracerebroventricular injection of the NMDA receptor agonist quinolinic acid in CD-1 mice results in the rapid onset of tonic-clonic convulsions which can be prevented by pretreatment with NMDA receptor antagonists. MDL 105,519 was able to completely inhibit the response to the chemoconvulsant with maximal effects occurring 5 min after i.v. administration with an ED_{50} value of 36.3 mg/kg (26.5–49.1). The approximate half-life of these effects was 3 h. ACEA 1021 was approximately 2.5-fold more potent in this model (ED_{50} = 14.7 mg/kg (9.2–22.3)) but of shorter duration ($t_{1/2}$ = 30 min). The channel blocker MK-801 was also evaluated in this paradigm and found to be extremely potent with an ED_{50} value of 0.08 mg/kg (95%

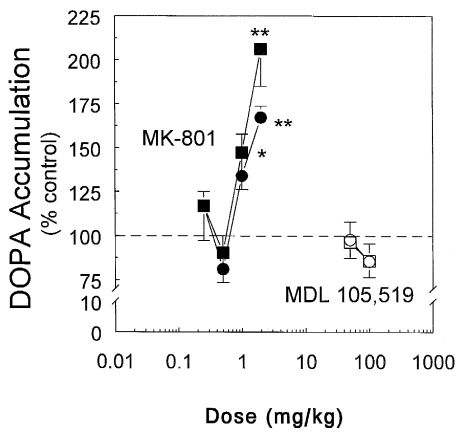


Fig. 6. Comparison of the effects of the NMDA receptor channel blocker MK-801 and the NMDA receptor glycine site antagonist MDL 105,519 on dihydroxyphenylalanine (DOPA) accumulation. Male CD rats were administered various doses of MK-801 or MDL 105,519 and DOPA accumulation was measured in the striatum (squares) and nucleus accumbens (circles). Values are expressed as the mean ± S.E.M percent of saline control (n = 5 for MDL 105,519 and n = 10 for MK-801). MK-801 (closed symbols) was administered at doses of 0.25, 0.5, 1.0, 2.0 mg/kg, i.p. with n = 4–5 rats in each group. MDL 105,519 (open symbols) was administered at doses of 50 mg/kg, i.p. (n = 4) and 100 mg/kg, i.p. (n = 5). Drug effects were analyzed by a one-way ANOVA followed by a post-hoc least-significant difference test (* $P < 0.05$, ** $P < 0.01$ vs. control).

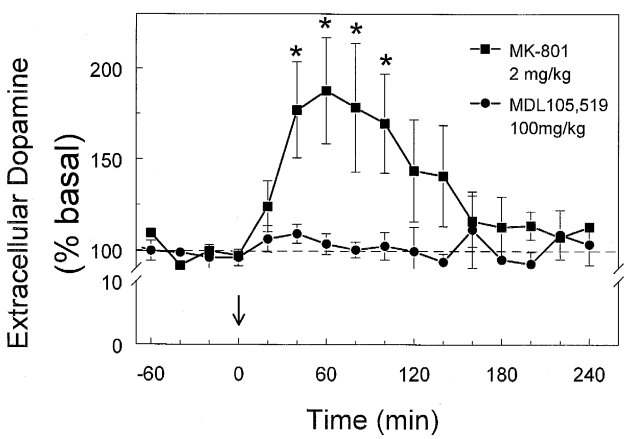


Fig. 7. Comparison of the effects of the NMDA receptor channel blocker MK-801 and the NMDA receptor glycine site antagonist MDL 105,519 on extracellular dopamine concentrations in the nucleus accumbens. Adult, male, CD rats were administered MK-801 (n = 4, 2 mg/kg, i.p.) or MDL 105,519 (n = 4, 100 mg/kg, i.p.) and extracellular dopamine concentrations were measured using in vivo microdialysis. The time of drug administration is indicated by the arrow. Values shown are the mean ± S.E.M. Drug effects were analyzed by repeated-measures two-way ANOVA followed by Tukey's test (* $P < 0.05$ vs. control).

confidence limits: 0.06–0.13). Both MDL 105,519 and ACEA 1021 were effective against electrically induced seizures in the CDF rat. The ED_{50} values obtained 5 min after i.v. dosing were 29.2 mg/kg (95% confidence limits: 21.2–41.3; MDL 105,519) and 46.3 mg/kg (95% confidence limits: 36.8–58.2; ACEA 1021). It is of interest that the relative potency of the two compounds varied considerably as a function of the type of test used for evaluation.

3.6. Anxiolytic action of MDL 105,519

NMDA receptor antagonists have previously been shown to exhibit effects in animal models predictive of potential anxiolytic activity. We have characterized potential anxiolytic activity of MDL 105,519 using a separation-induced vocalization model in neonatal, CD rats. MDL 105,519 was found to inhibit the ultrasonic vocalizations with an ED_{50} of 39.6 mg/kg (95% confidence limits: 20.9–207.4) by the i.p. route.

3.7. Effects on motor behavior

Motor coordination was measured in CD-1 mice 5 min after i.v. administration of NMDA receptor antagonists using the rotorod test. MDL 105,519 impaired rotorod performance with an ED_{50} value of 72.9 mg/kg (95% confidence limits: 52.0–107.0). As shown in Table 3, motor impairment is a common feature of NMDA receptor antagonists but occurs for each compound over markedly different dose ranges. We have used the ratio of the ED_{50}

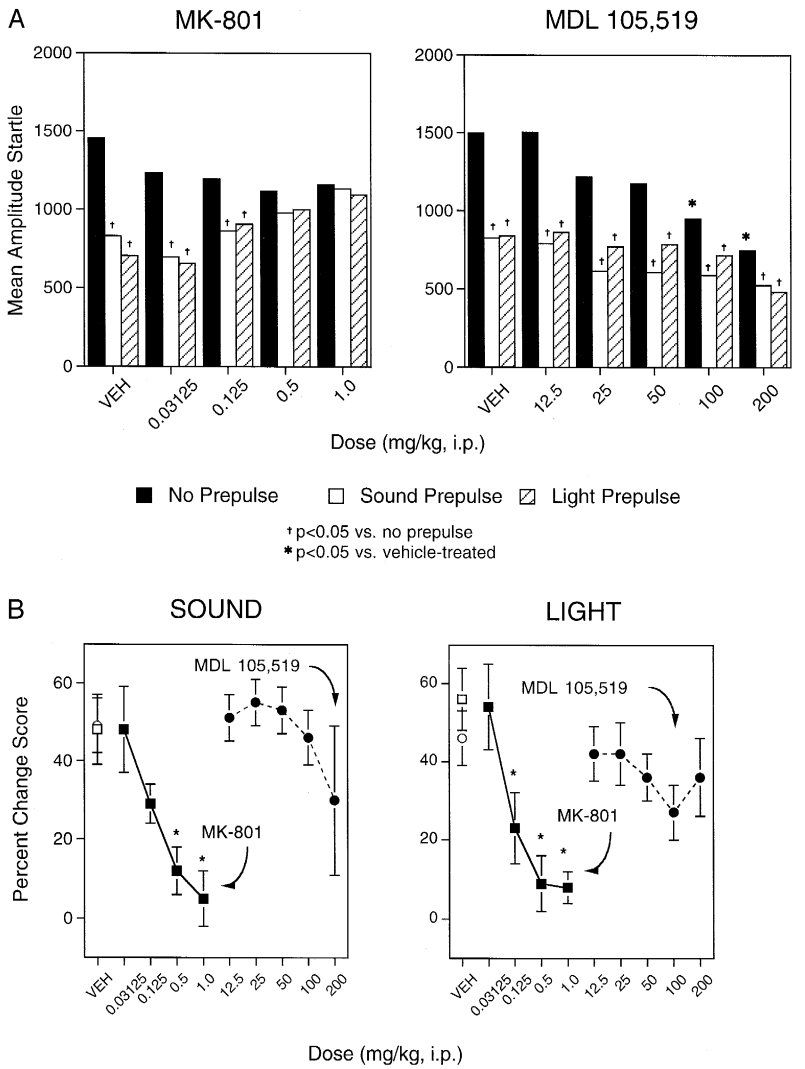


Fig. 8. Comparison of the effect of MDL 105,519 with MK-801 on prepulse inhibition of the startle reflex. Vehicle or various doses of MDL 105,519 or MK-801 were given intraperitoneally to adult, male, Wistar rats prior to startle reflex testing. (A) The amplitude of the acoustic startle reflex is shown by the filled bars, whereas the effect of either a sound- or light-prepulse stimulus is indicated by open bars and hatched bars, respectively. Significant changes in startle amplitude were determined using two-way ANOVA followed by Dunnett's test. * $P < 0.05$ no prepulse vs. control, † $P < 0.05$ significant difference vs. no prepulse condition. (B) The results are expressed as the percent change score vs. dose. The apparent change score is $100 \times$ (No Prepulse − Sound or Light Prepulse)/(No Prepulse). The percent change score normalizes the change score to the no-prepulse startle amplitude. * $P < 0.05$ drug vs. vehicle control.

value for each compound in the rotorod test divided by the ED_{50} value in the quinolinic acid seizure test to calculate a 'selectivity ratio'. The results are shown in Table 3. MDL 105,519 also produced motor impairment in neonatal, CD rats as indicated by a reduction in their ability to maintain their body position on an inclined plane (time on an inclined plane measure). The ED_{50} value (27.9 mg/kg (21.2–39.6)) in the inclined plane task was lower than that observed in the rotorod test using adult CD rats. This value is also less than the ED_{50} value for anxiolytic activity in the same animals.

3.8. Evaluation in biochemical and behavioral tests indicative of psychotomimetic liability

NMDA receptor glycine antagonists have been suggested to have a lower propensity for producing dose-limiting side effects. As many of the central nervous system effects are believed to be related to the pharmacological mechanism, it is curious that these events can be dissociated using different classes of NMDA receptor antagonists. Therefore, MDL 105,519 was evaluated in several models believed to be predictive of psychotomimetic liability. In each system, MDL 105,519 was compared to the channel blocker MK-801.

Psychomotor stimulation produced by NMDA receptor antagonists is believed to be related to the ability of these agents to increase the activity of dopaminergic pathways in the brain and in particular the mesolimbic (A10) system. No effect of MDL 105,519 (50, 100 mg/kg i.p.) was observed on either striatal or nucleus accumbens dopamine synthesis measured in CD rats by dihydroxyphenylalanine accumulation (Fig. 6). In contrast, comparative data are provided for MK-801, demonstrating a pronounced effect on both dopamine systems. Similarly, the high dose of MDL 105,519 (100 mg/kg, i.p.) was without effect on mesolimbic dopamine as monitored by microdialysis in the nucleus accumbens (Fig. 7), whereas the channel blocker MK-801 (2 mg/kg, i.p.) significantly increased extracellular mesolimbic dopamine concentrations. This dose of MK-801 is 4- to 10-fold higher than employed by previous investigators (e.g., Wedzony et al., 1993) but is identical to that used by Schmidt and Fadayel (1996).

The prepulse inhibition paradigm is performed in adult, male, Wistar rats and provides a quantitative behavioral estimate of a drug's ability to disrupt the gating of sensory information. Prepulse inhibition refers to the capacity for a subthreshold stimulus (the prepulse) to attenuate the magnitude of a subsequently elicited startle reflex. Agents which disrupt sensory gating such as NMDA receptor channel blockers reduce prepulse inhibition at doses consistent with their receptor blocking activity in vivo. A statistical analysis of the 'percent change scores' demonstrated that MDL 105,519 did not significantly disrupt sound or light prepulse inhibition, even at the 100 and 200 mg/kg doses which significantly depressed baseline startle (Fig. 8A and B). In contrast, MK-801 produced a complete suppression of light- and sound-induced prepulse inhibition at a dose of 1 mg/kg without a significant effect on the baseline startle response (Fig. 8A and B).

4. Discussion

MDL 105,519 has been identified as a selective antagonist for the glycine recognition site on the NMDA receptor complex. This compound successfully incorporates the structural features of two previously described classes of NMDA receptor glycine antagonists to form a highly potent and selective ligand for this binding site. Indole- or quinoline-containing dicarboxylic acids such as MDL 29,951 and MDL 100,748 (Baron et al., 1992; Harrison et al., 1990; Salituro et al., 1990) reveal the presence of a distal anionic binding moiety in the recognition site. Similarly, ligands with a distal aryl-containing substituent such as MDL 100,458 and MDL 102,288 (Salituro et al., 1991; Kehne et al., 1995; for reviews, see Baron et al., 1994 or Leeson and Iversen, 1994) indicate the presence of distal hydrophobic pockets. MDL 105,519 possesses both a distally located carboxylic acid function and an aromatic moiety conformationally fixed to assume the location of the respective side-chain residues in MDL 100,748 and MDL 100,458. This combination has led to a roughly 10–15-fold increase in in vitro potency at the recognition site, a further enhancement of selectivity, and improved brain penetration suggested by in vivo biochemical and anticonvulsant tests.

MDL 105,519 demonstrated low nM affinity for the glycine recognition site labeled by [^{3}H]glycine. MDL 105,519 is roughly equipotent to the most potent recently described glycine site antagonists including L-701,324 (Kulagowski et al., 1994) and ACEA 1021 (Woodward et al., 1995). MDL 105,519 displayed no significant affinity for non-NMDA glutamate receptors or other receptor types with selectivity for the NMDA receptor-associated glycine recognition site estimated at roughly 10 000-fold. The most prominent additional activity of MDL 105,519 was inhibition of binding to the benzodiazepine site with approximate IC_{50} of 100 μM. This is in contrast to ACEA 1021 which exhibits appreciable affinity for non-NMDA receptor sites (Woodward et al., 1995). It should be noted that while glycine site antagonism is the predominant activity of MDL 105,519 in vitro, in in vivo paradigms the tissue concentrations of MDL 105,519 are not known and therefore elements of the pharmacological profile may be contributed by other actions of the compound.

In broken membrane preparations, MDL 105,519 inhibited the binding of the open-channel blocker [^{3}H]TCP. This indicates that MDL 105,519 stabilizes the receptor in the closed configuration. This result is similar to that observed in the presence of glutamate and glycine recognition site antagonists (e.g., Baron et al., 1990, 1992; Bon-

haus and McNamara, 1988; Reynolds and Miller, 1988). Exogenous NMDA and glycine increase specific [^{3}H]TCP binding in a concentration-dependent fashion consistent with their ability to regulate channel opening. MDL 105,519 could prevent NMDA-mediated increases suggesting that this compound regulated the pore at a location distinct from the glutamate recognition site. In contrast, MDL 105,519 affected the potency but not the extent of glycine-evoked increases in [^{3}H]TCP binding consistent with a competitive action at the glycine recognition site.

Antagonism was also evident in the ability of MDL 105,519 to inhibit NMDA-elicited biochemical changes in vitro. MDL 105,519 inhibited NMDA-evoked increases in cGMP in cerebellar slices, cytosolic free Ca^{2+} rises in cerebellar granule cells, and Na^+ and Ca^{2+} currents in cortical neurons. The antagonism was shown to be non-competitive in nature, reversible by washing and prevented by inclusion of the glycine-mimetic D-serine. The measured affinity of the antagonist was approximately 30-fold less in the functional assays than assessed by occupancy measurements in the radioreceptor assays. This has been previously noted for other NMDA receptor antagonists and in the slice assay likely represents the contribution of endogenous glycine to the functional response (Baron et al., 1990, 1992). No quantitative determination of potency was made in the two biochemical tests utilizing cell monolayers; however, in all three preparations, concentrations of 1–2 μM MDL 105,519 were found to completely inhibit the observed response. As the cell culture-based assays included 2–10 μM added glycine during the period of NMDA exposure, it is likely that similar glycine concentrations were achieved by endogenous stores of glycine in the slice preparation.

MDL 105,519 functioned as an NMDA receptor antagonist in vivo. Systemic administration of this compound to mice led to an inhibition of the ability of harmaline to elevate cerebellar cGMP content. The doses required for achieving this biochemical effect were similar when the compound was given either by the intraperitoneal or the intravenous route. These results indicate that MDL 105,519 can penetrate the central nervous system and help to define a dose range in which the compound exerts NMDA receptor antagonistic effects. Comparison was made to the quinoxaline-containing NMDA receptor glycine antagonist ACEA 1021, which was also active against harmaline-induced elevations of cGMP and displayed potency roughly 17-fold greater than MDL 105,519.

MDL 105,519 and ACEA 1021 were also characterized for their activities in a variety of anticonvulsant and side-effect tests performed in rats and mice. Both compounds showed dose-dependent activity in chemical, electrical, and genetic seizure models. The relative potency of the two agents was dependent upon both the species and model chosen. For example, ACEA 1021 was approximately 3-fold more potent than MDL 105,519 versus audiogenic seizures and quinolinic acid-induced seizures in mice but was almost 2-fold less potent versus electrically induced seizures in rats. The pharmacological effect of MDL 105,519 appeared to be considerably longer lasting in both species than that of ACEA 1021. MDL 105,519 produced motor impairment at doses in excess of those producing anticonvulsant effects. A 'selectivity ratio' of 1.9 was calculated using the ratio of the ED_{50} value in the rotorod test of motor ability divided by the ED_{50} value determined in the quinolinic acid seizure test. In contrast, ACEA 1021 was 3-fold more potent in producing motor disruption than in inhibiting quinolinic acid-induced seizures. The 'selectivity ratio' for ACEA 1021 was 0.3. These results indicate that NMDA receptor glycine antagonists differ in their relative potency for different types of behavioral or pharmacological effects.

Previously we have described the anxiolytic-like effects of NMDA receptor antagonists in a separation-induced vocalization model in rats (Kehne et al., 1991). An interesting characteristic of this model is that it appears to differentiate among various types of NMDA receptor glycine antagonists (Kehne et al., 1995). Certain NMDA receptor glycine antagonists are classified as atypical anxiolytics based on potency ratios greater than unity in tests assessing anxiolytic activity relative to those measuring motoric effects. Thus, we have identified examples of NMDA receptor glycine antagonists with potent anxiolytic activity but which are devoid of muscle-relaxant activity (Kehne et al., 1995). In the same manner, a second class of NMDA receptor glycine antagonists has been defined which possess muscle-relaxant and anxiolytic activity at similar doses (Kehne et al., 1995). Selected members of the two types of NMDA receptor glycine antagonists show marked differences in anticonvulsant tests with only the latter class exhibiting measurable efficacy. MDL 105,519 was evaluated in this test battery and found to affect motoric behavior at doses slightly lower than those required for anxiolytic effects. These data are consistent with the profile developed for MDL 105,519 in anticonvulsant tests and strengthen the hypothesis that anticonvulsant and muscle-relaxant activities share a common pharmacological substrate whereas anxiolytic activity can be differentiated with particular structural modifications.

NMDA receptor antagonists have been shown in preclinical and clinical studies to disrupt sensory information processing. The animal experiments have provided insight into the potential neurochemical basis for this disruption and have linked these changes to alterations in dopaminergic transmission. For example, psychomotor stimulation produced by NMDA receptor antagonists has been shown to be sensitive to dopamine D_2 receptor antagonists (Hoffman, 1992; Ouagazzal et al., 1993), suggesting that enhanced dopaminergic transmission may play a role. The projections of the A10 dopamine nucleus to the nucleus accumbens are generally believed to mediate the psychomotor effects of central stimulants (French et al., 1991; Löscher and Honack, 1992). High doses of MK-801 do

enhance dopamine synthesis as measured in both the striatum and nucleus accumbens. In contrast, MDL 105,519 was without effect on dopamine synthesis. Similarly, the high dose of MDL 105,519 did not alter extracellular dopamine concentrations in the nucleus accumbens, whereas the channel blocker MK-801 significantly elevated extracellular mesolimbic dopamine.

The difference in neurochemical profile between the NMDA receptor channel blocker MK-801 and the NMDA receptor glycine antagonist MDL 105,519 is suggestive of the existence of pharmacological differences between NMDA receptors in various brain regions. Molecular biological studies indicate the presence of multiple NMDA receptor-encoding isoforms with distinct anatomical localization (Monyer et al., 1992) and pharmacology (Laurie and Seeburg, 1994; Buller et al., 1994). Consistent with such diversity of structure is an apparent diversity of function. For example, Schmidt and Fadayel (1996) have shown that MK-801 has no effect on extracellular dopamine concentrations in the striatum but increases levels in the nucleus accumbens and prefrontal cortex. Furthermore, these investigators describe qualitative differences in the pharmacological modulation of the increased extracellular dopamine concentrations in the two terminal fields (Schmidt and Fadayel, 1996). These results indicate that NMDA receptor ligand recognition sites in different brain regions show differing pharmacological characteristics and physiological roles.

The sensory disruption observed in man can also be modeled in rats by measuring a compound's ability to alter the modulating effect of a sensory stimulus on a simple reflex behavior (prepulse inhibition paradigm; Geyer et al., 1990; Hoffman and Ison, 1980). We have characterized the psychotomimetic liability of MDL 105,519 both behaviorally and neurochemically. Doses of MDL 105,519 several fold greater than those effective in other pharmacological models of NMDA receptor antagonism were ineffective in elevating mesolimbic dopamine turnover. Furthermore, although high doses of MDL 105,519 produced prominent muscle relaxation, as reflected in attenuation of the basal startle reflex, the effect of the prepulse stimulus was clearly evident. These neurochemical and behavioral data are in sharp contrast to those observed with NMDA receptor channel blockers such as CNS 1102, MK-801, and PCP and possibly indicate a lower psychotomimetic risk for NMDA receptor glycine antagonists.

In summary, we have described MDL 105,519 as a potent and selective ligand for the NMDA receptor-associated glycine recognition site. This compound is a noncompetitive antagonist of NMDA-elicited biochemical responses both in vitro and in vivo. The in vivo pharmacological profile indicates that this compound displays anticonvulsant, muscle-relaxant, and anxiolytic activity over a similar dose range. While interesting, these therapeutic targets would require potent oral activity, a prolonged duration of action, and a side-effect profile permitting chronic dosing. Oral bioavailability of MDL 105,519 has not been determined and current work is focusing on the use of MDL 105,519 and analogs as acute neuroprotective agents in cases of stroke or brain trauma. As psychotomimetic disturbances have been one of the prominent adverse events in stroke trials involving NMDA receptor antagonists (Muir and Lees, 1995), we are encouraged that neurochemical and behavioral tests indicate a low liability for psychotomimetic effects relative to NMDA receptor channel blockers. Confirmation of the superiority of this mechanistic approach relative to other NMDA receptor-based therapeutic agents will await exploration of the behavioral effects of these agents in man.

Acknowledgements

We are grateful to Amy L. Slone, Scott Meikrantz, David Janowick, Donald Matthews and Matthieu Poirot for technical assistance. Drs. David Rampe and Carl Rogers provided valuable advice for the electrophysiology experiments. Drs. Raymond Gross and Robert Farr provided important advice concerning synthesis of the molecules described.

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