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NEMATOCIDAL ACTIVITY OF MK-801 ANALOGS AND RELATED DRUGS

STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—A series of dibenzo[*a*,*d*]cycloalkenimines were evaluated for their affinity to the (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801) binding site in *Caenorhabditis elegans* membranes and their nematocidal activity. The (+)-MK-801 enantiomer (**1**) had a higher affinity (K_d = 240 nM) for its specific binding site and was a more potent nematocidal agent than the (−)-MK-801 enantiomer (**−1**). Ring expansion to form the dibenzo[*a*,*d*]cyclooctenimine analogs generally resulted in more potent compounds. The most potent of this series (**23**) was approximately 7-fold more potent than (+)-MK-801. A good correlation was established between binding affinities and nematocidal activity for all of the analogs that were tested. However, there was no correlation between binding to *C. elegans* membranes and affinity for mammalian MK-801 binding sites. Other noncompetitive inhibitors of the mammalian *N*-methyl-D-aspartate site were examined, and a series of diphenylguanidines were identified as potent competitive inhibitors of MK-801 binding to *C. elegans* membranes, in addition to displaying potent nematocidal activity. The most potent diphenylguanidine analog (**24**) was approximately 80-fold more potent than (+)-MK-801 in both its affinity for the MK-801 binding site and as a nematocidal agent. Molecular modeling studies support the hypothesis that the diphenylguanidines and MK-801 are binding to the same site and suggest that more potent compounds may be developed by effective modeling of the existing compounds.

Key words: anthelmintic; nematodes; *Caenorhabditis elegans*; MK-801 receptors

MK-801§ is a potent and selective noncompetitive antagonist of the NMDA subclass of glutamate receptors in mammalian tissues [1, 2]. Specific high-affinity MK-801 binding sites have been identified and characterized in rat brain preparations [1, 3, 4]. Electrophysiological and biochemical data suggest that MK-801 binds directly in the NMDA receptor channel complex and only when the channel has been opened by glutamate [5–7]. We recently reported that MK-801 has nematocidal activity, and this activity is mediated via a ligand–receptor interaction [8]. The pharmacological characteristics of the NMDA receptor complex described in mammalian neuronal tissue are distinct from those observed for the MK-801 binding site in nematode preparations. The mammalian binding site is modulated by magnesium ions, zinc and glycine [9–16], whereas the *Caenorhabditis elegans* MK-801 binding site is independent of these factors. Furthermore, polyamines potentiate the binding of [^{3}H]MK-801 to rat brain binding sites [17, 18] and inhibit binding to *C. elegans* binding sites [8]. The physiological significance of these interactions is not known.

A series of *N*,*N*′-diarylguanidines has been demonstrated to be noncompetitive NMDA antagonists that bind to the same site as MK-801 in rat brain tissue [19]. Since MK-801 is a potent nematocidal agent, we examined a series of diphenylguanidines to determine whether they too exhibit nematocidal activity. In this manuscript, we report the correlation between nematocidal activity and receptor binding affinities for a series of MK-801 analogs and diphenylguanidines.

MATERIALS AND METHODS

Materials. [^{3}H](+)-MK-801 (27.3 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Purity of [^{3}H](+)-MK-801 was confirmed to be greater than 96% using thin-layer chromatography on silica gel with a solvent system of chloroform:methanol (9:1). (+)- and (−)-MK-801 (5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate) were provided by Drs. T. Lyle and P. Anderson, Merck Research Laboratories, West Point, PA. All of the other MK-801 and diphenylguanidine analogs were obtained from the Merck Chemical Sample Collection (Rahway, NJ).

Membrane preparation. C. elegans, N2 strain was cultivated on NG agar plates covered with a lawn of *Escherichia coli*, as previously described [20]. Worms (all stages) were washed off the plates with 5 mM Trizma base, adjusted to pH 7.2 with HCl. The

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§ Abbreviations: MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; and NMDA, *N*-methyl-D-aspartate.

worms were washed once for 2 min at 1000 *g*, resuspended in buffer (approximately 20,000 worms/mL), and then broken up by homogenization in a Braun Homogenizer (Ace Scientific, New Brunswick, NJ) using 0.5 mm glass beads for 30 sec. The homogenate was centrifuged for 2 min at 1000 *g*, and the supernatant was centrifuged for 20 min at 28,000 *g*. The resulting pellet was resuspended in buffer and washed three more times by centrifugation at 28,000 *g* for 20 min in order to dilute cytoplasmic contaminants as well as possible. The final pellet was resuspended in Tris buffer and used immediately.

MK-801 binding. C. elegans membranes were incubated with [^{3}H]MK-801 at 22° for 15 min in the presence (nonspecific binding) or absence (total binding) of a 500-fold molar excess of unlabeled (+)-MK-801 in glass tubes (13 × 100 mm). The incubation was terminated by rapid filtration over Whatman GF/B filters (pre-soaked for 1 hr in 0.15% polyethylimine in order to minimize nonspecific binding) and rinsed with 15 mL (3 × 5 mL) of ice-cold Tris buffer. The filters were placed into glass vials containing 10 mL Aquasol II (New England Nuclear), and the radioactivity was determined by liquid scintillation spectrometry at 62% efficiency. At saturating concentrations of [^{3}H]MK-801, the nonspecific binding represented approximately 40% of the total counts. Specific binding was determined by subtracting nonspecific from total binding. The K_i values for MK-801 analogs and related compounds were determined as described by Cheng and Prusoff [21]. The membranes were incubated with 250 nM [^{3}H]MK-801 with increasing concentrations of the inhibitor. Under these conditions, 82% of the total [^{3}H]MK-801 binding was displaced by high concentrations of MK-801 (100 μM). High concentrations of the diphenylguanidines (**24–28**) inhibited specific [^{3}H]MK-801 binding to the same extent as MK-801.

Motility assay. Worms were rinsed off the agar plates with Tris buffer at 22°, washed twice by centrifugation at 1000 *g* for 2 min, and then resuspended in Tris buffer. Aliquots of the worms (50 μL, approximately 100 worms) were placed into 13 × 100 mm glass test tubes. The compounds to be tested were added to the worms in a final volume of 500 μL containing 1% dimethyl sulfoxide. After a 32 hr incubation at 22°, the number of worms still motile was determined by examination with a low power dissecting microscope. Greater than 90% of the worms continued to swim vigorously in the control tube.

Molecular modeling. Conformational searches on diphenylguanidines and geometry optimizations were performed using an empirical forcefield, OPTIMOL.* Other conformational searches were performed using DGEOM, an adaption of Crippen's distance geometry method [22] followed by geometry optimization using OPTIMOL. Conformations were selected using the program FLUSTER.† Comparison of MK-801 with diphenylguanidine structural types was done using the Steric and Electrostatic Alignment (SEAL) methods of Kearsley and Smith [23].

Protein assays. Protein concentrations were determined using the dye staining technique of Bradford [24] or in some cases following the procedure of Lowry *et al.* [25].

RESULTS

Inhibition of [^{3}H](+)*-MK-801 binding by MK-801 analogs.* A variety of MK-801 analogs were evaluated for their affinities in the [^{3}H](+)-MK-801 radioreceptor assay using a *C. elegans* membrane preparation. The specific binding of [^{3}H](+)-MK-801 was inhibited by (+)-MK-801 (**1**), in a concentration-dependent manner with a K_i value of 240 nM, whereas the (−)-5*S*,10*R*-enantiomer (−**1**) had a K_i of 1750 nM. Table 1 lists the affinities for two series of MK-801 analogs: the dibenzo[*a*,*d*]cyclohepten-5,10-imines (**1–11**) and dibenzo[*a*,*d*]cycloocten-6,12-imines (**12–23**). Substitution of electron withdrawing groups at the 3-position [either halogens (**2** and **3**) or SCF_3 (**4**)] increased or maintained the affinity for the *C. elegans* MK-801 binding site, whereas in the rat brain binding assay, the 3-trifluoromethyl-thio analog (**4**) is nearly inactive. Conversely, substitution with an amine in the 3-position (**5**) resulted in an 8-fold decrease of the binding affinity to the *C. elegans* binding site and no change in the affinity for the rat brain binding site [26].

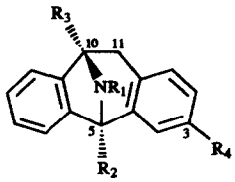
The most active MK-801 analogs evaluated were derived from a series of compounds with an expanded center ring. Molecular models suggest that the ring expansion of the cycloheptenimine ring results in considerable differences in the molecular topography [26]. The increased affinity of this series is demonstrated by comparison of (**7**), (**8**) and (**9**) with (**15**), (**20**) and (**23**) respectively. In each case, the binding affinity of the dibenzo[*a*,*d*]cyclooctenimines relative to the homologous dibenzo[*a*,*d*]cycloheptenimines was increased by at least 9-fold. Substitution of an allyl function at the bridge head nitrogen resulted in the compound (**23**) with the highest affinity for the *C. elegans* MK-801 binding site. This compound (**23**) was 7-fold more potent than (+)-MK-801 in the [^{3}H]MK-801 displacement assay, whereas the comparable compound in the cycloheptenimine series (**9**) was 40-fold less active than (+)-MK-801.

Modifications at other regions of the molecule resulted in decreased binding activity. *Endo*-hydroxylation at the C-10 position (**6**) led to inactivity in the *C. elegans* assay [83-fold less active than (+)-

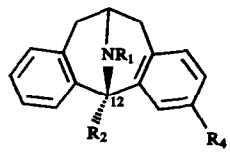
* OPTIMOL contains the MM2X and MMFF forcefields, which differ from MM2 principally in that lone pairs on heteroatoms are not used and in that electrostatic interactions take place between atom-centered charges, allowing proper treatment of charged systems. MM2X has been parameterized for a wide range of functional groups but shares many parameters with MM2. OPTIMOL has been developed at Merck by T. A. Halgren and other members of the Molecular Systems Department, Merck Research Laboratories.

† FLUSTER allows the selection of a user-determined number of structures with maximally dissimilar geometries and low energies based on the root mean squared deviation in the distances between selected atoms. FLUSTER has been developed at Merck Research Laboratories by S. K. Kearsley of the Molecular Systems Department.

Table 1. Inhibition of [^{3}H]MK-801 binding by MK-801 and MK-801 analogs



(1-11)

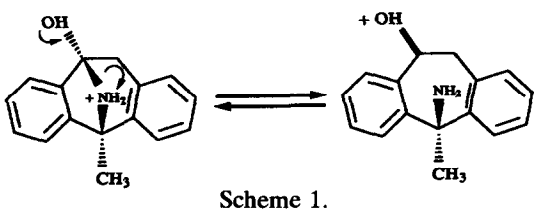


(12-23)

Compound	R_1	R_2	R_3	R_4	K_i (*C. elegans*) (nM)	K_i (rat)* (nM)
1 (+)-MK-801	H	CH_3	H	H	240	31
-1	H	CH_3	H	H	1,750	211
2	H	CH_3	H	Cl	130	11
3	H	CH_3	H	Br	200	80
4	H	CH_3	H	SCF_3	650	17,000
5	H	CH_3	H	NH_2	2,000	27
6	H	CH_3	OH	H	20,000	77
7	H	CH_2CH_3	H	H	900	45
8	CH_3	CH_3	H	H	9,000	610
9	$CH_2CH{=}CH_2$	CH_3	H	H	10,000	ND†
10	CH_2Ph	CH_2CH_3	H	H	10,000	ND
11	OH	CH_3	H	H	1,200	19,000
12	H	H		H	>20,000	5,100
13	H	CH_3		H	650	139
14	$CH_2C_6H_5$	CH_3		H	15,000	ND
15	H	CH_2CH_3		H	100	120
16	H	CH_3		Cl	145	110
17	OH	CH_3		H	20,000	ND
18	$COCH_3$	CH_3		H	1,500	ND
19	CH_3	Br		H	1,000	ND
20	CH_3	CH_3		H	250	1,200
21	CH_3	CH_2CH_3		H	180	2,040
22	CH_2CH_3	CH_3		H	110	ND
23	$CH_2CH{=}CH_2$	CH_3		H	35	ND

* Data reported in Ref. 26.
† Not determined.

MK-801, **1**]. This may be due to the expected sensitivity of these compounds to ring opening equilibria, as shown in Scheme 1. The equilibrium in Scheme 1 will be "driven" toward the ring opened form by protonation of the bridge-head amine; the pK_a of this amine lies in the range 9.5 to 10.5 (alkylation to a tertiary amine will lower the pK_a) and will therefore be expected to be protonated at physiological pH. Interestingly, **6** is only slightly less active than (+)-MK-801 in the rat brain membrane binding assay (Table 1).

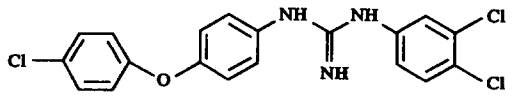


Scheme 1.

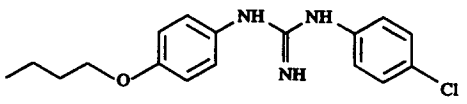
For comparison, Thompson *et al.* [26] report a 4-fold loss of activity for the 11-*exo*-OH compared with (+)-MK-801 and a 50-fold loss of activity for the *endo* isomer in the rat brain membrane assay. Leeson *et al.* [27] show similar effects on hydroxylation of the MK-801 derivatives.

As found by Thompson *et al.* [26] for the rat brain membrane receptors, there was increased affinity for the MK-801 binding site by compounds that have a C-12 methyl [compare **12** with **13** in which the addition of a methyl at C-12 produces a moderately active compound (K_i = 650 nM) from an inactive compound (K_i > 20,000 nM)]. The further addition of a methylene at the C-12 position led to a modest increase in binding for the cyclooctenimine (6.5-fold increase for **13** compared with **15**), which is not seen in the cycloheptenimine series; indeed there is a decrease in binding by nearly 4-fold when **7** is compared with (+)-MK-801. Contrary to the results obtained by Thompson *et al.* [26] for the rat brain membrane binding assay, alkylation at the ring nitrogen showed an increase in binding affinity in the *C. elegans* assay for the cyclooctenimines. The increases in binding were modest but were incremental at least to the addition of three carbon units (increases in binding of approximately 2 to 3-fold for each carbon added; compare **13**, **20**, **22** and **23**). A benzylic substituent at the ring nitrogen might be

Table 2. Inhibition of MK-801 binding by diphenylguanidine analogs



(24)



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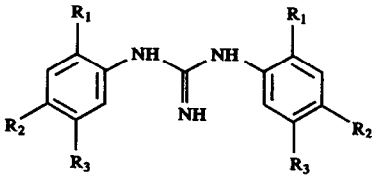

(26-31)

Compound	R_1	R_2	R_3	K_i (*C. elegans*) (nM)	K_i (rat)* (nM)
24				3	ND†
25				15	ND
26	H	H	H	40	ND
27	CH_3	H	H	40	10,700
28	OCH_3	H	H	40	1,600
29	CH_3	H	NO_2	750	ND
30	H	H	NO_2	900	ND
31	H	SO_2NH_2	H	2000	ND

* K_i values for compounds **27** and **28** were reported in Refs. 19 and 28, respectively.
† Not determined.

too large for this site, since it resulted in an inactive compound (**14**). Such structure–activity relationship (SAR) effects at the ring nitrogen are not seen for the cycloheptenimines; simple alkylation with methyl resulted in an inactive compound [(+)-MK-801 compared with **8**]. Further differences between these series were seen when the basicity of the ring nitrogen was altered. Cyclooctenimines showed a 13-fold decrease in binding when the ring nitrogen was acetylated (**18** compared with its ethyl analog, **22**) and were inactive when the nitrogen was hydroxylated, **17**. In contrast, binding of the cycloheptenimines was decreased only 5-fold when the ring nitrogen was hydroxylated, **11**. The symmetry of the cyclooctenimines may account for this surprising activity. These results emphasize the differences in structural requirements for active ligands between the MK-801 binding site in rat brain and *C. elegans*.

Inhibition of MK-801 binding by diphenylguanidines. A series of diphenylguanidines were evaluated as inhibitors of MK-801 binding to *C. elegans* membranes. As shown in Table 2, several of the diphenylguanidines were more potent than any of the MK-801 analogs that were evaluated. The most potent compounds tested were (**24**) and (**25**) with K_i values of 3 and 15 nM, respectively.[^{3}H]MK-801 binding was evaluated in the presence of different concentrations of (**24**) and, as shown in Fig. 1, the inhibition was of a competitive nature.

Correlation of nematocidal activity with affinity for the MK-801 binding site. The most potent MK-801 analogs and diphenylguanidines were also evaluated in the *C. elegans* motility assay in order to quantitate

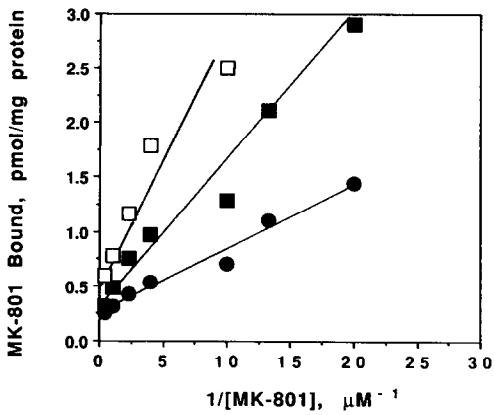

Fig. 1. Competitive inhibition of MK-801 binding by the *N,N'*-diarylguanidine (**24**). Specific MK-801 binding to *C. elegans* membranes was determined in the absence (●) or presence of 25 nM (■) or 20 nM (□) concentration of compound **24**. A double-reciprocal plot of 1/bound versus 1[MK-801] was used to analyze the data for competitive inhibition.

their nematocidal activity. As shown in Fig. 2, there was an excellent correlation between binding affinity (K_i values) and nematocidal activity (LD_{50} values). The most potent nematocidal compounds were **2, 3, 15, 16, 20–23** and **24–28**. These were also the compounds with the highest affinity for the MK-801 binding site. Conversely, the compounds with low

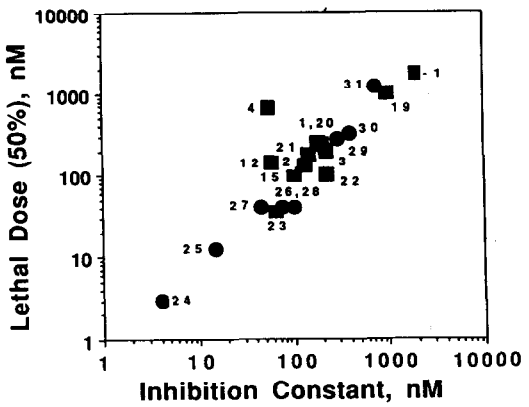

Fig. 2. Correlation between binding affinities of MK-801 analogs (■) and *N,N'*-diarylguanidines (●) and their biological potencies on *C. elegans* motility *in vivo*. Inhibition constants (K_i) were determined using the formula: $K_i = IC_{50}/(1 + c/K_d)$, where the IC_{50} is the concentration of the drug required to inhibit 50% of the specific binding (determined by log probit plots), and c is the concentration of [^{3}H]MK-801. The motility of *C. elegans* was determined as described in the text. The EC_{50} is the concentration of drug needed to produce immotility in 50% of the worms. The results are the mean values from two to four experiments, each one having at least three determinations (SE less than 18%). A correlation coefficient (r) of 0.825 was calculated by linear regression analysis. For the log–log plot, $r = 0.8175$. The number next to the data point represents the compound number provided in Tables 1 and 2. Compounds that had EC_{50} values greater than 1500 nM are not shown.

affinity for the MK-801 binding site were also the least potent nematocidal compounds. These results strongly support the concept that the nematocidal activity of the MK-801 and diphenylguanidine analogs is modulated via a ligand-binding site interaction.

Molecular modeling. The competitive inhibition of binding of (+)-MK-801 by the cycloheptenimines, cyclooctenimines and diphenylguanidines suggests that at least portions of these classes of molecules are interacting with similar regions of the binding protein and are presenting similar functionality at the binding site. Comparison of the structures of cycloheptenimines and the cyclooctenimines is trivial and was performed manually; the amine, protonated at pH 7, and the aromatic rings are clearly dominant features of these compounds and, as shown in Fig. 3, overlap very well. This overlap viewed along with the differences in the SAR discussed previously further highlights the effects subtle differences in structure may have on binding; the effects of increasing the ring size by one methylene although not leading to large structural differences do lead to striking differences in the SAR of these series.

Comparison of the structures of the cycloheptenimines and the diphenylguanidines is more challenging. Again the amine functionality and the guanidino group, both expected to be protonated at pH 7, and the aromatic rings are clearly dominant features. However, it is less clear how the more conformationally flexible diphenylguanidines can mimic the rigid "butterfly" structure of the cycloheptenimines. To investigate this superposition, a number of low energy conformations of **24** were generated, and each was automatically superposed with (+)-MK-801. Each superposition was scored according to the degree of overlap and the type of the atoms that were matched (steric and electrostatic overlap function). The highest scoring overlap is shown in Fig. 4. From Fig. 4 it is clear that the amine and the guanidino groups and the aromatic functionality overlap; similar functionality can be expected to be presented to the binding site in a similar way for these different structural classes.

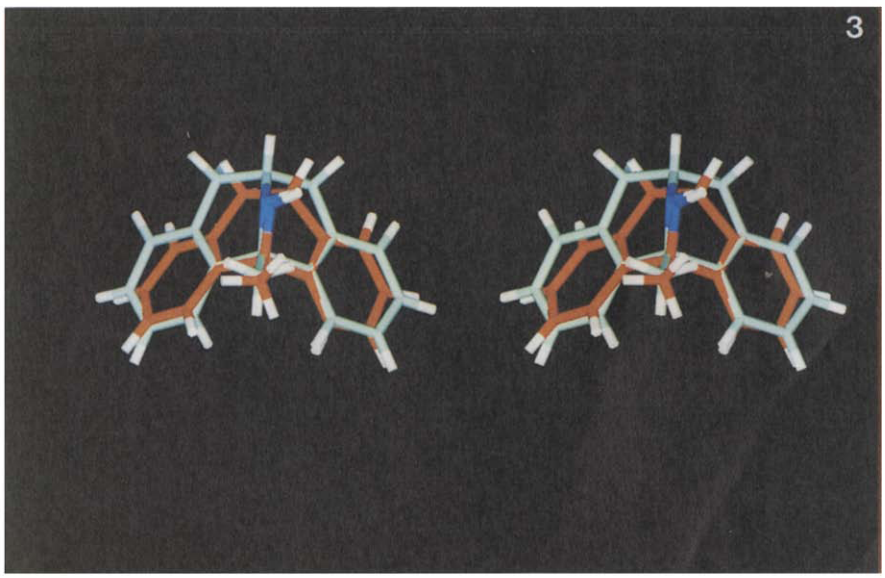

Fig. 3. Comparison of the structures of cycloheptenimines and the cyclooctenimines.

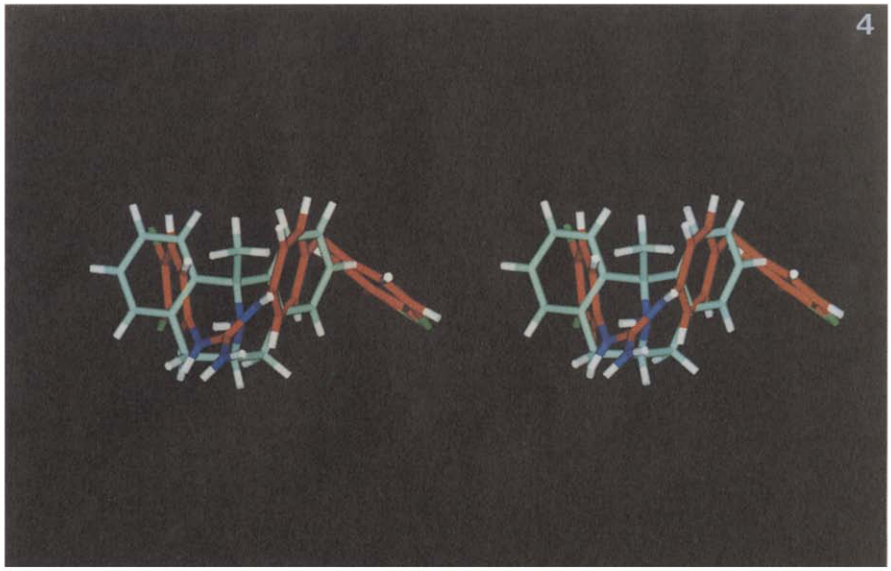

Fig. 4. The top scoring SEAL result from comparison of (+)-MK-801 and compound **24**.

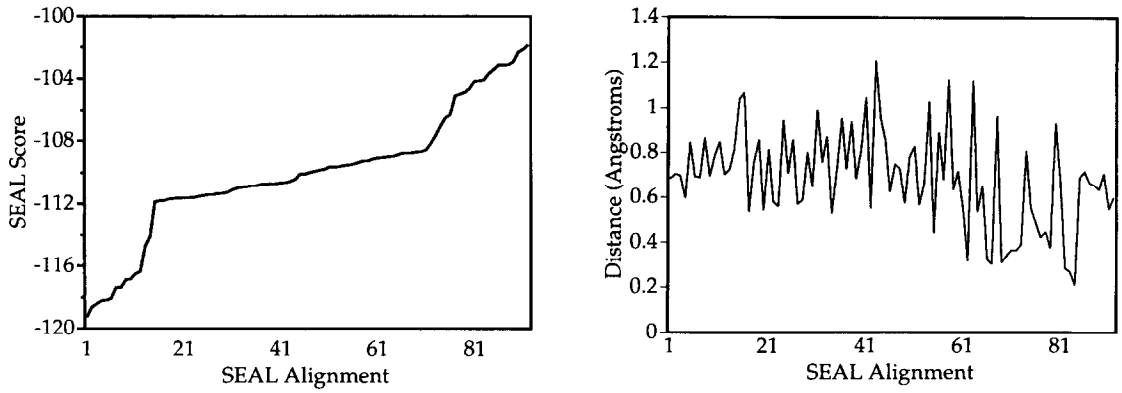

Fig. 5. (Left panel) Plot of the SEAL score vs alignment number for the top 100 results from the automatic comparison of (+)-MK-801 with the 50 structurally most dissimilar conformations of **24**. (Right panel) Plot of the distance (Å) between the amine N of (+)-MK-801 and the guanidino C of **24** for each of the top 100 SEAL alignments.

Figure 5 shows details of the results of the SEAL calculations. The first 10 alignments are the highest scoring (initial part of the curve in Fig. 5, left panel), and all of these structures show close overlap of the guanidino carbon and the amine nitrogen (between 0.5 and 0.8 Å; Fig. 5, right panel). In addition, for these alignments the aromatic rings of (+)-MK-801 and **24** overlap to a reasonable extent: the average distance between the centroids of the aromatic rings of (+)-MK-801 and their counterparts in **24** are 1.1 and 1.4 Å, respectively. The conformations of diphenylguanidines that were used in the SEAL study are shown in Fig. 6 on a contour plot produced from a systematic search of the guanidino N—C torsions of **24** using OPTIMOL (the torsions were changed in increments of 15° and at each step the geometry was optimized). All conformations used in the SEAL calculation correspond to low energy points on the torsional surface. The central region of the contour plot (which corresponds to the low energy "S" and "W" regions found for robenidine [29]), although of low energy, represents extended conformations placing the aromatic rings of the diphenylguanidines too far apart to overlap with (+)-MK-801.

DISCUSSION

MK-801 is a nematocidal compound that binds specifically and with high affinity to *C. elegans* membranes [8]. In this study, a series of MK-801 analogs has been evaluated, and a strong correlation

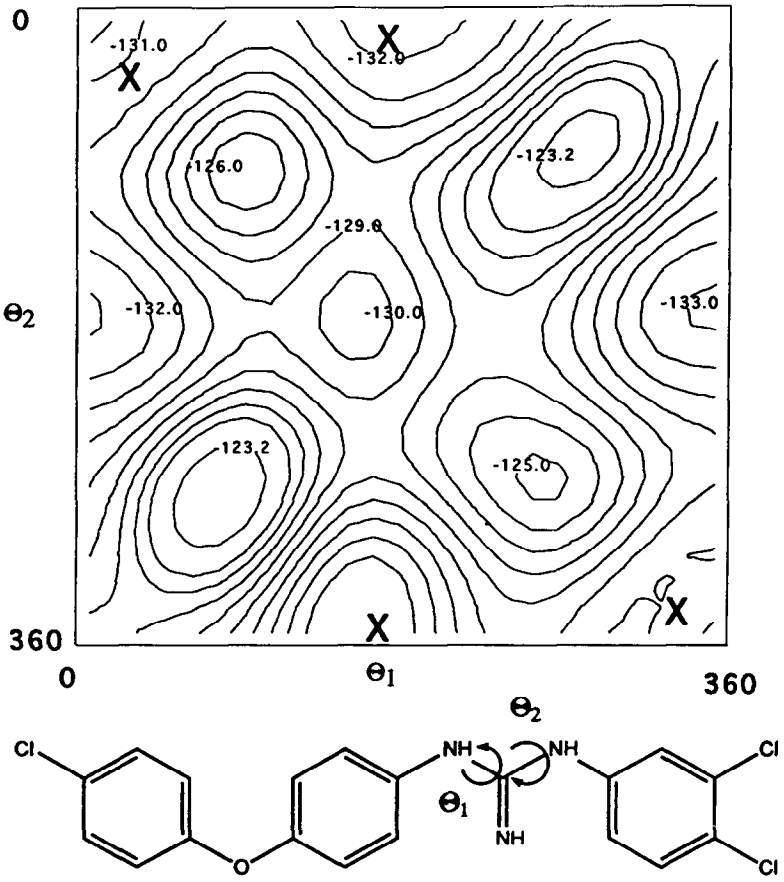

Fig. 6. Contour map (OPTIMOL) of **24** for the torsions Θ_1 and Θ_2. The symbol **X** marks the conformations for **24** that scored in the top 100 SEAL alignments.

between the nematocidal activity and affinity for the MK-801 binding site has been established, consistent with the hypothesis that the ligand-binding site interaction mediates the nematocidal activity. There are many pharmacological distinctions between the MK-801 binding site in *C. elegans* and mammalian neuronal tissue. First, polyamines inhibit MK-801 binding to *C. elegans* membranes [8] and stimulate the binding to rat brain membrane preparations [17, 18]. Second, MK-801 binding to rat brain receptors is modulated by glycine, serine and zinc [9–16], and the *C. elegans* binding site is unaffected by these agents [8]. Furthermore, a large number of MK-801 analogs have been synthesized and evaluated in the rat brain binding assay [26, 27, 30], and there is no correlation between binding affinities for the *C. elegans* and rat brain binding site. The pharmacological differentiation of binding requirements between *C. elegans* and mammalian brain suggests that this binding site is a viable target for the development of anthelmintic drugs that will distinguish host and parasite targets.

To identify more potent nematocidal compounds acting at the MK-801 binding site, various other compounds known to interact with the mammalian MK-801 binding site were examined. It had been reported that *N,N'*-disubstituted guanidines represent a new class of noncompetitive NMDA channel blockers interacting at the same binding site as MK-801 in neuronal tissue [19, 28]. Consequently, a series of diphenylguanidines was tested as nematocidal compounds and as inhibitors of MK-801 binding to *C. elegans* membranes. Several of these compounds had potent nematocidal activity, and the correlation between nematocidal activity and binding affinities suggests that the bioactivity is mediated via the ligand-binding site interaction. The most potent diphenylguanidine examined in this study is a competitive inhibitor of MK-801 binding to *C. elegans* membranes, and molecular modeling results are consistent with the hypothesis that both classes of compounds are structurally similar and likely to be interacting at the same binding site. Interestingly, Angelo *et al.* [31] described the antifilarial activity of a structurally similar series of diphenylguanidines (*N*-[4-[[4-alkoxy-3-[(dialkylamino)methyl]phenyl]amino] - 2 - pyrimidinyl] - *N'* - phenylguanidines). These compounds have excellent antifilarial activity against adult *Litomosoides carinii* worms; however, they lacked activity against the circulating immature forms of the worms and consequently were not pursued. It is not known whether the antifilarial activity of these compounds is mediated via an interaction with an MK-801

binding site. However, the results presented in this paper demonstrate that the nematode MK-801 binding site is pharmacologically distinct from the mammalian binding site and that molecular modeling may facilitate the development of novel compounds useful in the treatment of a broad spectrum of nematocidal infections.

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