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Characterization of Interactions of 4-Nitrophenylpropyl-*N*-alkylamine with Sigma Receptors

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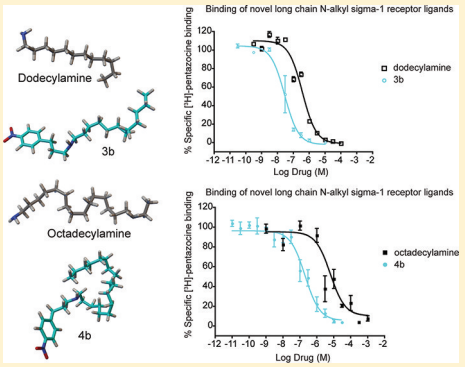
Supporting Information

ABSTRACT: Sigma receptors are small membrane proteins implicated in a number of pathophysiological conditions, including drug addiction, psychosis, and cancer; thus, small molecule inhibitors of sigma receptors have been proposed as potential pharmacotherapeutics for these diseases. We previously discovered that endogenous monochain *N*-alkyl sphingolipids, including D-*erythro*-sphingosine, sphinganine, and *N*,*N*-dimethylsphingosine, bind to the sigma-1 receptor at physiologically relevant concentrations [Ramachandran, S., et al. (2009) *Eur. J. Pharmacol. 609*, 19−26]. Here, we investigated several *N*-alkylamines of varying chain lengths as sigma receptor ligands. Although the K_I values for *N*-alkylamines were found to be in the micromolar range, when *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl derivatives of butylamine (**1a** and **1b**, respectively), heptylamine (**2a** and **2b**, respectively), dodecylamine (**3a** and **3b**, respectively), and octadecylamine (**4a** and **4b**, respectively) were evaluated as sigma receptor ligands, we found that these compounds exhibited nanomolar affinities with both sigma-1 and sigma-2 receptors. A screen of high-affinity ligands **2a**, **2b**, **3a**, and **3b** against a variety of other receptors and/or transporters confirmed these four compounds to be highly selective mixed sigma-1 and sigma-2 ligands. Additionally, in HEK-293 cells reconstituted with K_v1.4 potassium channel and the sigma-1 receptor, these derivatives were able to inhibit the outward current from the channel, consistent with sigma receptor modulation. Finally, cytotoxicity assays showed that **2a**, **2b**, **3a**, and **3b** were highly potent against a number of cancer cell lines, demonstrating their potential utility as mixed sigma-1 and sigma-2 receptor anticancer agents.



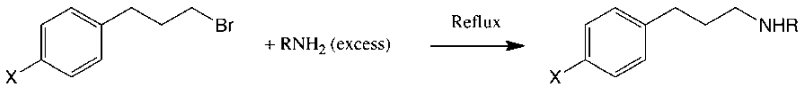
Two subtypes of the sigma receptor have been described, sigma-1 and sigma-2, which can be distinguished by their pharmacological profiles, selectivity, functions, subcellular locations, and molecular weight. The sigma-1 receptor was cloned in 1996[1] and has since been extensively characterized, while very little is known about the sigma-2 receptor that has not yet been cloned. The sigma-1 receptor is a membrane protein of 25.3 kDa and has been recognized in recent years as a small molecule operated chaperone important for the regulation of the passage of Ca^{2+} from the ER to the mitochondria.[2] Additionally, sigma ligands can also modulate the function of various voltage-gated ion channels, including potassium (K^+),[3] sodium (Na^+),[4] calcium (Ca^{2+}),[5] and chloride (Cl^-) channels,[6] in a manner independent of G proteins or phosphorylation. A direct physical interaction between the sigma-1 receptor and voltage-gated potassium channels[3] and that between the sigma-1 receptor and the acid-sensing ion channel ASIC 1a[7] have also been demonstrated, leading to the suggestion that the sigma-1 receptor is a regulatory subunit for certain ion channels.

Depending on cell types, the sigma-1 receptor has been localized to different subcellular regions, including the endoplasmic reticulum (ER),[8,9] the mitochondria-associated ER membrane (MAM),[2] and the ER cisternae proximal to the plasma membrane,[10] in focal adhesion contacts,[11] at the plasma membrane,[3] and at the nuclear membranes.[12] Functional modulation of the sigma-1 receptor may be achieved pharmacologically by synthetic small molecules and those present endogenously such as progesterone,[13] *N*,*N*-dimethyltryptamine,[14] and/or sphingolipids.[8,15] Therefore, the plethora of physiological outcomes resulting from treatments with sigma receptor ligands may be explained by the differential subcellular localization and wide tissue distribution of the sigma-1 receptor. For a review of the sigma-1 receptor as an interorganelle signaling modulator, see Su et al.[16]

Both sigma receptor subtypes are expressed at high densities in many human tumors and cancer cell lines.[17−20] These tumors and cancer cell lines include those from the breast, lung, colon, ovary, prostate, and brain.[19,21−23] The elevated levels of both sigma receptor subtypes in many cancer cell lines have led to a concerted effort in the development of sigma receptor targeting anticancer agents and imaging tools.[24−26] For example, small molecule sigma-1 receptor antagonists, BD-1047 and BD-1063, have been reported to inhibit cancer cell survival, while the agonists, (+)-SKF-10,047 and (+)-pentazocine,

Received: April 1, 2011
Revised: July 25, 2011
Published: July 26, 2011

Scheme 1



1a) X = H, R = *n*Butyl
1b) X = NO_2, R = *n*Butyl
2a) X = H, R = *n*Heptyl
2b) X = NO_2, R = *n*Heptyl
3a) X = H, R = *n*Dodecyl
3b) X = NO_2, R = *n*Dodecyl
4a) X = H, R = *n*Octadecyl
4b) X = NO_2, R = *n*Octadecyl

abrogated these effects.[25] Additionally, a sigma-1 receptor antagonist, rimcazole, was shown to initiate tumor-selective and caspase-dependent apoptosis, which could be rescued by the agonist (+)-pentazocine.[27] Sigma-2 receptor ligands, including siramesine, SV119, and SW43, have also been used to augment conventional chemotherapeutic agents in a preclinical model of pancreatic cancer.[26] Interestingly, the sigma-1 and sigma-2 receptors are suggested to have opposite cellular function because inhibition of the sigma-1 receptor (by treatment with antagonists) and activation of the sigma-2 receptor (with sigma-2 receptor agonists) resulted in antiproliferative effects against cancer cells.[28] Sigma-1 and sigma-2 receptor ligands have also been aggressively pursued in the area of tumor imaging in light of their elevated levels of expression in cancer cells. For example, Mach and co-workers compared the nonselective high-affinity radioligand *N*-[1-(4′-[^{18}F]fluorobenzyl)piperidin-4-yl]-3-bromophenylacetamide ([^{18}F]FBPBPA) with 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) and 5-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IUdR) in a tumor imaging study of nude mice with tumors grown from mouse mammary adenocarcinoma cell line 66. They confirmed receptor-specific uptake of the tracer (~25%) in the tumor and in the brain (~60%) with the following order of tumor uptake: [^{18}F]FDG > [^{18}F]FBPBPA > [^{125}I]IUdR.[17] Additionally, the tumor:blood and tumor:muscle ratios were larger for [^{18}F]FBPBPA than for both [^{18}F]FDG and [^{125}I]IUdR. These studies demonstrated the utility for sigma-1 receptor imaging agents as tools for anticancer therapy. Several recent reviews are focused on the development and use of sigma ligands for these purposes.[29,30]

Structure−activity studies of sigma-1 receptor ligands have mainly focused on *N*,*N*-diarylalkylamine[31] and *N*,*N*-dialkylphenylpropylamine molecules[14] but in general have overlooked long chain *N*-alkylamines such as tridemorph,[32] D-*erythro*-sphingosine,[15] *N*,*N*-dimethylsphingosines,[15] and glycosphingolipids such as galactosylceramide and lactosylceramide[8] that also bind to the sigma-1 receptor. To expand our knowledge of 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. In addition, we report the selectivity of these *N*-alkylamine derivatives compared to more than 40 other membrane receptor and transporter targets. Finally, their utility as potential anticancer agents was analyzed through multiplex cytotoxicity assays measuring growth inhibition of various cancer cell lines.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and utilized without further purification. [^{3}H]-(+)-Pentazocine and [^{3}H]-ditolylguanidine (DTG) were purchased from Perkin-Elmer Life Sciences (Wellesley, MA).

Chemical Syntheses. *Synthesis of 1-Bromo-3-(4-nitrophenyl)propane.* The preparation of 1-bromo-3-(4-nitrophenyl)propane from 1-bromo-3-phenylpropane was performed using the method of Hajipour et al.[33] Briefly, P_2O_5 and silica gel were ground at room temperature for 1 min to homogeneity. The P_2O_5/silica gel mixture (1 mmol) and 1-bromo-3-phenylpropane (1 mmol, 0.199 g) were combined and ground for 30 s before 0.5 mL of 65% HNO_3 was added. Additional grinding was conducted until TLC (9:1 *n*-hexane/ethyl acetate) showed the complete disappearance of 1-bromo-3-phenylpropane (~6 min). The product was extracted from the silica gel with ethyl acetate (10 mL), washed with H_2O (10 mL), and dried with anhydrous $MgSO_4$. Solvent was evaporated under reduced pressure, and the product was purified by column chromatography (9:1 *n*-hexane/ethyl acetate). 1-Bromo-3-(4-nitrophenyl)propane was obtained as a yellow oil (0.74 mmol, 0.18 g, 74%).

Synthesis of N-Alkylamine Derivatives (Scheme 1). In a round-bottom flask equipped with a magnetic stirrer and a condenser, 2 mmol of 1-bromo-3-phenylpropane (0.4 g) or 1-bromo-3-(4-nitrophenyl)propane (0.49 g) and 10 mmol of the appropriate amine (5 equiv) were refluxed in ethanol (10 mL) until TLC (9:1 *n*-hexane/ethyl acetate) showed the complete disappearance of the 1-bromo-3-phenylpropane or 1-bromo-3-(4-nitrophenyl)propane (3 h). The reaction was quenched with water and the mixture extracted three times with 5 mL of ethyl acetate. The combined extracts were dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The purification of the product was performed by column chromatography using silica gel and a 9:1 *n*-hexane/ethyl acetate mixture.

Structural Characterization of N-Alkylamine Derivatives. Yields refer to isolated products after column chromatography. Products were characterized by ^{13}C, ^{1}H, and IR spectroscopy and mass spectrometry. All ^{1}H NMR spectra were recorded at 300 MHz in $CDCl_3$ relative to TMS (0.00 ppm), and IR spectra were recorded on a Shimadzu 435 IR spectrometer at the Research Institute of Petroleum Industry (Tehran, Iran).

*N-(3-Phenylpropyl)butan-1-amine (**1a**).* Yellow oil. Yield: 0.30 g, 78%, 1.5 mmol. IR (KBr): 1664 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.21−7.08 (m, 5H), 2.4 (m, 6H), 2.00 (m, 4H), 1.34 (m, 4H), 0.96 (t, 3H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$): δ 138.1, 128.9, 128.2, 126.1, 49.6, 49.4, 33.4, 32.8, 29.6, 20.2, 13.8. MS (CI): m/z 191 (100, M$^+$). Anal. Calcd for $C_{13}H_{21}N$: C, 81.62%; H, 11.06%; N, 7.32%. Found: C, 81.50%; H, 11.20%; N, 7.20%.

dx.doi.org/10.1021/bi2004872|*Biochemistry* 2011, 50, 7568−7578

N-[3-(4-Nitrophenyl)propyl]butan-1-amine (**1b**). Red oil. Yield: 0.33 g, 70%, 1.4 mmol. IR (KBr): 1664 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.21 (d, J = 8.3 Hz, 2H), 7.55 (d, J = 8.3 Hz, 2H), 2.4 (m, 6H), 2.00 (m, 4H), 1.34 (m, 4H), 0.96 (t, 3H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$): δ 148.1, 145.2, 130.3, 124.0, 49.6, 49.5, 32.7, 30.8, 29.5, 20.1, 13.8. MS (CI): m/z 236 (100, M^+). Anal. Calcd for $C_{13}H_{20}N_2O_2$: C, 66.07%; H, 8.53%; N, 11.85%. Found: C, 65.90%; H, 8.70%; N, 11.80%.

N-(3-Phenylpropyl)heptan-1-amine (**2a**). Yellow oil. Yield: 6.0 g, 100%, 25.7 mmol. IR (KBr): 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40−7.28 (m, 5H), 2.62−2.55 (m, 6H), 1.95 (m, 3H), 1.38−1.10 (m, 10H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 148.1, 145.2, 130.3, 122.1, 49.9, 49.5, 31.8, 30.8, 30.5, 27.1, 22.6, 14.1. MS (CI): m/z 233 (100, M^+). Anal. Calcd for $C_{16}H_{27}N$: C, 82.34%; H, 11.66%; N, 6.00%. Found: C, 82.40%; H, 11.70%; N, 6.10%.

N-[3-(4-Nitrophenyl)propyl]heptan-1-amine (**2b**). Red oil. Yield: 0.57 g, 100%, 2 mmol. IR (KBr): 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.21 (d, J = 8.6 Hz, 2H), 7.55 (d, J = 8.6 Hz, 2H), 2.62−2.55 (m, 6H), 1.95 (m, 3H), 1.38−1.10 (m, 10H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 142.1, 128.8, 126.0, 49.9, 49.5, 31.8, 30.8, 30.5, 27.1, 22.6, 14.1. MS (CI): m/z 278 (100, M^+). Anal. Calcd for $C_{16}H_{26}N_2O_2$: C, 69.03%; H, 9.41%; N, 10.06%. Found: C, 68.80%; H, 9.60%; N, 9.60%.

N-(3-Phenylpropyl)dodecan-1-amine (**3a**). Oil. Yield: 0.5 g, 82%, 1.6 mmol. IR (KBr): 1661 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40−7.29 (m, 5H), 2.58 (m, 6H), 1.98 (m, 3H), 1.30 (m, 20H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 142.1, 128.8, 127.6, 126.4, 49.9, 49.5, 30.7, 30.5, 29.6, 29.5, 29.3, 27.0, 22.7, 14.1. MS (CI): m/z 303 (100, M^+). Anal. Calcd for $C_{21}H_{37}N$: C, 83.10%; H, 12.29%; N, 4.61%. Found: C, 82.90%; H, 12.50%; N, 4.50%.

N-[3-(4-Nitrophenyl)propyl]dodecan-1-amine (**3b**). Yield: 0.55 g, 78%, 1.6 mmol. IR (KBr): 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.21 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 2.56 (m, 6H), 2.01 (m, 3H), 1.30 (m, 20H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 148.1, 145.2, 130.3, 124.0, 49.6, 49.5, 30.7, 30.5, 29.6, 29.5, 29.3, 27.0, 22.7, 14.1. MS (CI): m/z 348 (100, M^+). Anal. Calcd for $C_{21}H_{36}N_2O_2$: C, 72.37%; H, 10.41%; N, 8.04%. Found: C, 72.10%; H, 10.50%; N, 8.00%.

N-(3-Phenylpropyl)octadecan-1-amine (**4a**). Yield: 0.62 g, 80%, 1.6 mmol. IR (KBr): 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.28 (m, 5H), 2.58 (m, 6H), 2.0 (m, 3H), 1.30 (m, 32H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 142.1, 128.8, 127.6, 126.4, 49.9, 49.5, 30.7, 30.5, 29.6, 29.5, 29.3, 27.0, 22.7, 14.1. MS (CI): m/z 387 (100, M^+). Anal. Calcd for $C_{27}H_{49}N$: C, 83.65%; H, 12.74%; N, 3.61%. Found: C, 83.70%; H, 12.80%; N, 3.50%.

N-[3-(4-Nitrophenyl)propyl]octadecan-1-amine (**4b**). Yield: 0.80 g, 92%, 1.85 mmol. IR (KBr): 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.21 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 2.56 (m, 6H), 2.01 (m, 3H), 1.30 (m, 32H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 148.1, 145.2, 130.3, 124.0, 49.6, 49.5, 30.7, 30.5, 29.6, 29.5, 29.3, 27.0, 22.7, 14.1. MS (CI): m/z 432 (100, M^+). Anal. Calcd for $C_{27}H_{48}N_2O_2$: C, 74.95%; H, 11.18%; N, 6.47%. Found: C, 74.80%; H, 11.40%; N, 6.70%.

Overexpression and Purification of the Sigma-1 Receptor from *Escherichia coli*. The guinea pig sigma-1 receptor was expressed and purified as previously described[34] using the pMal P2X plasmid (New England Biolabs, Ipswich, MA) encoding the guinea pig sigma-1 receptor fused to the maltose binding protein (MBP) at its N-terminus and carried a hexahistidine epitope tag at its C-terminus. Proteins were expressed in *E. coli* strain BL21(DE3) (Novagen, Madison, WI). Cells were grown to an OD_{600} of 0.7 before being induced with 0.6 mM IPTG for 4 h at 37 °C. The collected *E. coli* pellet was resuspended in buffer I [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM 2-mercaptoethanol, and 1 mM EDTA], and the cell suspension was sonicated using a Branson soniWer 250 employing a 1 cm probe (output of 50%, 2 s bursts, 1 s lag) for 15 min on ice. The cell lysate was centrifuged at 100000*g* for 1 h to separate total particulate and soluble proteins. The particulate fraction was extracted with a 4:1 ratio of Triton X-100 to total protein (w/w) for 3 h with stirring at 4 °C. The extracted material was centrifuged at 100000*g* for 1 h, and the supernatant was diluted with a volume of buffer I that reduces the Triton X-100 (TX-100) concentration to 0.5−1% before being loaded onto an amylose column (New England Biolabs). After the resin had been washed once with 5 column volumes of buffer II [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.5% TX-100] and once with 3 column volumes of buffer III [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, and 0.5% TX-100], the MBP−sigma-1 receptor fusion protein was eluted with 3 column volumes of buffer IV [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 10 mM maltose, and 0.5% TX-100].

The pure MBP−sigma-1-receptor fusion protein was cleaved with Factor Xa protease (Novagen, Madison, WI) in a volume of 5 mL at room temperature for 24−48 h and the cleavage monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The sigma-1 receptor from the Factor Xa cleavage was purified with HIS-Select HC Nickel affinity gels (Sigma, St. Louis, MO) in a batch format with overnight incubation on Ni^{2+} beads at 4 °C and then with three washes each lasting 15 min in buffer V [50 mM Na_2HPO_4 (pH 8), 200 mM NaCl, and 0.5% TX-100] and eluted for 1 h in buffer VI [50 mM Na_2HPO_4 (pH 8), 200 mM NaCl, 250 mM imidazole, and 0.5% TX-100] at room temperature. Centrifugation for each step of the Ni^{2+} purification was performed at 5000 rpm for 1 min at room temperature in a benchtop microcentrifuge.

Preparation of Guinea Pig Liver Membranes (GPLM) and Rat Liver Membranes (RLM). Membranes were prepared as described previously[35] from frozen tissues (Pel Freez Biologicals, Rogers, AR). The liver tissue was homogenized (10 mL of buffer/g of wet tissue) by four bursts of 10 s each using a brinkman polytron (American Laboratory Trading Inc., East Lyme, CT) on setting 6 in ice-cold sodium phosphate buffer (10 mM, pH 7.4) containing 0.32 M sucrose and a cocktail of protease inhibitors [20 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, 100 μM phenylmethanesulfonyl fluoride (PMSF), 100 μM benzamidine, and 1 mM EDTA]. The membrane suspension after homogenization was centrifuged for 10 min at 17000*g*, and the supernatant from this first centrifugation was further centrifuged at 100000*g* to collect the membrane fraction. The pellet from the second high-speed spin was resuspended in the same buffer described above, snap-frozen, and stored at −80 °C at a protein concentration of 10 mg/mL.

Competitive Displacement of [^{3}H]-(+)-Pentazocine and [^{3}H]DTG Binding. Binding of competition ligand to the pure sigma-1 receptor and the sigma-1 receptor from guinea

pig liver membranes (GPLM) was performed as described previously.[34] Binding was conducted in 50 mM Tris-HCl (pH 8.0) in a total volume of 100 μL containing 50−100 ng of the pure sigma-1 receptor, 10 nM [^{3}H]-(+)-pentazocine, and various concentrations of the inhibitors to be tested. After incubation at 32 °C for 60 min, the reaction was terminated by rapid filtration through glass fiber filters (Whatman GF/B, Whatman, Maidstone, U.K.), using a Brandel cell harvester (Brandel, Gaithersburg, MD). The glass fiber filters were presoaked in 0.5% polyethyleneimine (PEI) for at least 1 h at room temperature. Filters were washed four times with 4 mL of ice-cold 50 mM Tris-HCl (pH 8.0). Radioactivity was quantified by liquid scintillation (Ultima Gold, Perkin-Elmer, Waltham, MA) counting using a liquid scintillation counter (model 1600CA, Packard Instrument Co., Downers Grove, IL). The affinity determinations for the sigma-2 receptor were performed in rat liver membranes (RLM) in a manner similar to that described above using 20 nM [^{3}H]DTG as the radioligand with 100 nM (+)-pentazocine added to selectively mask the sigma-1 receptor binding sites in these assays. IC_{50} values were then converted to K_I values using the Cheng−Prusoff correction with the equation $K_I = IC_{50}/(1 + [L]/K_D)$, where [L] is the ligand concentration of the radioactive molecule and K_D is the previously determined dissociation constant [10 nM for [^{3}H]-(+)-pentazocine and 20 nM for [^{3}H]DTG].

Alternate Receptor and Transporter Target Determination. K_I determinations for receptor binding to alternate targets were performed by the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH PDSP) directed by B. L. Roth at the University of North Carolina (Chapel Hill, NC) and Project Officer J. Driscol at NIMH (Bethesda, MD). For detailed methodology, please refer to the PDSP website (http://pdsp.med.unc.edu/). A default concentration of 10 μM of *N*-alkylamine derivatives was chosen for the primary screening against the binding of a radioligand to the target receptor and/or transporter. Selected receptor and transporter targets with affinities for the sigma-1 receptor, their specific radioligand, and concentrations are listed in the order shown in Table 3. The protein source used in these assays for the sigma-1 receptor was a rat brain homogenate. For the sigma-2 receptor, PC12 cells were used. For the other membrane receptors and/or transporters, either transient or stably transfected cells were used, e.g., COS-7, HEK-293, CHO, and NIH3T3. The total bound radioactivity was estimated from quadruplicate wells containing no test or reference compound and adjusted to 100%; nonspecifically bound radioactivity was assessed from quadruplicate wells containing the suitable reference compound (10 μM) and adjusted to 0%. The average bound radioactivity in the presence of the test compound (10 μM) quadruplicate determinations was then expressed on a percentage scale. The percentage inhibition of radioligand binding was calculated as follows: % inhibition = 100% − % radioactivity bound.

The secondary assays for determining the K_I values were performed only for targets with ≥50% inhibition in the primary screen. The radioligands used in the secondary screen were the same as in the primary screen.

Patch Clamp Recordings. A pIRES bicistronic mammalian expression plasmid (BD Biosciences Clonetech, Mountain View, CA) was constructed that carried the rat $K_v1.4$ in the first multiple-cloning site and the rat sigma-1 receptor in the second multiple-cloning site. Human embryonic kidney (HEK-293) cells were cultured on 60 mm dishes and transfected with 3 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 2 μg of pIRES K_vS1R and 0.3 μg of pEGFP (BD Biosciences Clonetech) following the manufacturers' instructions. Thirty-six to forty-eight hours after transfection, whole cell K^+ currents were recorded from GFP positive HEK cells under voltage clamp using standard patch clamp techniques,[36] using patch pipettes filled with 120 mM KCl, 10 mM NaCl, 10 mM EGTA, 2 mM $MgCl_2$, 2−4 mM MgATP, 0.3 mM NaGTP, and 10 mM HEPES (pH 7.3). The external solution consisted of 130 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4) at room temperature (22−24 °C). Patch pipettes with resistances from 1 to 3 MΩ were fabricated from borosilicate glass (Garner Glass Co., Claremont, CA). K^+ channels are activated by a voltage step from −120 to 20 mV for 250 ms. An Axopatch 200B patch clamp amplifier interfaced with a personal computer running PCLAMP 7 (Axon Instruments, Foster City, CA) was used for the acquisition of data. Drugs were applied by a gravity feed system at a flow rate of 1−3 mL/min. Series resistance compensation was used for cells with series resistance values higher than 10 MΩ. We were able to achieve 80−90% series resistance compensation with such cells.

Multiplex Cytotoxicity Assays against a Panel of Cancer Cell Lines. The multiplex cytotoxicity assays on an array of cancer cell lines were performed at the Small Molecule and Medicinal Chemistry Screening Facility at the University of Wisconsin School of Medicine and Public Health.

Compound Handling. Working stocks were prepared at a final concentration of 100× in anhydrous DMSO. Serial dilutions were made in DMSO in 96-well polypropylene plates using the Precision XS liquid handler (BioTek, Inc., Winooski, VT). Compounds were divided equally into the corresponding wells of a 384-well plate in all four quadrants using a Biomek FX liquid handler with a 96-channel pipetting head (Beckman-Coulter Inc., Miami, FL). Compounds were stored at −20 °C in 100% DMSO until the day of the assay(s). Freeze−thaw cycles were minimized to a maximum of 10 per plate.

Cytotoxicity Assays. All cell lines were maintained as previously reported.[37] Cells were harvested by trypsinization using 0.25% trypsin and 0.1% EDTA and then counted in a Cellometer Auto T4 cell counter (Nexcelom LLC, Lawrence, MA), before dilution for assay plating. Cell plating, compound handling, and assay setup were performed as previously reported,[37] except that cells were plated in 50 μL volumes in 384-well clear bottom, tissue culture plates (Corning-Costar, Cambridge, MA). Compounds were added from the 384-well compound stock plates at a 1:100 dilution using a Biomek FX liquid handler equipped with a 384-channel head (Beckman Coulter Inc.). Acetoxymethyl ester (Calcein AM, Molecular Probes, Eugene, OR) reagent and ethidium homodimer-1 (EthD-1, Molecular Probes) in a volume of 30 μL, 10 μM CAM, and 100 μM EthD-1 were added, and the cells were incubated for 30 min at 37 °C. Plates were read for emission by using the appropriate wavelengths for Calcein AM and EthD-1 following the manufacturers' instructions. Fifteen microliters of CellTiter-Glo reagent (Promega Corp., Madison, WI) was added and the mixture incubated for 10 min at room temperature with gentle agitation to lyse the cells. Each plate was reread for luminescence to confirm the inhibition observed in the fluorescent Calcein AM/EthD-1 assay.

Data Analysis. IC_{50} values for cytotoxicity assays were determined for Calcein AM, the ratio of live to dead cells (Calcein AM/EthD-1), and CellTiter-Glo using XLfit 4.0 as previously reported by Langenhan et al.[37] The IC_{50} determined using the best-fit curve for dosage response from any of the three assays is reported as the final IC_{50}. The best-fit curve is defined as the curve that has the lowest standard error. If the standard error exceeds 10% of the IC_{50}, the assay was deemed a failure and repeated.

RESULTS

N-Alkylamines Bind to the Sigma-1 Receptor. The discovery that D-*erythro*-sphingosine binds to the sigma-1 receptor with relatively high affinity[15] prompted us to study monochain sphingosine-like *N*-alkylamine molecules to determine the relationship between sigma-1 receptor affinity and the number of alkyl carbons on the molecule. We performed competitive displacement of [^{3}H]-(+)-pentazocine binding to the pure guinea pig sigma-1 receptor[34] and found that affinities of *N*-alkylamines for the sigma-1 receptor increased in the series (Table 1). The data show a positive correlation between the number of carbons and affinity for the sigma-1 receptor from butylamine to dodecylamine. However, a further increase in carbon chain length yielded a decrease in affinity above dodecylamine (Table 1).

Table 1. K_I Values of *N*-Alkylamine for the Pure Sigma-1 Receptor

compound	K_I (μM)
butylamine	477 ± 67
amylamine	167 ± 35
heptylamine	21 ± 9
octylamine	6 ± 3
dodecylamine	0.2 ± 0.02[a]
tetradecylamine	1.0 ± 0.03
hexadecylamine	3.0 ± 0.36
octadecylamine	8.5 ± 5.5[a]

[a]Values obtained from ref 15.

Addition of *N*-3-Phenylpropyl and *N*-3-(4-Nitrophenyl)propyl to *N*-Alkylamines Resulted in an Increase in Affinity for Sigma-1 and Sigma-2 Receptors. The presence of a nitrogen has been reported to be an important pharmacophoric element for sigma-1 receptor binding.[38] Furthermore, *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl substitution on the obligate nitrogen of sigma-1 receptor ligands was previously shown to enhance the affinity of sigma-1 receptor ligands.[39] Using the alkylation of alkylhalide methodology depicted in Materials and Methods (Scheme 1), butylamine, heptylamine, dodecylamine, and octadecylamine were selected to highlight the effect of adding the *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl moiety on the affinity of these compounds for the sigma-1 receptor. Binding curves for heptylamine and heptylamine derivatives are summarized in Figure 1A, and K_I values are listed in Table 2. While heptylamine has an affinity of 21 ± 9 μM, the addition of the *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl shifted the competitive displacement curves 3−4 orders of magnitude to the left (**2a**, 18 ± 14 nM; **2b**, 7.5 ± 1 nM), indicating a dramatic increase in affinity for each compound (Figure 1A). Similarly, affinities of the other six *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl derivatives for the pure sigma-1 receptor were all higher than those of their *N*-alkylamine counterparts (Table 2). Figure 1B illustrates the relationship between carbon chain length (plotted on the abscissa) and the inverse of the K_I of *N*-alkylamines and their derivatives (plotted on the ordinate). Interestingly, the addition of the pharmacophore moiety was most dramatic with butylamine and less so with dodecylamine, suggesting a combined effect of the aromatic feature and the aliphatic hydrocarbon moieties.

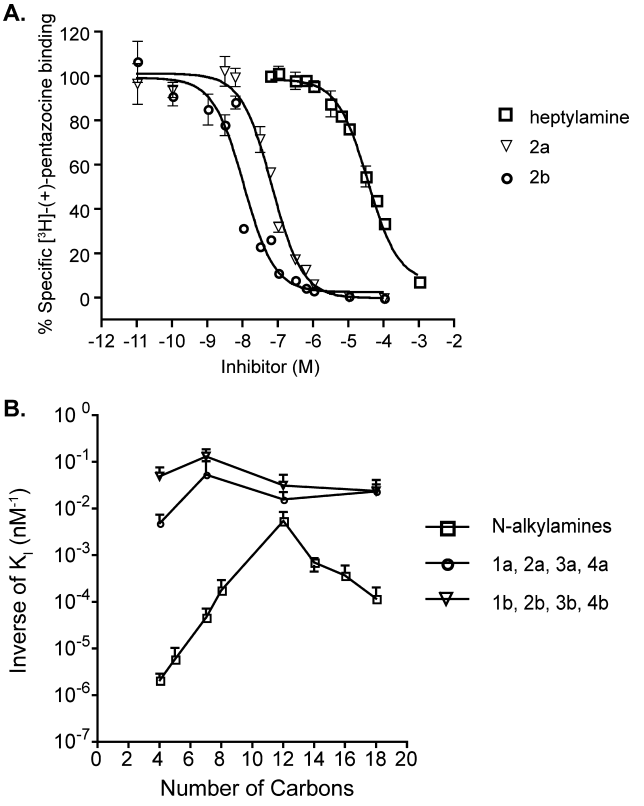


Figure 1. Interaction of *N*-alkylamine and *N*-alkylamine derivatives with the pure sigma-1 receptor. (A) Representative competitive displacement of [^{3}H]-(+)-pentazocine binding curves for heptylamine and its analogues (**2a** and **2b**) on the pure sigma-1 receptor. Data points were obtained in triplicate, and 10 μM haloperidol was used to assess nonspecific binding. Error bars represent the mean ± the standard error of the mean (SEM) (n = 3). (B) Reciprocals of K_I values of *N*-alkylamines and their derivatives at the pure sigma-1 receptor (K_I values are listed in Table 2) plotted vs the number of carbon atoms. The vertical axis is shown in log scale, illustrating the orders of magnitude change in affinity when the *N*-3-phenylpropyl or *N*-3-(4-nitrophenyl)propyl group was substituted on the nitrogen of the *N*-alkylamines. Error bars represent the mean ± SEM (n = 3).

In guinea pig liver membranes (GPLM), K_I values for shorter chain *N*-alkylamine derivatives were comparable to the K_I values obtained with the pure sigma-1 receptor in that all exhibited nanomolar affinities (Table 2). For longer chain *N*-alkylamine derivatives, however, values obtained from GPLM were in the micromolar range (Table 2). Because the longer chain *N*-alkylamines (**3a**, **3b**, **4a**, and **4b**) are structurally similar to lipids, the apparent low affinities of these molecules may be attributable to their partitioning in membranes, consequently reducing the free ligand concentrations available for binding. As was demonstrated previously for D-*erythro*-sphingosine,[15] *N*-alkylamine derivatives also showed a competitive binding

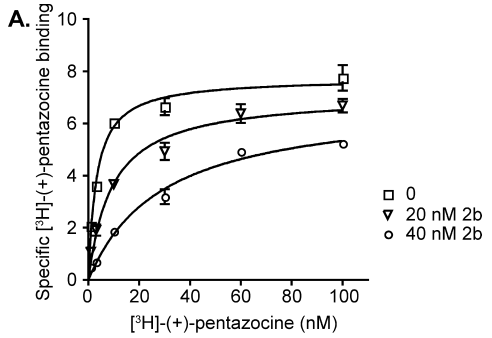
dx.doi.org/10.1021/bi2004872 | *Biochemistry* 2011, 50, 7568−7578

Table 2. K_I Values of *N*-Alkylamine Derivatives for the Pure Sigma-1 Receptor and the Sigma-1 and Sigma-2 Receptors in Membranes

	K_I (μM)			
compd	pure sigma-1 receptor	sigma-1 receptor sites[a]	sigma-2 receptor sites[b]	sigma-2/sigma-1[c]
1a	0.20 ± 0.02	0.073 ± 0.002	0.015 ± 0.01	2
1b	0.020 ± 0.005	0.003 ± 0.0005	0.022 ± 0.01	7
2a	0.018 ± 0.014	0.015 ± 0.009	0.033 ± 0.01	2
2b	0.0075 ± 0.001	0.010 ± 0.002	0.011 ± 0.005	1
3a	0.063 ± 0.011	5.3 ± 0.1	2.1 ± 1.0	0.4
3b	0.032 ± 0.022	5.1 ± 0.2	2.4 ± 1.0	0.5
4a	0.042 ± 0.002	34.4 ± 0.6	20.0 ± 2.0	0.6
4b	0.042 ± 0.007	26.9 ± 0.7	9.0 ± 0.4	0.3

[a]Sigma-1 site K_I values were determined using guinea pig liver membranes. [b]Sigma-2 site K_I values were determined using rat liver membranes in the presence of 100 nM (+)-pentazocine. [c]Ratio values were obtained from the K_I value of sigma-1 and sigma-2 sites.

mechanism against [^{3}H]-(+)-pentazocine binding to the sigma-1 receptor in GPLM (Figure 2). As depicted, the presence of



C.

	(+)-pentazocine	
2b (nM)	K_D (nM)	Bmax (pmol/mg)
0	3.1	7.7
20	9.2	7.2
40	29.1	6.9

Figure 2. Binding mechanism of *N*-alkylamine derivatives. (A) Saturation binding of [^{3}H]-(+)-pentazocine in the presence of increasing concentrations of **2b**. Data points were obtained in duplicate, and the nonspecific binding (in the presence of 10 μM haloperidol) was subtracted from the total binding (without haloperidol). (B) Scatchard transformation of the binding curves shown in panel A. (C) B_{max} and K_D values of [^{3}H]-(+)-pentazocine in the presence of 20 and 40 nM **2b**.

increasing concentrations of **2b** caused the K_D of [^{3}H]-(+)-pentazocine to change dramatically, from 3 nM for the control to 9 and 29 nM with the addition of 20 and 40 nM **2b**, respectively. On the other hand, the B_{max} remained unchanged (Figure 2D), characteristic of a competitive binding mechanism.

We also determined the affinities of **1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4a**, and **4b** for the sigma-2 receptor in rat liver membranes (RLM) 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. Again, K_I values of shorter chain *N*-alkylamine derivatives (**1a**, **1b**, **2a**, and **2b**) determined for the sigma-2 receptor were in the nanomolar range, while values of longer chain derivatives (**3a**, **3b**, **4a**, and **4b**) were all in the micromolar range. *N*-Alkylamine derivatives were fairly nonselective between the sigma-1 and sigma-2 receptors, indicating that the two receptor subtypes may share some similarities in their binding characteristics for monochain lipidlike molecules.

2a and 2b Are Selective Sigma-1 and Sigma-2 Receptor Ligands. To determine whether *N*-alkylamine derivatives are selective for sigma-1 and sigma-2 receptors, we selected four of the eight synthetic *N*-alkylamine derivatives (**2a**, **2b**, **3a**, and **3b**) and submitted them to the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH PDSP) to screen for alternate receptor and transporter targets. Figure S-2 of the Supporting Information shows a heat map of the data from a primary screen of 44 membrane receptors and transporters (shown on the vertical axis) tested with **2a**, **2b**, **3a**, or **3b** (shown on the horizontal axis). The values in each box correspond to the percent inhibition at 10 μM (a default concentration) of **2a**, **2b**, **3a**, or **3b** against a radioligand that binds to the membrane receptor or transporter being tested. In the illustration of these data, the more intense red color represents a greater level of inhibition and the beige color indicates a lower level of inhibition. From the primary screen results, there were number of protein targets to which *N*-alkylamine derivatives bind.

Receptor and transporter targets identified to be ≥50% inhibited at 10 μM in the primary screen were subjected to a secondary assay to determine the K_I values for these targets. While the primary screen showed numerous targets that were inhibited by ≥50% at 10 μM **2a**, **2b**, **3a**, or **3b**, the K_I values obtained in the secondary screen showed that these four molecules have much lower affinities for alternate receptors and/or transporters than for the sigma-1 and sigma-2 receptors. This is especially true for **2b** because there are only two other targets, 5HT1A and D4 receptors, with K_I values below 50 nM. In addition, for **3b**, 5HT1A was the only other target demonstrating a K_I value comparable to those of both the sigma-1 and sigma-2 receptors. Together, the data from the primary and secondary screens showed that of the four *N*-alkylamine derivatives tested, **2b** and **3b** are high-affinity and

dx.doi.org/10.1021/bi2004872 | *Biochemistry* 2011, 50, 7568–7578

Table 3. K_I Values of 2a, 2b, 3a, and 3b for Alternate Receptor and/or Transporter Targets[a]

		K_I (μM)			
receptor or transporter	radioligand [concn (nM)]	2a	2b	3a	3b
Sig-1R	[^{3}H]-(+)-pentazocine (3 nM)	0.09	0.02	1.5	0.20
Sig-2R	[^{3}H]DTG (3 nM)	0.25	0.014	3.6	0.27
5HT1A	[^{3}H]-8-OH-DPAT (0.5 nM)	0.13	0.05	>10.0	0.04
5HT1B	[^{3}H]GR127543 (0.3 nM)	>10.0	>10.0	>10.0	>10.0
5HT2B	[^{3}H]LSD (1 nM)	>10.0	1.1	>10.0	7.9
5HT7	[^{3}H]LSD (1 nM)	>10.0	0.9	>10.0	>10.0
α-1A	[^{3}H]prazosin (0.7 nM)	>10.0	>10.0	>10.0	>10.0
α-1D	[^{125}I]HEAT (0.1 nM)	>10.0	6.9	>10.0	9.0
β-2	[^{125}I]iodopindolol (0.1 nM)	>10.0	>10.0	>10.0	>10.0
β-3	[^{125}I]iodopindolol (0.1 nM)	>10.0	>10.0	>10.0	>10.0
D1	[^{3}H]SCH233930 (0.2 nM)	>10.0	>10.0	>10.0	>10.0
D4	[^{3}H]-*N*-methylspiperone (0.3 nM)	0.39	0.24	>10.0	1.8
H1	[^{3}H]pyrilamine (0.9 nM)	>10.0	9.2	>10.0	>10.0
H2	[^{3}H]tiotidine (3 nM)	>10.0	0.6	>10.0	3.9
H3	[^{3}H]-α-methylhistamine (0.4 nM)	>10.0	>10.0	>10.0	>10.0
M1	[^{3}H]QNB (0.5 nM)	>10.0	1.9	>10.0	>10.0
M2	[^{3}H]QNB (0.5 nM)	>10.0	3.2	>10.0	5.6
M3	[^{3}H]QNB (0.5 nM)	>10.0	1.1	>10.0	3.3
M4	[^{3}H]QNB (0.5 nM)	>10.0	1.4	>10.0	4.6
M5	[^{3}H]QNB (0.5 nM)	>10.0	0.3	>10.0	3.6
NET	[^{3}H]nisoxetine (0.5 nM)	>10.0	4.0	>10.0	>10.0
SERT	[^{3}H]citalopram (0.5 nM)	>10.0	1.1	>10.0	0.7

[a]Sig-1R, sigma-1 receptor; Sig-2R, sigma-2 receptor; 5HT, 5-hydroxytryptamine/serotonin receptor (subtypes 1A, 1B, 2B, and 7); α, α-adrenergic receptor (subtypes 1A and 1D); β, β-adrenergic receptor (subtypes 2 and 3); D, dopamine receptor (types 1 and 4); H, histamine receptor (types 1−3); M, muscarinic acetylcholine receptor (types 1−5); NET, norepinephrine transporter; SERT, serotonin transporter.

relatively high-selectivity mixed sigma-1/sigma-2 receptor ligands.

It is important to note that the K_I values of the four *N*-alkylamine derivatives for the sigma-1 and sigma-2 receptors measured by NIMH PDSP were slightly different from our determinations (compare Tables 2 and 3). The difference in the affinities of *N*-alkylamine derivatives obtained from NIMH PDSP and our results is likely due to the difference in the source of the receptors; NIMH PDSP used RLM as a source of sigma-1 receptor and PC12 cells for sigma-2 receptor, while our standard sources of sigma-1 and sigma-2 receptors were GPLM and RLM, respectively. Nonetheless, K_I values for **2a**, **2b**, **3a**, and **3b** obtained from NIHM PDSP confirmed our general conclusion that an electron-withdrawing moiety (4-nitro) is an important structural element of high-affinity sigma-1 and sigma-2 receptor ligands.

1a and 2a Functionally Inhibit the K_v1.4 Potassium Channel Current. The sigma-1 receptor has been shown to modulate the activity of ion channels such as K^+, Na^+, Ca^{2+}, and Cl^- channels.[3−6] To test the functional property of the *N*-3-phenylpropyl derivatives on a known effector of the sigma-1 receptor, we coexpressed the K_v1.4 potassium channel and the sigma-1 receptor in human embryonic kidney (HEK-293) cells. Coexpression of both the K_v1.4 potassium channel and the sigma-1 receptor was achieved by the use of a pIRES vector that carried both the K_v1.4 and sigma-1 receptor sequences. Potassium currents were recorded using the whole cell patch clamp technique. Examples of channel current traces from the control samples with no drug treatment and current traces that resulted from the application of **1a** and **2a** are shown in Figure 3A. **1a** and **2a** at concentrations up to 300 μM did not significantly inhibit the K_v1.4 potassium channel, with the average level of inhibition being 21% for butylamine and 15% for heptylamine (Figure 3B). However, the *N*-3-phenylpropyl derivatives of butylamine and heptylamine showed profound inhibition of the K_v1.4 potassium channel at the same concentrations. The IC_{50} for inhibition of the K_v1.4 potassium channel was 50 μM for **1a** and 10 μM for **2a**. This dramatic improvement in the ability of both **1a** and **2a** to inhibit the K_v1.4 potassium channel is consistent with their increase in apparent affinity for the sigma-1 receptor.

***N*-Alkylamine Derivatives Are Cytotoxic against a Number of Cancer Cell Lines.** Because both the sigma-1 and the sigma-2 receptors are overexpressed in a number of human tumors and cancer cell lines,[19] **2a**, **2b**, **3a**, and **3b** were tested for their cytotoxicity against multiple types of cancer cells. Three different cytotoxicity assays were used, Calcein AM, CellTiter-Glo, and EthD-1, with concentrations of *N*-alkylamine derivatives ranging from 1 to 100 μM.[37,40] All three assays produced similar results; however, only IC_{50} values from CellTiter-Glo are reported in Table 4. A three-dimensional illustration of the reciprocal of IC_{50} in micromolar is plotted for the sake of clarity. Because we (and others) have shown that the sigma-1 receptor is predominantly in the ER and ER cisternae[2,10,41] and the binding site is highly likely to be intraluminal in the ER, the micromolar concentrations of *N*-alkylamine derivatives required for cytotoxicity responses in these assays may be due to interference from membrane partitioning of the drugs as they pass through multiple layers of biologic membranes before accessing the functional sigma-1 receptor binding site. Nevertheless, **3b** was found to be highly cytotoxic to the majority of cancer cell lines, whereas **3a** treatments were significantly less effective. The cytotoxicity patterns observed for **3b** indicate a general mechanism of toxicity that is

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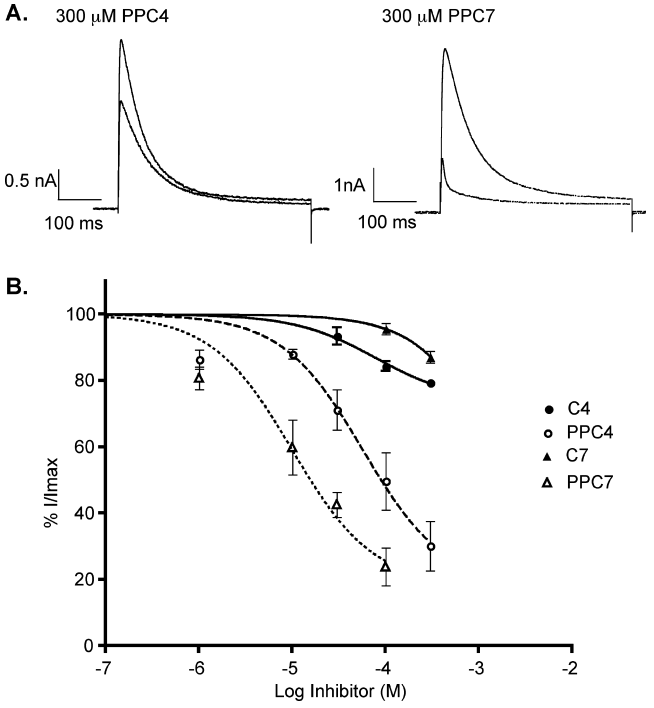

Figure 3. Inhibition of the $K_v1.4$ potassium channel by **1a** and **2a**. (A) Representative K^+ channel current traces from three experiments showing inhibition of the $K_v1.4$ potassium channel by *N*-3-phenylpropyl derivatives of *N*-alkylamines. HEK-293 cells were transfected with both the sigma-1 receptor and $K_v1.4$ potassium channel. K^+ channels were activated by 400 ms pulses to 20 mV from a resting potential of −80 mV. (B) Concentration response curves of butylamine, heptylamine, and their *N*-3-phenylpropyl derivatives. The leftward shift of the response curve for the *N*-3-phenylpropyl derivatives corresponds with the relative increase in affinity for the sigma-1 receptor.

Table 4. Summary of the Cancer Cell Cytotoxicity Measurements with 2a, 2b, 3a, and 3c[a]

	IC_{50} (μM)			
cell line	**2a**	**2b**	**3a**	**3b**
MDA-MB-231	33 ± 2	30 ± 2	54 ± 3	15 ± 1
MCF-7	20 ± 1	21 ± 1	50 ± 18	12 ± 1
MCF-10A	70 ± 5	53 ± 6	53 ± 18	15 ± 1
NCI-H460	45 ± 5	38 ± 4	55 ± 3	18 ± 2
A549	83 ± 11	55 ± 5	87 ± 70	29 ± 1
H1299	39 ± 6	28 ± 1	39 ± 0.1	13 ± 1
HCT-15	18 ± 12	>100	>100	25 ± 290
HT-29	40 ± 1	30 ± 1	42 ± 21	14 ± 0.2
SK-OV-3	>100	73 ± 8	60 ± 7	16 ± 1
Du145	25 ± 1	29 ± 1	51 ± 4	17 ± 0.4
SF-268	63 ± 2	40 ± 3	60 ± 14	16 ± 1

[a]IC_{50} values were obtained from CellTiter-Glo assays measuring ATP, an indicator of metabolically active cells. IC_{50} values ± standard errors are presented. MDA-MB-231 and MCF-7, human breast adenocarcinoma; MCF-10A, human immortalized breast cell line; NCI-H460, human lung carcinoma; A549, human lung adenocarcinoma; H1299, human non-small cell lung carcinoma; HCT-15, human colorectal adenocarcinoma; HT-29, human colorectal adenocarcinoma; SK-OV-3, human ovary adenocarcinoma; Du145, human prostate carcinoma; SF-268, human central nervous system glioblastoma.

dependent on the presence of the 4-nitro moiety and agrees with the enhanced targeting of sigma receptors.

In contrast, **2a** and **2b** exhibited some selectivity in cytotoxicity against a number of cancer cell lines. For example, **2a** and **2b** were relatively more potent against MCF-7 and MDA-MB-231, both of which are human mammary adenocarcinoma, than against MCF-10A, a nontumorigenic mammary epithelial cell line. These results are consistent with a previous report indicating that the sigma-1 receptor levels in breast cancer cells are elevated compared to those of normal cells.[42] However, a simple correlation between toxicity and expression levels of the sigma receptor may not provide a complete explanation of the data because a lung cancer cell line, H1299, that was previously shown to have increased levels of sigma-1 receptor[18,43] was insensitive to **2a** and **2b**. Nevertheless, a similar toxicity pattern of **2a** and **2b** across the panel of cancer cell lines suggests that these two compounds are likely to function through the same mechanism that is likely to involve sigma receptors.

Because sigma-1 and sigma-2 receptor ligands have been shown to induce apoptosis through the activation of caspases,[26,27] **2a**, **2b**, **3a**, and **3b** were assessed for their caspase activation potential (this work was performed at the University of Wisconsin—Madison SMMCF). These measurements showed that none of the four compounds activated caspases 3/7, 8, and 9 (data not shown). Rimcazole has been previously shown to activate caspases 3/7 in MCF-7 and MDA-MB-468 mammary carcinoma;[27] contrary to earlier reports, in our experiments, rimcazole was not found to activate caspases 3/7 in any cancer cell lines tested, including MCF-7. It is therefore unclear whether the cytotoxic effect of **2a**, **2b**, **3a**, and **3b** can be attributed to apoptosis or another currently unknown mechanism of cell death.

DISCUSSION

The binding of tridemorph,[15,32] (2*R*,5*R*)-2-butyl-5-heptylpyrrolidine,[44] and monochain sphingolipids[15] to the sigma-1 receptor warranted the current systematic study of the features of sphingosine-like molecules that strengthen their binding affinities for the sigma-1 receptor. Upon testing a series of *N*-alkylamines for their affinity for the sigma-1 receptor, we found a dependence on hydrocarbon chain length, whereby dodecylamine exhibited maximal binding. 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 for the sigma-1 receptor.[14,31,45−47] Furthermore, electron-withdrawing groups such as a *p*-nitro substitution on the phenyl ring have also been shown to enhance the affinity of a ligand.[48] In agreement with these earlier reports, we found that both *N*-3-phenylpropyl and *N*-3-(4-nitrophenyl)propyl derivatives of *N*-alkylamines have affinities 2−6 orders of magnitude higher than those of their corresponding amines for the pure sigma-1 receptor. The enhancement in affinity with the addition of *N*-3-phenylpropyl or *N*-3-(4-nitrophenyl)propyl groups is more profound with shorter chain length butylamine than with longer chain length dodecylamine for the pure sigma-1 receptor (Table 2). Studies by Glennon[31] proposed a pharmacophore for high-affinity sigma-1 receptor ligands to be composed of two hydrophobic regions flanking a central nitrogen atom. The primary hydrophobic region is situated optimally five carbons (6−10 Å) from the nitrogen. The secondary hydrophobic site is located closer to the obligate nitrogen than the primary site and is separated from the nitrogen by 2.5−3.9 Å, approximately the

dx.doi.org/10.1021/bi2004872 | *Biochemistry* 2011, 50, 7568−7578

length of three 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). The results with butyl- and heptylamine derivatives (**1a**–**2b**) correlate well with these earlier studies by Glennon et al., who showed that compounds containing an aliphatic hydrocarbon chain of four to eight carbons can bind to the sigma-1 receptor in the primary hydrophobic region of the Glennon model.[31] The data presented here provide further support for the conclusion that the sigma-1 receptor binding site(s) can accommodate molecules with an aliphatic hydrocarbon chain with as many as 18 carbons (octadecane) with a preference for 12 carbons (dodecane). Because the longer carbon chain of *N*-alkylamines bears resemblance to lipids such as D-*erythro*-sphingosine, we propose that the alkyl chain intercalates against the transmembrane (TM) regions of the sigma-1 receptor, possibly the TM2 region.

Our earlier results showed that sphingosine, *N*,*N*-dimethylsphingosine, and sphinganine weakly interact with the sigma-2 receptor;[15] therefore, it was somewhat unexpected that *N*-alkylamine derivatives also bind to the sigma-2 receptor with K_I values comparable to those obtained for the sigma-1 receptor in GPLM (Table 2). These data reflect a continuing challenge in the field to design selective sigma-2 receptor ligands.

Together, these systematic binding studies of binding of *N*-alkylamines to the pure sigma-1 receptor and *N*-alkylamine derivatives to both the sigma-1 and the sigma-2 receptors have expanded our understanding of features of both sigma binding sites that may have been neglected since the discovery that tridemorph (Figure S-1 of the Supporting Information) binds with high affinity to the sigma-1 receptor. Further studies are needed to identify the binding region of the alkyl chain moiety of these molecules to improve our understanding of the essential structural features of sigma-2 receptors.

Both sigma-1 and sigma-2 receptors are overexpressed in numerous cancer cell lines, and sigma ligands inhibit cancer cell growth presumably by activating caspase-dependent apoptosis.[18−22,26,27,42] Multiplex cytotoxicity assays revealed that **2a**, **2b**, **3a**, and **3b** inhibited growth of a number of cancer cell lines, including breast (MCF-7 and MDA-MB-231), lung (NCI-H460, A549, and H1299), prostate (Du145), ovarian (SK-OV-3), colorectal (HCT-15 and HT-29), and central nervous system (SF-268). Whether *N*-alkylamine derivatives in these studies inhibit cancer cell growth via the activation of caspases remains unclear because rimcazole, shown previously to enhance the activity of caspases 3 and 7,[27] did not give a positive caspase signal in our studies. Functional characterization of **1a** and **2a** showed that these molecules actively inhibit outward current from the $K_v1.4$ potassium channel with relative IC_{50} values that closely correlate with the relative binding affinities at the sigma-1 receptor. There has been a focus in recent years directed at understanding the mechanism by which the cellular membrane electrical potential affects cancer cell growth and proliferation; for example, numerous reports showed that progression through the cell cycle is dependent on the translocation of ions across the plasma membrane (for a review, see ref 49). Thus, pharmacological blockade of ion channel activity may lead to inhibition of cell proliferation. The coupling of the sigma-1 receptor to multiple ion channels may provide a mechanism by which sigma ligands are cytotoxic to cancer cells. Of further importance is the finding that *N*-alkylamine derivatives in this study, including **2a**, **2b**, **3a**, and **3b**, are highly selective for the sigma-1 and sigma-2 receptors. Therefore, *N*-alkylamine derivatives may be valuable tools for the functional and biochemical studies of both sigma receptor subtypes and may prove useful as potential diagnostic and/or anticancer therapeutic agents.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Structure of tridemorph (Figure S-1) and results of the primary alternate target screening (Figure S-2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was supported by National Institutes of Health Grants MH065503 and GM-33138 (to A.E.R.), the Retina Research Foundation Edwin and Dorothy Gamewell Professorship (to A.E.R.), the University of Wisconsin—Madison Advanced Opportunity Fellowships (AOF), and the Gates Millennium Scholarship (to U.B.C.). The cytotoxicity assays were performed at the University of Wisconsin—Madison Small Molecule and Medicinal Chemistry Facility and were funded by the Wisconsin Alumni Research Foundation (WARF). The alternate target assays were conducted by the National Institute of Mental Health's Psychoactive Drug Screening Program (Department of Pharmacology, School of Medicine, University of North Carolina, CB 7365, 8032 Burnett-Womack Building, Chapel Hill, NC).

■ ACKNOWLEDGMENTS

We acknowledge invaluable discussion during these experiments with Dr. Lianwang Guo (Department of Pharmacology, University of Wisconsin—Madison). We thank Dr. Nicholas Cozzi (Department of Pharmacology, University of Wisconsin—Madison) for arranging the alternate target screenings with NIMH PDSP, Noel Peters and Dr. F Michael Hoffmann from the University of Wisconsin—Madison Small Molecule and Medicinal Chemistry Screening Facility, Dr. Meyer B. Jackson (Department of Neuroscience, University of Wisconsin—Madison) for his expert advice on the electrophysiology experiments, and Dr. Jay Yang (Department of Anesthesiology, University of Wisconsin—Madison) for his assistance with the three-dimensional graphical depictions of *N*-alkylamine and *N*-alkylamine derivatives.

■ ABBREVIATIONS

DMT, *N*,*N*-dimethyltryptamine; DTG, ditolylguanidine; ER, endoplasmic reticulum; GPLM, guinea pig liver membranes; IP_3R-3, inositol trisphophate receptor type 3; KO, knockout; MAM, mitochondria-associated ER membrane; MBP, maltose binding protein; NIMH PDSP, National Institute of Mental Health's Psychoactive Drug Screening Program; SBDL, steroid-binding domain liked; SKF-10,047, *N*-allyl-normetazocine; TM, transmembrane; RLM, rat liver membranes; TLC, thin layer chromatography; SMMCSF, Small Molecule and Medicinal Chemistry Screening Facility.

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