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The Ligand Binding Region of the Sigma-1 Receptor: Studies Utilizing Photoaffinity Probes, Sphingosine and N-Alkylamines

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Abstract

The sigma-1 receptor is a 26 kDa endoplasmic reticulum resident membrane protein that has been shown to have chaperone activity in addition to its promiscuous binding to pharmacological agents. Ligand binding domain(s) of the sigma-1 receptor have been identified using the *E. coli* expressed and purified receptor protein and novel radioiodinated azido photoaffinity probes followed by pro-teolytic and chemical cleavage strategies. The outcome of these experiments indicates that the sigma-1 receptor ligand binding regions are formed primarily by juxtaposition of its second and third hydrophobic domains, regions where the protein shares considerable homology with the fungal enzyme, sterol isomerase that is essential for the biosynthesis of ergosterol. Data indicate that these hydrophobic steroid binding domain like (SBDL) regions on the sigma-1 receptor are likely to interact selectively with N-alkyl amines such as the endogenous sphingolipids and with synthetic N-alkylamines and N-aralkylamines derivatives. A proposed model for the sigma-1 receptor is presented.

Keywords

Sigma-1 receptor; photoaffinity labeling; ligand binding region; shingosine; N-alkylamines

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I. THE SIGMA-1 RECEPTOR PROTEIN

Homology

The 223 amino acid sigma-1 receptor cloned from various mammalian species including guinea pig liver [1], human placental choriocarcinoma cells [2], rat brain [3], and mouse kidney [4] shares 90% sequence identity and 95% similarity. While the sigma-1 receptor has no sequence homology to any existing mammalian proteins, it shares 30.3% sequence identity and 66.4% similarity to ERG2, a fungal gene encoding a C8-C7 sterol isomerase from *Magnaporthe grisea*, *Saccharomyces cerevisiae*, and *Ustilago maydis* [1]. ERG2 is an enzyme that catalyzes the isomerization of C8(9) [1] double bond in the B-ring of sterols to the C7(8) position, an essential step in steroid biosynthesis of yeast and fungi. Despite the high sequence homology between the sigma-1 receptor and yeast sterol isomerase, overexpression of the sigma-1 receptor failed to rescue an ERG2 deficient strain of the yeast *Saccharomyces cerevisiae* [1]. The ERG2 functional equivalent in mammals is called the emopamil-binding protein (EBP) which shares very little sequence homology to either the sigma-1 receptor or ERG2 but was able to rescue the sterol isomerase deficient strain of yeast [5].

Membrane Topology of the Sigma-1 Receptor

Similar to the yeast sterol isomerase, the hydrophobicity plot of the sigma-1 receptor primary protein sequence has identified the presence of three hydrophobic domains: amino acid residues 11–29, 91–109 and 176–194 (Fig. (1, I, II and III) respectively) [6]. Originally, the sigma-1 receptor was thought to contain a single trans-membrane (TM) region (hydrophobic region I) [1], however, recent data from two separate groups support a two TM model for the sigma-1 receptor [7, 8].

The first model was proposed by Aydar *et al.* [7] using antibody accessibility studies directed against the separate C and N terminal GFP fusion constructs of the sigma-1 receptor which were overexpressed in *Xenopus* oocytes. The results indicated that both N and C terminal GFP tags could be accessed by antibody only after permeabilization of the oocyte membranes suggesting that both the N and C termini were intracellular Fig. (1). Furthermore, using a surface biotin labeling approach, Aydar *et al.* [7] predicted that residues 30–80 (the region between hydrophobic segments I and II) were extracellular Fig. (1). Thus, hydrophobic regions I and II were suggested to be TM segments I and II with a 50 amino acid extracellular loop and a 123 amino acid intracellular C-terminus.

The second model was proposed by Hayashi and Su [8], who used protease protection methodologies and immunocytochemistry with sequence specific antibodies against different regions of the sigma-1 receptor, overexpressed in Chinese hamster ovary (CHO) cells. In these experiments, the sigma-1 receptor was exclusively localized to the endoplasmic reticulum (ER) and both N- and C-termini were topologically predicted to be inside the ER lumen Fig. (1). The precise reason(s) for the topological difference in the two models is currently unclear.

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The Sigma-1 Receptor Ligand Binding Site

The majority of the homologous residues between the sigma-1 receptor and sterol isomerase occurs in the second and the third hydrophobic domains of the sigma-1 receptor and the sterol-binding 'pocket' of the sterol isomerase [1, 9]. For example, 75% of the amino acids in the second hydrophobic domain of the sigma-1 receptor are identical in sequence to the sterol-binding 'pocket' in the fungal isomerase [9]. Thus the second and third hydrophobic regions have been variously referred to as steroid binding domain (SBD) I and II [1] or SBD-like (SBDL) I and II [10] respectively. Mutagenesis experiments on recombinant sigma-1 receptors have further led to elucidation of different domains involved in constituting the binding site. In one study, the sigma-1 receptor carrying one, two or three amino acid substitutions to alanine in the second hydrophobic domain were expressed in *Xenopus* oocytes [11]. The expression levels of the mutants were not significantly different but the binding properties of the sigma-1 receptor radioligands [^{3}H]-(+)-pentazocine and [^{3}H]-NE-100 with the mutants were concluded to be different as compared to the wild-type receptor although no clear explanation for these differences was provided [11]. These data suggested that residues in the second TM domain are important for ligand binding. A splice variant of the sigma-1 receptor was identified in a Jurkat T leukemia cell line that lacked exon 3 (corresponding to amino acids 119–149 in the protein) from the sigma receptor open reading frame [12]. This splice variant when expressed in Jurkat cells was found to be nonfunctional in ligand binding assays [12]. These data further indicated that regions in the C-terminal domain were either structurally important or were also essential for ligand binding.

Based on these observations and the observation that most of sigma ligands are positively charged, the authors predicted that anionic amino acid residues located in the C-terminal domain were essential for sigma-1 receptor ligand binding [13]. Two different approaches were used to test this hypothesis: 1) chemical modification of anionic residues with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide led to a marked reduction of ligand binding activity; 2) each anionic amino acid in the C-terminal region was individually mutated to a glycine and the influence of each individual mutation on ligand binding was assessed. These studies identified two amino acids, D126 and E172, which when mutated to glycine completely abolished [^{3}H]-haloperidol binding to the sigma-1 receptor (see Fig. (1)) [13].

The sigma-1 receptor has previously been reported to bind to progesterone [14]. A study performed by Palmer *et al.* [14] showed that the myc tagged sigma-1 receptor peptides 161–180 and 191–210 were able to bind to cholesterol-immobilized beads. Individual mutation of tyrosine 173 201 206 to serine prevented the peptides from binding to these beads suggesting that these amino acids are important for cholesterol binding.

Sigma-1 Receptor Photolabels

In addition to the mutagenesis work, several photoaffinity labels have been utilized for covalent derivatization of the sigma-1 receptor. Early photolabeling studies were reported on the sigma-1 receptor with tritiated derivatives as shown in Fig. (2): [^{3}H]-azido-ditolyl-guanidine ([^{3}H]-DTG (**I**), [^{3}H]-azido-emopamil (**II**), and [^{3}H]-haloperidol (**III**). We have utilized novel carrier free radioiodinated photoprobes to define the sigma-1 receptor ligand

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binding site using biochemical approaches. The sigma-1 specific photo-probe [^{125}I]-iodoazidococaine ([^{125}I]-IACoc) Fig. (2, IV) was found to specifically insert into aspartate 188 in the C-terminal domain of the sigma-1 receptor [15]. Additionally, truncation of 15 amino acids or more from the C-terminus of the sigma-1 receptor resulted in a loss of [^{125}I]-IACoc photolabeling [15].

Access to the purified guinea pig sigma-1 receptor in our laboratory [16] also advanced binding site determination efforts. Using protease digestion of the pure sigma-1 receptor with EndoLysC and subsequently by CNBr cleavage, it was found that a morpholine-based photoprobe [^{125}I]-iodoazidofenpropimorph Fig. (2, [^{125}I]-IAF, V) identified specific photoinsertion in both SBDLI and SBDLII regions [10]. Based on these results, two additional photoaffinity labels (Fig. (2), [^{125}I]-N-IACoc, **VI**; [^{125}I]-IAC44, **VII**) were synthesized. In the cocaine derived photoprobe [^{125}I]-N-IACoc, the photactivatable moiety was placed at the opposite end of the cocaine molecule compared to [^{125}I]-IACoc Fig. (2, IV) [17] while [^{125}I]-IAC44 was derived from the structure of fenpropimorph and provided a more flexible backbone [17]. [^{125}I]-N-IACoc was found to specifically label the SBDLI region in TM2 of the sigma-1 receptor while [^{125}I]-IAC44 labeled both regions: SBDLI and SBDLII [17]. In a separate study, a unique sulfhydryl cleavable, radioactive photo-crosslinker, [^{125}I]-IABM Fig. (2, VIII), was shown to intramolecularly tether together the SBDLI and SBDLII domains of the sigma-1 receptor [18]. The 8 Å crosslinking radius of this probe [18] indicated that these two domains were at a distance of 8 Å or closer in the native structure of the sigma-1 receptor. These data are summarized in Fig. (3) and support a current model of the sigma-1 receptor ligand binding site proposed by Fontanilla *et al.* [17] Fig. (4).

CONCLUSIONS

The sigma-1 receptor ligand binding region is formed primarily by juxaposition of the second and third hydrophobic domains as determined by radioiodinated photoaffinity approaches, analysis of specific photoprobe covalently labeled peptides and the use of a sulfhydryl specific radioiodinated photoactive compound. This conclusion is broadly supported by radioligand binding analyses of selective amino acids mutations and sequence deletions of the receptor. Because of the rather promiscuous binding to pharmacological agents, the term “binding region” is currently more appropriate for the sigma-1 receptor rather than the term, “binding site”. Atomic structures of the sigma-1 receptor with various types of ligands bound will provide important clarifications of this issue. Although not discussed in this review it is highly likely that the native structure of the sigma-1 receptor occurs as a dimer and/or a higher order oligomer. Whether oligomeric forms are important for the proper in situ functioning of the receptor are unknown at present but would be important areas of further experimentation.

II. INTERACTION OF N-ALKYL AMINES WITH SIGMA RECEPTORS

Interactions of Sphingosine and Derivatives with the Sigma-1 Receptor

Sigma receptors have been previously reported to be associated with lipid containing microdomains. The sigma-1 receptor was found to be present in cholesterol enriched,

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detergent insoluble lipid rafts of the ER in NG108 neuroblastoma cells where they were shown to be important for the compartmentalization of ER synthesized lipids [19]. In the ER lipid droplets, the sigma-1 receptor co-localized with caveolin-2, a cholesterol binding protein. Treatment with the sigma-1 receptor ligand (+)-pentazocine resulted in translocation of sigma-1 receptors from the ER lipid microdomains to cytoplasmic domains [19]. Furthermore, treatment of Chinese hamster ovary (CHO-K1) cells with sigma ligands enhanced the appearance of the sigma-1 receptor in focal adhesions [20]. In rat primary hippocampal cultures, sigma-1 receptors were shown to form galac-toceramide enriched lipid rafts and promote differentiation of oligodendrocytes [21].

The sigma-2 receptor has also been shown to localize to lipid raft fractions enriched in a raft marker flotillin 2 [22]. In MCF-7 and T47D breast tumor cells, sigma-2 receptor ligands CB-184 and BD737 caused increases in ceramide and sphingomyelin levels [23]. This has led to the suggestion that the sigma-2 receptor may have effects on cell growth and apoptosis through the sphingolipid pathway.

(2*R*-trans)-2-Butyl-5-heptylpyrrolidine Fig. (5, 1), isolated from the culture broth of *Streptomyces longispororuber* has been shown to be a high affinity sigma-1 receptor ligand [24] which inhibited [^{3}H]-(+)-pentazocine binding with an IC_{50} of 2 nM [25] and also exhibited high affinity for the dopamine (D_2) receptor [24]. Another structurally similar ligand, tridemorph, Fig. (5, 2), is an agricultural insecticide that inhibits the yeast sterol isomerase and has a high affinity for the sigma-1 receptor in guinea pig liver membranes as well as on the pure sigma-1 receptor [16, 26]. Both of these compounds share a common long alkyl chain extending from a secondary or tertiary nitrogen atom. Based on the structures of these compounds, alkyl amines such as dodecylamine and stearylamine Fig. (5, 3 and 4 respectively) were tested for inhibition of [^{3}H]-(+)-pentazocine binding to the *E. coli* purified sigma-1 receptor. Dodecylamine and stearylamine were found to inhibit [^{3}H]-(+)-pentazocine binding to the pure sigma-1 receptor with K_d values of 183 ± 20 nM and 8.5 ± 5.4 μM, respectively Fig. (5).

D-erythro sphingosine Fig. (5, 5), the major cellular sphingolipid base, conforms to the structural motif of having a long alkyl chain with a positively charged nitrogen atom. D-erythro sphingosine and its derivatives are important endogenous lipid mediators variously involved in multiple signal transduction pathways [27]. To determine if sphingosine and other similar compounds bind to the sigma-1 receptor, they were tested for inhibition of [^{3}H]-(+)-pentazocine binding to guinea pig liver membranes and the purified guinea pig sigma-1 receptor [25]. D-erythro sphingosine has two asymmetric chiral carbon atoms (2 and 3) and can exist in four possible stereoisomers; (D-erythro (2*S*,3*R*), D-threo (2*R*,3*R*), L-erythro (2*R*,3*S*) and L-threo (2*S*,3*S*)); we only used the D-erythro and L-threo Fig. (5, 6) stereoisomers of sphingosine, *N*,*N*′-dimethyl sphingosine Fig. (5, 7) and the C4 saturated analogue dihy-drosphingosine (D-erythro-sphinganine, Fig. (5, 8)). D-erythro-sphingosine, *N*,*N*′-dimethyl sphingosine, L-threo-sphingosine and sphinganine inhibited [^{3}H]-(+)-pentazocine binding to the purified sigma-1 receptor with K_d values of 140 ± 23 nM, 115 ± 30.0 nM, 18 ± 2.5 nM and 73 ± 9.5 nM respectively and to guinea pig liver membranes with K_d values of 13 ± 3.1 μM, 2.8 ± 0.7 μM, 8.3 ± 2.7 μM and 35 ± 8.5 μM respectively. For all four compounds that inhibited [^{3}H]-(+)-pentazocine binding, the K_d values for inhibition

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were lower by an order of magnitude on the pure sigma-1 receptor as compared to guinea pig liver membranes Table 2. These results are likely to be due to partitioning of the lipids into the membranes thus reducing the free concentration available for receptor interaction. Selectivity of binding was not observed between the D-erythro Fig. (5, 5) or L-threo Fig. (5, 6) stereoisomers of sphingosine however; the affinity of **(6)** was an order of magnitude higher than **(5)**. Sphinganine also showed affinities comparable to sphingosine in both guinea pig liver membranes and the pure sigma-1 receptor Table 2. Finally, D-erythro sphingosine showed a competitive binding mechanism against [^{3}H]-(+)-pentazocine [25].

Sphingosine can be phosphorylated to sphingosine-1 phosphate by the enzyme sphingosine kinase [28]. However, we found that sphingosine-1 phosphate did not inhibit the [^{3}H]-(+)-pentazocine binding to the sigma-1 receptor [25]. Moreover, the acylated derivatives of sphingosine, ceramide and ceramide-1 phosphate also failed to inhibit [^{3}H]-(+)-pentazocine binding to the sigma-1 receptor. High affinity sphingosine binding to the sigma-1 receptor raises the possibility that sphingosine and methylated derivatives of sphingosine may be endogenous regulators of the sigma-1 receptor.

Detection of Sphingosine in Sigma-1 Receptor Immunoprecipitates

The specific binding of sphingosine and not sphingosine-1 phosphate to the sigma-1 receptor raised the interesting possibility that sphingosine is an endogenous regulator of the sigma-1 receptor. An unequivocal demonstration of sphingosine interaction with the sigma-1 receptor *in situ* would be to capture sphingosine and the sigma-1 receptor in a complex. This strategy has been used previously for the nuclear receptor, transcription factor steroidogenic factor (SF-1) where it was shown that sphingosine could be immunoprecipitated with SF-1 from a H295R adrenocortical cell line [29]. This study utilized mass spectrometric methods for the detection of sphingosine in SF-1 immunoprecipitates [29, 30].

For the sigma-1 receptor we employed a similar approach using overexpression in Human embryonic kidney (HEK-293) cells followed by immunoprecipitation of cell homogenates with specific antibody against the sigma-1 receptor. The immunoprecipitated sigma-1 receptor was processed for mass spectrometric detection of sphingosine using methods reported previously [31]. As shown in Fig. (6), significant levels of sphingosine were detected in the sigma-1 receptor immunoprecipitates indicating that in a resting cellular state sphingosine occurs bound at the sigma-1 receptor. This interaction would also be expected to occur in other cells containing putatively large endogenous pools of sphingosine such as MCF-7 cells and PC12 cells [32]. Moreover, it would be of interest to examine the dynamics of endogenous bound sphingosine on the sigma-1 receptor in presence of small molecule sigma-1 receptor regulators such as pentazocine, haloperidol and *N*,*N*′-dimethyltryptamine [33]. The phosphorylated analog of sphingosine, sphingosine-1 phosphate was also detected in the same immunoprecipitates Fig. (6), however, its levels were significantly lower validating the ligand binding results and suggesting specificity for the interaction of the sigma-1 receptor with sphingosine.

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Interactions of 4-Nitrophenylpropyl-N-Alkylamine Derivatives with Sigma Receptors

Synthesis of N-alkylamine Derivatives—To expand our knowledge regarding the interaction of sphingosine-like molecules with the sigma-1 receptor, we designed, synthesized, and evaluated *N*-alkylamine derivatives with the goal of developing high affinity and selective sigma receptor ligands (Scheme 1). In addition, we have reported their utility as potential anti-cancer agents through multiplex cytotoxicity assays measuring growth inhibition of various cancer cell lines [34]. Utilizing competitive displacement of [^{3}H]-(+)-pentazocine binding to the pure guinea pig sigma-1 receptor [16] it was found that the affinities of *N*-alkylamines for the sigma-1 receptor increased in the series up to 12 carbons, or dodecylamine Table 1, and decreased with longer carbon chain length (to 18 carbons).

Addition of N-3-Phenylpropyl and N-3-(4-Nitrophenyl)Propyl to N-Alkylamines Resulted in an Increase in Affinity with Both Sigma-1 and Sigma-2 Receptors

The presence of an ionizable nitrogen has been reported to be an important pharmacophoric element for sigma-1 receptor binding [35]. Furthermore, *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl substitution on the obligate nitrogen of sigma-1 receptor ligands enhances their affinity [17]. Thus, we have generally categorized the basic pharmacophore for sigma-1 receptor ligands to be a structure containing an aromatic feature (either indole or phenyl) linked to an obligate ionizable nitrogen by a propyl chain; the structure is largely represented by *N-3*-phenylpropylamine. Using the alkylation methodology shown in (Scheme 1), butylamine, heptylamine, dodecylamine, and octadecylamine were selected to highlight the effect of adding the pharmacophore on the affinity of these compounds for the sigma-1 receptor Fig. (7). While heptylamine (K_i value was 21 ± 9 μM Table 1), the addition of the *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl (Fig. (7), **10a** and **10b, respectively**) shifted the competitive displacement curves 3 to 4 orders of magnitude to the left (10a, 18 ± 14 nM; 10b, 7.5 ± 1 nM). Similarly, affinities for the other six *N*-3-phenylpropyl and *N*-4-(3-nitrophenyl)propyl derivatives at the pure sigma-1 receptor Fig. (7) were higher than their *N*-alkylamine counterparts Table 1. Interestingly, the addition of the pharmacophore moiety was more dramatic in butylamine than in dodecylamine, suggesting a combined effect of the pharmacophore and the aliphatic hydrocarbon moieties.

For ligand binding assays performed with guinea pig liver membranes (GPLM), the K_i values for shorter chain *N*-alkylamine derivatives (**9a**, **9b**, **10a**, and **10b)** were comparable to their K_i values on the pure sigma-1 receptor; however, for longer chain *N*-alkylamine derivatives K_i values (**11a**, **11b**, **12a**, and **12b**) in GPLMs were much higher, i.e. their apparent affinities were much lower on membrane preparations. (Fig. (7), Table 3). Since the longer chain *N*-alkylamines are structurally similar to lipids (as with sphingosine), the apparent lower affinities of these molecules when utilizing membranes may be attributable to their partitioning in membranes thus resulting in less free compound to be available for binding. *N*-alkylamine derivatives showed a competitive binding mechanism against [^{3}H]-(+)-pentazocine for the sigma-1 receptor in GPLM similar to that observed for D-erythro-sphingosine [25, 34].

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We also determined the affinities of **9a**, **9b**, **10a**, **10b**, **11a**, **11b**, **12a**, and **12b** for the sigma-2 receptor in rat liver membranes [36] using the competitive displacement of [^{3}H]-DTG binding. In these assays, 100 nM cold (+)-pentazocine was used to block the sigma-1 receptor sites Fig. (7). *N*-alkylamine derivatives were fairly non-selective between the sigma-1 and sigma-2 receptor indicating that the two receptor subtypes may share some similarities in their binding characteristics for monochain lipid-like molecules. It is interesting to note that *N*-alkylamine derivatives with an alkyl chain length shorter than eight carbons**,** e.g. **9b**, **10a** were slightly more selective (by 1 to 7 fold) for the sigma-1 receptor than the sigma-2 receptor in membranes and those with longer chain length above eight carbons, e.g. **11a**, **11b**, **12a**, **12b**, favored the sigma-2 receptor sites (by 2 to 3 fold). However, compounds **9a**, **10b** did not adhere to this rule, **10b** had the same affinity at both sigma-1 and sigma-2 sites while **9a** had a higher affinity at the sigma-2 receptor.

CONCLUSIONS

Earlier studies based on structure-activity relationships of known sigma-1 receptor ligands indicated that there is a requirement for at least one arylalkyl substitution on a nitrogen atom for high affinity to the sigma-1 receptor [37]. In our lab we compared the structure of a number of high affinity sigma-1 receptor ligands; these studies suggested that the minimal pharmacophore for sigma-1 receptor binding is a phenylalkylamine structure [33, 38]. In addition, an electron-withdrawing group such as a *para*-nitro substitution on the phenyl ring also enhanced the affinity of the ligand [15]. We found that *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl derivatives of *N*-alkylamines have 2 to 6 orders of magnitude higher affinity than their corresponding amines for the pure sigma-1 receptor. This increase in affinity was dramatic for the short carbon chain compounds (compare butylamine with 9a and 9b, and heptylamine with 10a and 10b), but lesser for the longer chain compounds dodecyl- and octadecylamine (Table 1 and Fig. (7)). Glennon *et al.* [37] proposed that a pharmacophore for high affinity sigma-1 receptor ligands is composed of two hydrophobic regions flanking a central nitrogen atom [37]. The primary hydrophobic region was proposed to be situated optimally at five carbons (6 – 10 Å) from the nitrogen. The secondary hydrophobic site was proposed to be located closer to the obligate nitrogen than the primary site and is separated from the nitrogen by 2.5 – 3.9 Å, approximately the length of 3 carbons. It is likely that the *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl moieties of *N*-alkylamine derivatives occupy the secondary hydrophobic site (closer to the nitrogen) but whether the primary hydrophobic region in the Glennon model could accommodate aliphatic hydrocarbon chain such as octadecylamine remains unclear. Because the longer aliphatic hydrocarbon chain of *N*-alkylamines bears resemblance to lipids such as D-*erythro*-sphingosine, we propose that the alkyl chain intercalates between the transmembrane (TM) regions of the sigma-1 receptor between TMI and TMII.

The discovery that sphingosine and sphingosine like compounds but not sphingosine -1-phosphate bound with reasonable affinity to the sigma-1 receptor is worth noting. In situ, the intracellular balance between sphingosine and the sphingosine-1-phosphate is controlled in part by sphingosine kinases. Sphingosine itself is often associated with apoptotic signaling pathways where as sphingosine-1-phophate is associated with progrowth signaling pathways. Since we have already shown that an in situ population of the sigma-1 receptor

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can in fact occur in complex with sphingosine, it is possible that the functional activity of the sigma-1 receptor in certain intracellular membrane environments is regulated by one or more sphingosine kinases that phosphorylate the specifically Sigma-1 receptor bound sphingosine thereby releasing the functional effects of sphingosine on the receptor. In this regard it would be worth investigating whether the sphingosine that is specifically bound to the sigma-1 receptor can serve as a substrate for sphingosine kinases. Further, is there a molecular complex that forms between the sigma-1 receptor/sphingosine and a sphingosine kinase?

III. OVERALL SUMMARY

Taken together, identification of the specifically and covalently labeled peptides from the pure sigma-1 receptor using radioiodinated photoprobe ligands and the systematic binding studies of *N*-alkylamines and *N*-aralkylamines to the membrane bound and the pure sigma-1 receptor have expanded our understanding of the main features of the receptor binding region. Further detailed structural biology studies are needed in the future to identify the precise locations and atomic details of ligand binding interactions with the sigma-1 receptor and the manner by which the alkyl chain moieties of these molecules interact with the SBDL1 and SBDLII regions. It is likely that a specific interaction of the endogenous lipid, sphingosine, with the sigma-1 receptor plays an important regulatory role for the receptor. Intracellular signal transduction mechanisms which involve the concerted conversion of the sigma-1 receptor bound sphingosine to sphingosine-1-phosphate in specific membrane microdomains may be important for cellular homeostasis. This possibility needs to be further evaluated for a better understanding of the regulatory role(s) of the sigma-1 receptor on cellular functions.

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ABBREVIATIONS

DTG	Ditolylguanidine
ER	Endoplasmic reticulum
GPLM	Guinea pig liver membranes
[^{125}I]-IACoc	[^{125}I]-iodoazidococaine
[^{125}I]-IAF	[^{125}I]-iodoazidofenpropimorph
SBDL	Steroid-binding domain liked
TM	Transmembrane

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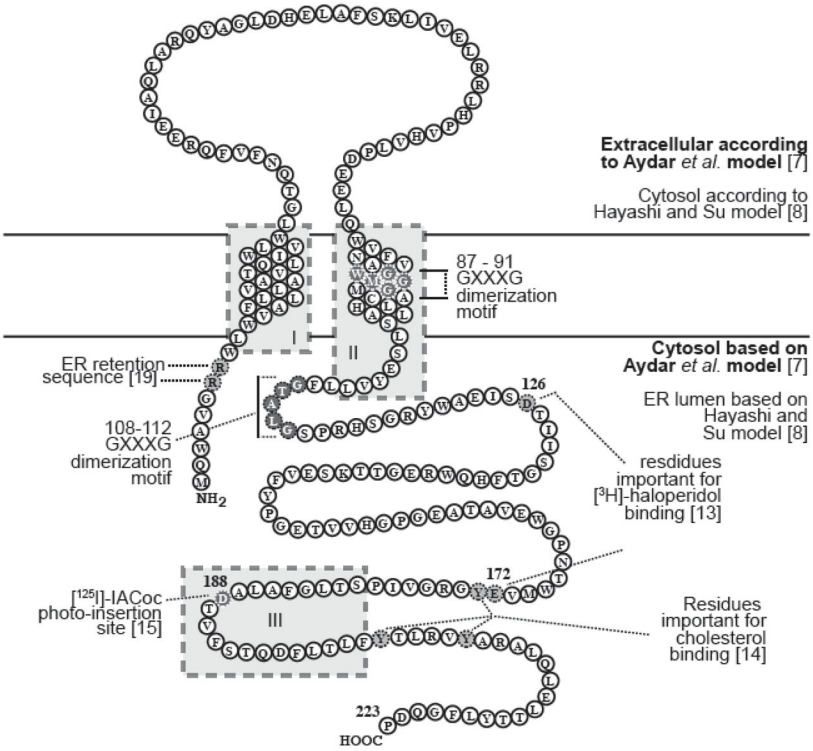

Fig. (1). Topological model of the sigma-1 receptor

The model of the sigma-1 receptor reported by Aydar *et al*. [7] indicated that the N and C-termini were cytosolic in Xenopus oocytes. Hayashi and Su [8] reported a similar model but showed that the sigma-1 receptor N and C-termini were found to be in the ER lumen in chinese hamster ovary cells. Shaded boxes indicate hydrophobic regions based on the hydropathy analysis: **I**, TMI (aa 11–29); **II** (aa 91–109), and **III** (aa 176 – 194). Hydrophobic region **I** corresponds to TMI, hydrophobic region **II** corresponds to TMII and part of SBDLI, and region III corresponds to SBLDII. ER retention sequence (shaded); putative GXXXG dimerization sequences (dark shaded); critical amino acids required for haloperidol binding (shaded) [39]; amino acids required for cholesterol binding (shaded); and the amino acid identified by [^{125} I]-IACoc photoaffinity labeling (shaded) [15].

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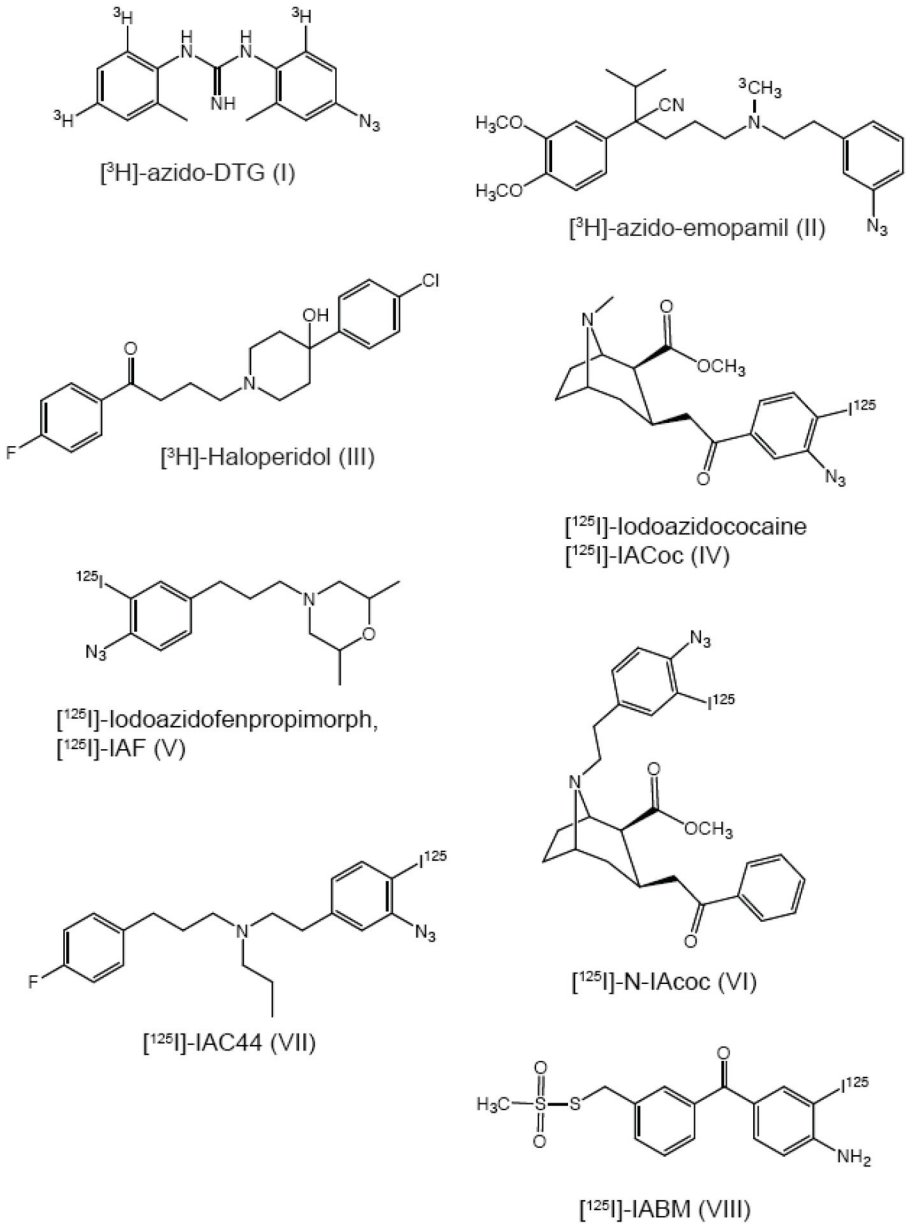

Fig. (2). Photoprobes used for identification of the sigma-1 receptor

Several photoaffinity labels have been developed for the sigma-1 receptor. [^{3}H]-azido-DTG (**I**) was the first sigma receptor photolabel used that identified a 29 kDa band in guinea pig brain membranes. The probe [^{3}H]-azido-emopamil (azidopamil) (**II**) identified two peptides with molecular weights of 27 and 22 kDa in guinea pig liver microsomes that were later cloned and characterized as the sigma-1 receptor and the sterol C8-C7 isomerase respectively. [^{3}H]-Haloperidol (**III**) – itself reported a photoprobe – was shown to label the yeast C8-C7 sterol isomerase. The aryl ketone moiety of haloperidol is photoactive and haloperidol could be used as photolabel without the need to modify the structure of the compound. The photoprobes [^{125}I]-Iodoazidococaine ([^{125}I]-IACoc, **IV**), [^{125}I]-Iodoazidofenpropimorph ([^{125}I]-IAF, **V**), [^{125}I]-N-IACoc (**VI**), [^{125}I]-IAC44 (**VII**) and

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[^{125}I]-IABM (**VIII**) have been developed in the Ruoho laboratory. Notice the opposite placement of the photo-moiety between [^{125}I]-IACoc (**IV**) and [^{125}I]-N-IACoc (**VI**). [^{125}I]-IACoc (**IV**) labeled aspartate 188 (SBDLII region) and [^{125}I]-N-IACoc (**VI**) labeled SBDLI regions. [^{125}I]-IAF (**V**) and [^{125}I]-IAC44

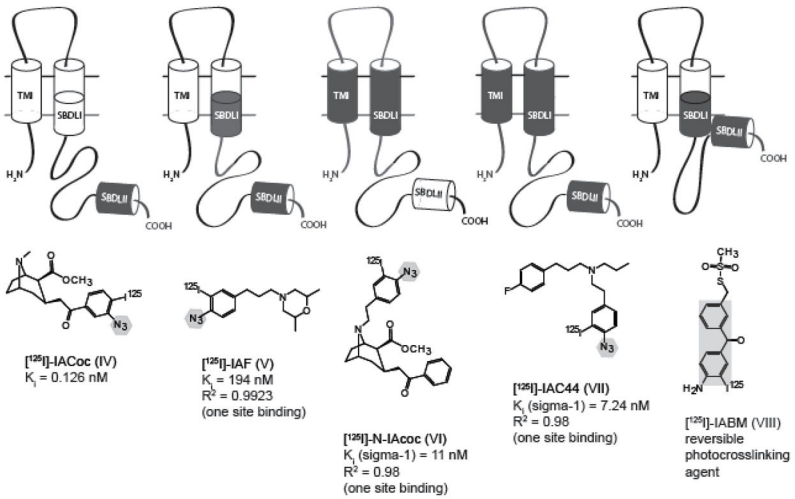

Fig. (3). Summary of the photolabeling results using radio-iodinated photoprobes

Shown are graphical models of the sigma-1 receptor with the regions labeled by the corresponding photoprobes highlighted in black. [^{125}I]-IACoc photoincorporated at aspartate 188 in the SBDLII region, [^{125}I]-N-IACoc labeled SBDLI regions, both [^{125}I]-IAF and [^{125}I]-IAC44 equally labeled SBDLI and SBDLII, and [^{125}I]-IABM reversibly crosslinked SBDLI and SBDLII and further allowed transfer of radiolabel from one region to the other. This strongly supports the juxtaposition of SBDLI and SBDLII Fig. (4). R^2 values are the correlation coefficient of the raw data fitted to a one-site binding model. Literature references to these probes are given in the main text.

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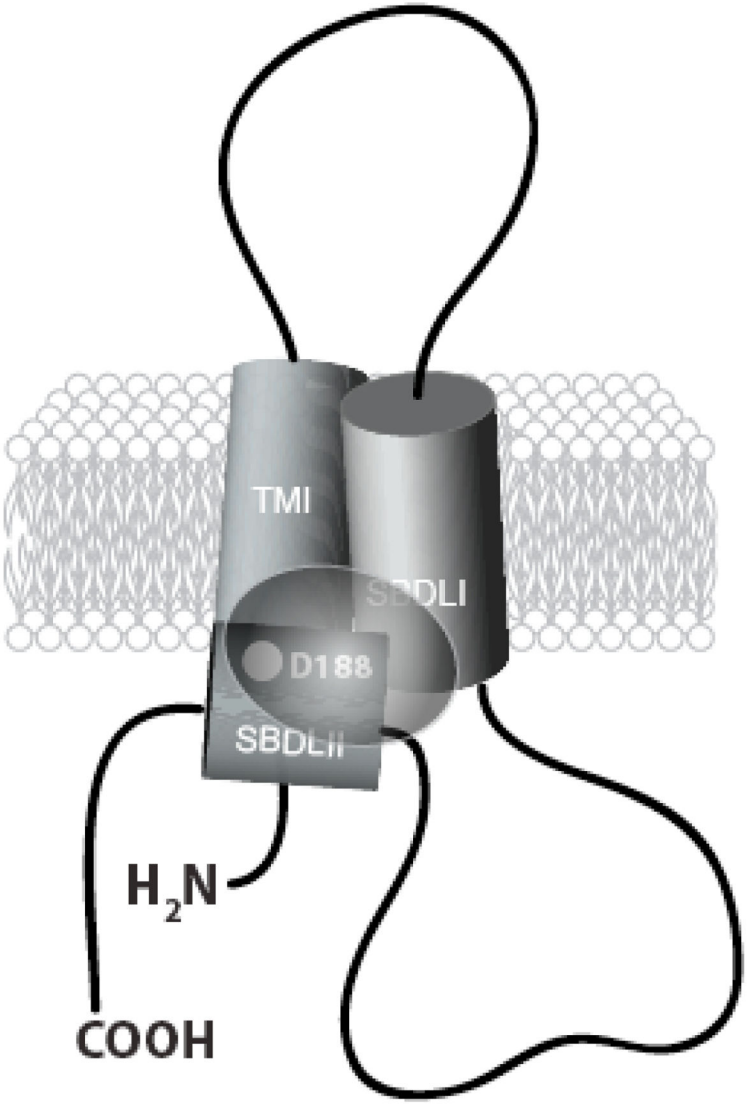

Fig. (4). Current model of the sigma-1 receptor ligand binding region

A current sigma-1 receptor ligand binding model supported by multiple photoaffinity labeling studies from the Ruoho laboratory with the pure sigma-1 receptor [10, 15, 17, 40]. The three hydrophobic regions in the sigma-1 receptor sequence, TM (transmembrane) domain I, II and the third hydrophobic domain are represented by cylindrical segments. The steroid binding domain like (SBDL) I region (part of TM II) and the SBDLII region along with possible contributions from TM I constitute the sigma-1 receptor ligand binding pocket and is expected to be part of the binding site for the sphingosine-like molecules based on photoaffinity labelings (refer to Fig. (2) for the structures of photoprobes) [10, 15, 17, 40].

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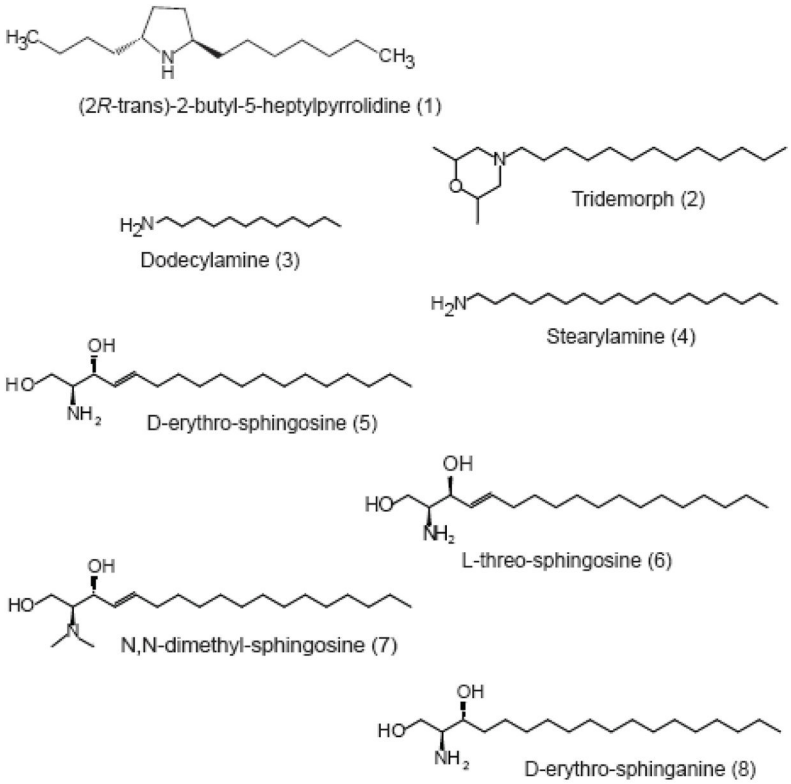

Fig. (5).
Structures of sphingolipids and structurally related molecules.

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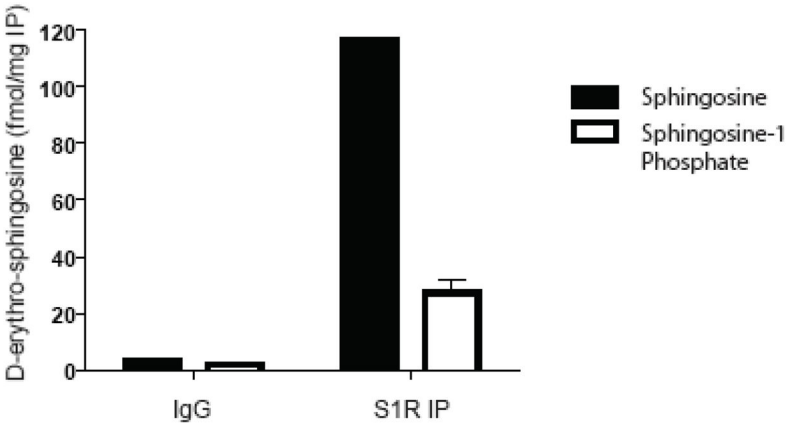

Fig. (6). ***In situ*** **detection of a sigma-1 receptor/sphingosine complex**

Homogenates from human embryonic kidney (HEK-293) cells overexpressing the sigma-1 receptor were used in an immunoprecipitation experiment with the polyclonal sigma-1 receptor antibody (rabbit IgG immunoprecipitation, IgG; sigma-1 receptor antibody immunoprecipitation, (S1R IP)) [16]. The data demonstrate the specific interaction between D-erythro-sphingosine and sigma-1 receptor. Levels of D-erythro-sphingosine were 101.0 ± 9.4 and sphingosine-1 phosphate were 27.1 ± 11.7 (mean ± SEM, n = 3 experiments each in tripli-

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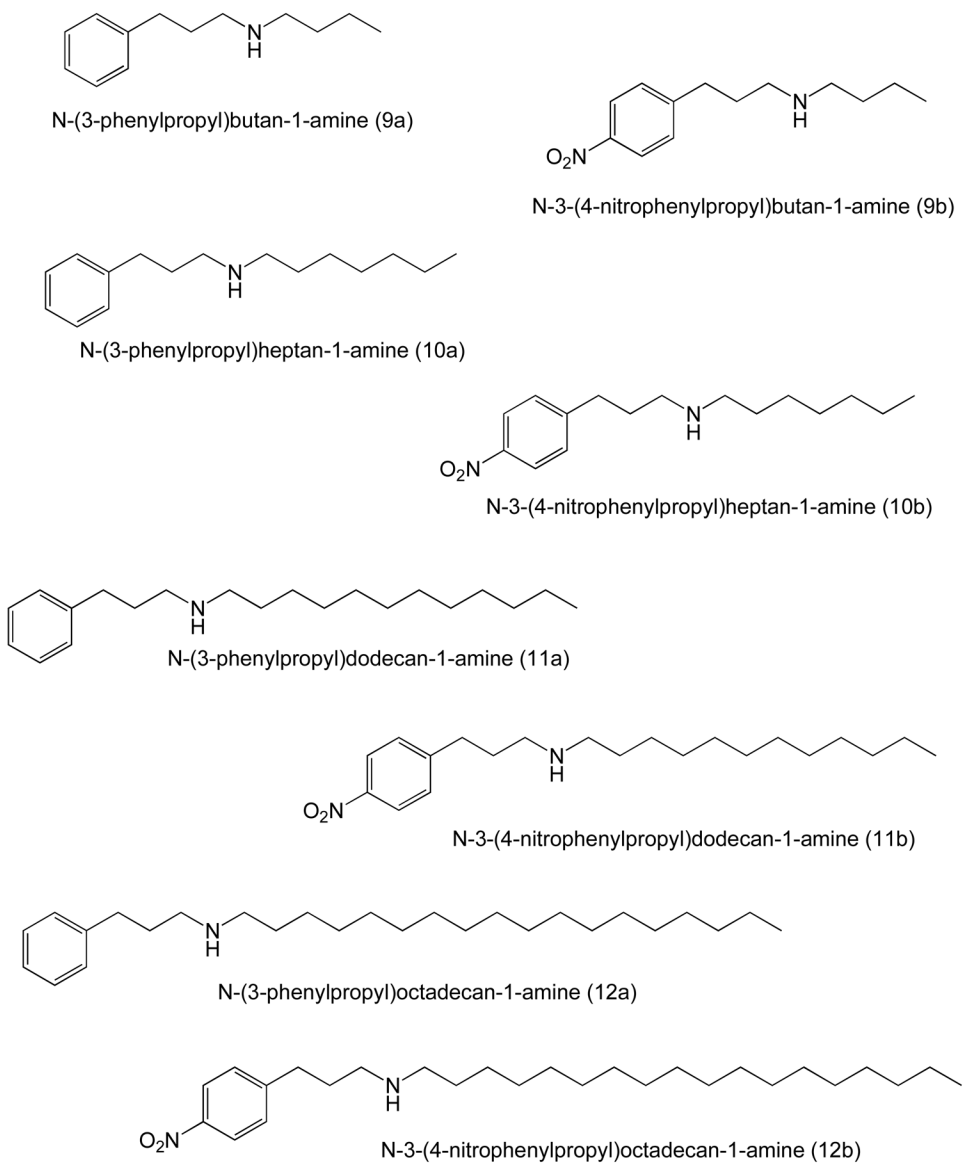
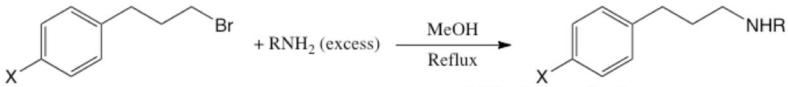


Fig. (7).
Chemical structures of N-alkylamine derivatives.



9a) X = H, R = *n*-Butyl
9b) X = NO_2, R = *n*-Butyl
10a) X = H, R = *n*-Heptyl
10b) X = NO_2, R = *n*-Heptyl
11a) X = H, R = *n*-Dodecyl
11b) X = NO_2, R = *n*-Dodecyl
12a) X = H, R = *n*-Octadecyl
12b) X = NO_2, R = *n*-Octadecyl

Synthetic Scheme I.
Synthesis of N-alkylamine derivatives

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Table 1

Summary of the binding affinity of N-alkylamines for the *E. coli* purified sigma-1 receptor. All K_i values were obtained from Chu *et al.* [34]

Compounds	K_i (9M) for the pure sigma-1 receptor
Butylamine	477 ± 67
Amylamine	167 ± 35
Heptylamine	21 ± 9.0
Octylamine	6.0 ± 3.0
Dodecylamine	0.2 ± 0.02
Tetradecylamine	1.0 ± 0.03
Hexadecylamine	3.0 ± 0.04
Octadecylamine	8.5 ± 5.5

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Table 2

Affinity of sphingolipids and related molecules (mean ± SEM, n = 3).

Compounds	Affinity (9M)
1	0.002 (IC50)[1]
2	0.00004 ± 0.00002[2], 0.031 ± 0.006[3]
3	0.18 ± 0.02[4]
4	8.5 ± 5.4[4]
5	0.140 ± 0.023[4]
6	0.018 ± 0.0025[4]
7	0.115 ± 0.030[4]
8	0.073 ± 0.009[4]

[1]Values were obtained from Kumagai *et al.* 2000 [24].

[2]Values were obtained from Moebius *et al.* 1997 [26].

[3]Values were obtained from Ramachandran *et al.* 2007 [16].

[4]Values were obtained from Ramachandran *et al.* 2009 [25].

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Table 3

Summary of K_i values obtained for N-alkylamine derivatives for the *E. coli* purified sigma-1 receptor, and the sigma-1 and sigma-2 receptors in guinea pig liver and rat liver membranes respectively (mean ± SEM, n = 3).

	K_i (:M)		
Compounds	**Pure Sigma-1 receptor**	**Sigma-1 sites[a]**	**Sigma-2 sites[b]**
9a	0.20 ± 0.02	0.073 ± 0.002	0.015 ± 0.01
9b	0.02 ± 0.005	0.003 ± 0.0005	0.022 ± 0.01
10a	0.018 ± 0.014	0.015 ± 0.009	0.033 ± 0.01
10b	0.0075 ± 0.001	0.010 ± 0.002	0.011 ± 0.005
11a	0.063 ± 0.011	5.3 ± 0.1	2.1 ± 1.0
11b	0.032 ± 0.022	5.1 ± 0.2	2.4 ± 1.0
12a	0.042 ± 0.002	34.4 ± 0.6	20.0 ± 2.0
12b	0.042 ± 0.007	26.9 ± 0.7	9.0 ± 0.4

[a]Values obtained using guinea pig liver membranes.

[b]Values obtained using rat liver membranes. All K_i values were obtained from Chu *et al.* [34].