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Synthesis and *in vitro* Evaluation of Novel Indole-Based Sigma Receptors Ligands

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To investigate the molecular features involved in sigma (σ) receptors binding, a series of compounds based on indole scaffolds were synthesized and their chemical structures were confirmed by ^{1}H-NMR, IR, and elemental analysis. Their affinity toward σ_1 and σ_2 receptor subtypes was evaluated. 1-{[4-(2-phenylethyl)piperazin-1-yl]methyl}-3-methyl-1*H*-indole 3b had a high affinity to σ_1 receptors, while three compounds, 1-{3-[4-(substitutedphenyl)piperazin-1-yl]propyl}-1*H*-indole derivatives 4a–c had shown high affinity and selectivity for σ_2 receptors. Cytotoxicity of the compounds was demonstrated on cancer cell lines from liver (HUH7), breast (MCF7), and colon (HCT-116) cancer cell lines. Compound 1c (3-{[4-(3,4-dichlorobenzyl)piperazin-1-yl]methyl}-1*H*-indole) showed significant cell growth inhibitory activity on the selected cancer cell lines.

Key words: cytotoxicity assay, drug design, indole, piperazine, radio ligand binding assay, radioligands, sigma ligands

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The class of σ receptors is subdivided into at least two subtypes, which are termed σ_1 and σ_2 receptors. To date, the σ_1 receptor is pharmacologically well characterized because of the receptor sequence information and availability of selective σ_1 ligands. The 223-amino acid σ_1 receptor with two transmembrane-spanning regions (1,2) has been purified and cloned from several species, including mouse, rat, guinea pig, and human (3–7). The σ_1 receptors bind structurally diverse classes of compounds, including diverse psychotherapeutic agents, drugs of abuse such as cocaine and methamphetamine and steroid hormones such as progesterone. The general pharmacophoric element appears to be an *N*-alkyl, *N,N*-dialkyl, or *N*-arylalkyl amine (8). The protein corresponding to σ_2 sites has not yet been cloned. In comparison with the σ_1 receptor, it appears to be slightly smaller in size (σ_1: 25–29 kDa, σ_2: 18–22 kDa) (9,10). Pharmacological experiments reveal that σ_2 receptors may be lipid raft proteins that affect calcium signaling via sphingolipid products. Unlike σ_1 receptors, σ_2 receptors do not appear to translocate. Both the subtypes of σ receptors are highly expressed on tumor cell lines from human and rat cancer tissues. However, malignant tumor cells show a higher expression of σ_2 receptors than quiescent tumor cells. The overexpression of σ_2 receptors in human and murine tumors suggests that σ_2 receptors may be a biomarker of tumor cell proliferation (11–13). Owing to the lack of availability of detailed protein structural information and truly selective σ_2 ligands, the pharmacological characterization of the σ_2 subtype with regard to its mechanism of action and biochemical role in various biological effects has been very limited.

Therefore, ligands interacting with σ receptors are of interest for example as atypical antipsychotics (14,15), antidepressants (16), anticocaine agents (17–19), and antitumor agents (20–23). Thus, selective σ_1 and σ_2 agonists and antagonists may be potentially useful drugs for treatment of several pathologic conditions such as psychiatric disorders, cocaine abuse, memory and learning disorders, dyskinesia and dystonic reactions induced by classical antipsychotic drugs, cancer and tumor diagnosis. Several compounds binding σ_1 receptors with high affinity and selectivity have been discovered, whereas σ_2 receptor ligands generally have poor selectivity over σ_1 receptors and new σ_2 ligands are needed to define the structural features that may improve their affinity and selectivity.

Glennon *et al.* (24) reported on the structure affinity relationships of a series of phenylalkylamine derivatives with respect to their binding at σ_1 receptors and elaborated the features of these compounds being important for high σ_1 receptor binding. According to the proposed two-dimensional model, two hydrophobic substituents in different distances from a basic nitrogen atom, which is supposed to bind to a proton donor site (Asp 126 and/or Glu 172) of the receptor, are required for a high σ_1 receptor affinity (Figure 1). Additionally, 1,4-disubstituted piperazine derivatives are described as high affinity σ receptor ligands in the literature (25–28). Here, we report the design of compounds according to the Glennon's pharmacophore model as well as synthesis, their binding affinities to the σ_1 and σ_2 receptors, and their effect on the inhibition of cancer cell lines from liver (HUH7), breast (MCF7), and colon (HCT-116) samples.

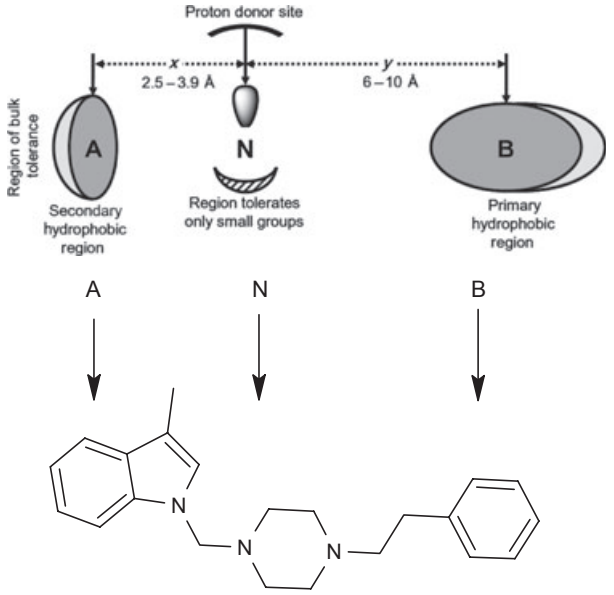


Figure 1: Pharmacophore model of σ_1 receptor ligands (24) and hypothetical binding of the compound **3b** to the σ_1 receptor.

Materials and Methods

Chemistry

Melting points (°C) were determined by using a Mettler Toledo FP62 capillary melting point apparatus (Mettler-Toledo, Greifensee, Switzerland) and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Spectrum One series FTIR apparatus (Version 5.0.1) (Perkin Elmer, Norwalk, CT, USA), using potassium bromide pellets; the frequencies are expressed in cm^{-1}. The 1H NMR spectra were recorded with a Varian Mercury-400 FT-NMR spectrometer (Varian Inc., Palo Alto, CA, USA), using tetramethylsilane as the internal reference, with chloroform-$CDCl_3$ or dimethylsulphoxide-DMSO-d6 as solvents, the chemical shifts are reported in parts per million (ppm). Elemental analyses were performed on LECO 932 CHNS (Leco-932, St. Joseph, MI, USA) instrument and were within ± 0.4% of the theoretical values.

General procedure for the preparation of 3-{[4-(substitutedphenyl/benzyl)piperazin-1-yl]methyl}-1H-indole (1a–c)

ndole (2 mmol, 235 mg) was dissolved in 20-mL ethanol–water (1:1) solution; formalin (3 mmol) and substituted phenyl piperazine (2 mmol) were added. The mixture was stirred in room temperature and the reaction monitored by TLC in benzene/methanol (9:1) and toluene/ethyl acetate/DEA (75:25:1). At the end of the reaction, the crude precipitate was filtered and purified by recrystallization or column chromatography.

3-{[4-(4-fluorophenyl)piperazin-1-yl]methyl}-1*H*-indole (1a)[a]

Crystalized from ethanol-water. Yield: 63%; white solid. Mp 166.8 °C. IR (KBr, cm^{-1}): 3128 (N-H), 3094–2756 (C-H). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 8.23 (bs,1H,indole N-H), 7.78 (d,1H,indole H_4), 7.33 (d,1H,indole H_7), 7.24 (d, 2H, phenyl H_3, H_5), 7.21 (s,1H, indole,H_2), 6.96 (d, 2H, phenyl H_2, H_6), 6.87–6.84 (m, 2H, indole H_5, H_6), 3.78 (s,2H, C-CH_2-N), 3.11 (t,4H, piperazine H_3, H_5), 2.67 (t,4H, piperazine H_2, H_6). ^{13}C-NMR (400 MHz, DMSO, ppm): 150.99, 136.23, 128.74, 127.51, 124.53, 120.82, 118.97, 118.54, 118.31, 115.20, 111.23, 110.57 (aromatics), 53.09 (C-CH2-N), 52.41 (piperazine C_3, C_5) 48.18 (piperazine C_2, C_6). Anal. Cacld. for $C_{19}H_{20}FN_3$: C, 73.76; H, 6.52; N, 13.58. Found: C, 73.69; H, 6.50; N, 13.50.

3-{[4-(2,5-difluorobenzyl)piperazin-1-yl]methyl}-1*H*-indole (1b)

Purified by column chromatography (SiO_2, AcOEt/n-hexane 1:2). Yield: 23%; yellowish solid. Mp 126.7 °C. IR (KBr, cm^{-1}): 3057 (N-H), 2935–2814 (C-H). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 8.13 (bs,1H,indole N-H), 7.72 (d,1H,indole H_4), 7.35 (dd,1H,indole H_7), 7.26 (s,1H, indole,H_2), 7.21–6.89 (m,5H, indole H_5, H_6, phenyl), 3.74 (s,2H, C-CH_2-N), 3.54 (s, 2H, N-CH_2-Ph) 2.53 (bs,8H,piperazine). Anal. Cacld. for $C_{20}H_{21}F_2N_3$: C, 70.36; H, 6.20; N, 12.31. Found: C, 70.27; H, 6.15; N, 12.26.

3-{[4-(3,4-dichlorobenzyl)piperazin-1-yl]methyl}-1*H*-indole (1c)

Purified by column chromatography (SiO_2, AcOEt/n-hexane 1:2). Yield: 20%; yellowish solid. Mp 107 °C. (KBr, cm^{-1}): 3435 (N-H), 2933–2820 (C-H). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 8.48 (bs,1H,indole N-H), 7.71 (d,1H,indole H_4), 7.39 (d,1H,indole H_7), 7.34 (s,1H, indole,H_2), 7.32–7.05 (m,5H, indole H_5, H_6, phenyl), 3.74 (s,2H, C-CH_2-N), 3.41 (s, 2H, N-CH_2-Ph) 2.45 (bs,8H,piperazine). Anal. Cacld. for $C_{20}H_{21}Cl_2N_3$: C, 64.18; H, 5.65; N, 11.23. Found: C, 64.08; H, 5.62; N, 11.20.

General procedure for the preparation of 1,3-di-{[4-(substitutedphenyl)piperazin-1-yl]methyl}-1H-indole (2a)

For disubstituted derivative, indole (2 mmol), formalin (6 mmol), and 4-fluorophenyl piperazine (4 mmol) were refluxed in ethanol for 4 h. The precipitate was filtered, washed with cold ethanol, dried and purified by recrystallization.

1,3-di-{[4-(4-fluorophenyl)piperazin-1-yl]methyl}-1*H*-indole (2a)

Recrystallization from ethanol-water. Yield: 28.5%; white solid. Mp 192.8 °C. (KBr, cm^{-1}): 2938–2836 (C-H). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.76 (d,1H,indole H_4), 7.47 (d,1H,indole H_7), 7.26 (s,1H, indole,H_2), 7.23–6.89 (m, 10H, indole H_5, H_6 + phenyl), 4.86 (s,2H, N-CH_2-N), 3.78 (s,2H, C-CH_2-N), 3.10–3.05 (m,8H, piperazine H_3, $H_{3'}$, H_5, $H_{5'}$), 3.58–2.70 (m, 8H, piperazine H_2, $H_{2'}$, H_6, $H_{6'}$). Anal. Cacld. for $C_{30}H_{33}F_2N_5$: C, 71.83; H, 6.63; N, 13.96. Found: C, 71.75; H, 6.59; N, 13.94.

General procedure for the preparation of 1-{[4-(substitutedphenyl/phenylethyl)piperazin-1-yl]methyl}-3-methyl-1H-indole (3a–b)

To a solution of 3-methylindol (2.2 mmol, 300 mg) in ethanol (20 mL), formalin (3 mmol) and substituted phenyl piperazine

(2.2 mmol) were added. The mixture was refluxed 4 h, and the formed precipitate was filtered, dried and if necessary recrystalized from appropriate solvent.

1-{[4-(4-fluorophenyl)piperazin-1-yl]methyl}-3-methyl-1*H*-indole (3a)

Crystalized from ethanol-water. Yield: 50.6%; white solid. Mp 109.5 °C. IR (KBr, cm^{-1}): 3045–2788 (C-H). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.55 (d,1H,indole H_4), 7.42 (d,1H,indole H_7), 7.21 (t, 1H, indol H_6), 7.12 (t,1H, indole H_5), 6.93 (d, 2H, phenyl H_3, H_5), 6.90 (s,1H, indole H_2), 6.81 (dd, 2H, phenyl H_2, H_6), 4.80 (s,2H, N-CH_2-N), 3.08 (t,4H, piperazine H_3, H_5), 2.69 (t,4H, piperazine H_2, H_6), 2.33 (s, 3H, -CH_3). ^{13}C-NMR (400 MHz, $CDCl_3$, ppm): 158.65, 156.27, 148.14, 137.48, 129.17, 126.33, 121.92, 119.20, 118.25, 115.72, 111.15, 110.01 (aromatics), 67.86 (C-CH_2-N), 50.72(piperazine C_3, C_5), 50.33 (piperazine C_2, C_6), 9.83 (-CH_3). Anal. Cacld. for $C_{20}H_{22}FN_3$: C, 74.28; H, 6.86; N, 12.99. Found: C, 74.25; H, 6.75; N, 12.96.

1-{[4-(2-phenylethyl)piperazin-1-yl]methyl}-3-methyl-1*H*-indole (3b)

Crystalized from ethanol-water. Yield: 39%; white solid. Mp 122.9 °C. IR (KBr, cm^{-1}): 3022–2763 (C-H). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.54 (d, 2H, indole H_4), 7.40 (d, 1H, indole H_7), 7.28–7.08 (m, 7H, indol H_5, H_6 + phenyl), 6.92 (s,1H, indole H_2), 4.76 s, 2H, N-CH_2-N), 2.75. Anal. Cacld. for $C_{22}H_{27}N_3$: C, 79.24; H, 8.16; N, 12.60. Found: C, 79.20; H, 8.15; N, 12.56.

General procedure for the preparation of 1-{3-[4-(substitutedphenyl)piperazin-1-yl]_ propyl}-1H-indole (4a–c)

To a solution of substituted phenyl piperazine (5 mmol) in 10 mL of acetone was added 7.5 mL of a 25% solution sodium hydroxide. Thirty minutes later, 1-bromo-3-chloropropane (5.5 mmol) was added carefully to minimize its mixing with aqueous layer. The mixture was stirred slowly for 22 h with a magnetic stirrer. The organic phase was then separated, and the solvent was removed under vacuum. A mixture of indole (2.5 mmol) and 87% w/v solution KOH (7.5 mmol) in DMSO (30 mL) was stirred at room temperature for 1 h. Reaction mixture was cooled in ice-water bath to 0 °C, and 1-(3-Chloropropyl)-4-(substituted phenyl)piperazine in DMSO (10 mL) was added dropwise. The stirring was continued at room temperature for 20–30 h. After addition of water (50 mL) and extraction with Et_2O, the organic layer was washed with water and dried over anhydrous Na_2SO_4. The solvent was evaporated and the oily residue was purified by column chromatography (SiO_2, AcOEt/n-hexane 1:2) to give 1-{3-[4-(substituted phenyl)piperazin-1-yl]propyl}-1H-indole as an oil.

1-{3-[4-(4-fluorophenyl)piperazin-1-yl]propyl}-1*H*-indole *(4a)*

Yellowish oily residue was purified by column chromatography (SiO_2, AcOEt/n-hexane – 1/2). Rf 0.19 (SiO2, AcOEt/n-hexane 1:1). Yield: 18%. IR (KBr, cm^{-1}): 3022–2763 (C-H), 1245 (C=C). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm) 6.86–7.64 (m, 10H, indole + phenyl), 4.24 (t,2H,indoleN-CH_2-CH_2-CH_2), 3.13 (t,4H, piperazine H_3, H_5), 2.57 (t, 4H, piperazine H_2, H_6), 2.33 (t, 2H, CH_2-CH_2-CH_2-Npiperazine), 2.03 (q, 2H, CH_2-CH_2-CH_2). ^{13}C-NMR (400 MHz, DMSO, ppm): 158.79, 155.45, 148.62, 136.40, 129.31, 128.73, 121.57, 119.49, 117.70, 117.64, 116.01, 115.79, 110,43, 101.13 (aromatics), 55,13 (indoleN-CH_2-CH_2-CH_2), 55.14 (piperazine C_3, C_5), 49.65 (piperazine C_2, C_6), 43.95 (CH_2-CH_2-CH_2-N piperazine), 27,67 (CH_2-CH_2-CH_2). Anal. Cacld. for $C_{21}H_{24}FN_3$: C, 74.75; H, 7.17; N, 12.45. Found: C, 74.71; H, 7.13; N, 12.46.

1-{3-[4-(2-fluorophenyl)piperazin-1-yl]propyl}-1*H*-indole (4b)

Yellowish oily residue was purified by column chromatography (SiO_2, AcOEt/n-hexane – 1/2). Rf 0.21 (SiO2, AcOEt/n-hexane 1:1). Yield: 15%. IR (KBr, cm^{-1}): 3010–2755 (C-H), 1225 (C=C). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 6.84–7.65 (m, 10H, indole + phenyl), 4.22 (t,2H,indoleN-CH_2-CH_2-CH_2), 3.10 (t,4H, piperazine H_3, $H_{3'}$, H_5, $H_{5'}$), 2.55 (t, 4H, piperazine H_2, $H_{2'}$, H_6, $H_{6'}$), 2.32 (t, 2H, CH_2-CH_2-CH_2-Npiperazine), 2.05 (q, 2H, CH_2-CH_2-CH_2). Anal. Cacld. for $C_{21}H_{24}FN_3$: C, 74.75; H, 7.17; N, 12.45. Found: C, 74.70; H, 7.15; N, 12.26.

1-{3-[4-(phenyl)piperazin-1-yl]propyl}-1*H*-indole (4c)[b]

Yellowish oily residue was purified by column chromatography (SiO_2, AcOEt/n-hexane – 1/2). Rf 0.20 (SiO2, AcOEt/n-hexane 1:1). Yield: 17%. IR (KBr, cm^{-1}): 3011–2788 (C-H), 1240 (C=C). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): 7.00–7.64 (m, 11H, indole + phenyl), 4.20 (t,2H,indoleN-CH_2-CH_2-CH_2), 3.11 (t,4H, piperazine H_3, $H_{3'}$, H_5, $H_{5'}$), 2.54 (t, 4H, piperazine H_2, $H_{2'}$, H_6, $H_{6'}$), 2.32 (t, 2H, CH_2-CH_2-CH_2-Npiperazine), 2.07 (q, 2H, CH_2-CH_2-CH_2). Anal. Cacld. for $C_{21}H_{25}N_3$: C, 78.96; H, 7.89; N, 13.15. Found: C, 78.91; H, 7.83; N, 13.16.

Receptor binding studies

Materials and general procedures

Guinea pig brains and rat livers were commercially available (Harlan-Winkelmann, Germany). Homogenizer: Elvehjem Potter (B. Braun Biotech International). Centrifuge: High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Finnigan). Filter: Printed Filtermat Type B (Perkin Elmer) presoaked in 0.5% aqueous polyethylenimine for 2 h at room temperature before use. The filtration was carried out with a MicroBeta FilterMate-96 Harvester (Perkin Elmer). The scintillation analysis was performed using Meltilex (Type A) solid scintillator (Perkin Elmer). The solid scintillator was melted on the filtermat at a temperature of 95 °C for 5 min. After solidification of the scintillator at room temperature, the scintillation was measured using a MicroBeta Trilux scintillation analyzer (Perkin Elmer). The counting efficiency was 20%.

Membrane preparation for the σ_1 assay (29–32)

Five guinea pig brains were homogenized with the potter (500–800 rpm, 10 up-and-down strokes) in six volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 × *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 23 500 × *g* for 20 min at 4 °C. The pellet was resuspended in 5–6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23 500 × *g* (20 min, 4 °C). This procedure was repeated twice. The final pellet

was resuspended in 5–6 volumes of buffer, the protein concentration was determined according to the method of Bradford (33) using bovine serum albumin as standard, and subsequently the preparation was frozen (−80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

Performing of the σ_1 assay (29–32)

The test was performed with the radioligand [^{3}H]-(+)-pentazocine (42.5 Ci/mmol; Perkin Elmer). The thawed membrane preparation (about 75 μg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and buffer (50 mM TRIS, pH 7.4) in a total volume of 200 μL for 180 min at 37 °C. The incubation was terminated by rapid filtration through the presoaked filtermats by using the cell harvester. After washing each well five times with 300 μL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was placed on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at room temperature. The bound radioactivity trapped on the filters was counted in the scintillation analyzer. The non-specific binding was determined with 10 μM unlabeled (+)-pentazocine. The K_d-value of the radioligand [^{3}H]-(+)-pentazocine is 2.9 nM.

Membrane preparation for the σ_2 assay (29–32)

Two rat livers were cut into small pieces and homogenized with a potter (500–800 rpm, 10 up-and-down strokes) in six volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 × g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31 000 × g for 20 min at 4 °C. The pellet was resuspended in buffer (50 mM TRIS, pH 8.0) and incubated at room temperature for 30 min. After the incubation, the suspension was centrifuged again at 31 000 × g for 20 min at 4 °C. The final pellet was resuspended in buffer, the protein concentration was determined according to the method of Bradford (33) using bovine serum albumin as standard, and subsequently the preparation was frozen (−80 °C) in 1.5 mL portions containing about 2-mg protein/mL.

Performing of the σ_2-assay (29–32)

The test was performed with the radioligand [^{3}H]-di-*o*-tolylguanidine (50 Ci/mmol; ARC). The thawed membrane preparation (about 100 μg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]-di-*o*-tolylguanidine, 500 nM (+)-pentazocine and buffer (50 mM TRIS, pH 8.0) in a total volume of 200 μL for 180 min at room temperature. The incubation was terminated by rapid filtration through the presoaked filtermats using a cell harvester. After washing each well five times with 300 μL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was placed on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at room temperature. The bound radioactivity trapped on the filters was counted in the scintillation analyzer. The non-specific binding was determined with 10 μM unlabeled ditolylguanidine. The K_d-value of the radioligand [^{3}H]-ditolylguanidine is 17.9 nM.

Data analysis

All experiments were carried out in triplicate using standard 96-well multiplates (Diagonal). The IC_{50}-values were determined in competition experiments with six concentrations of the test compounds and were calculated with the program GraphPad Prism® 3.0 (GraphPad Software) by nonlinear regression analysis. The K_i-values were calculated according to Cheng and Prusoff (34). The K_i-values are given as mean values + SEM from three independent experiments.

Cytotoxicity studies

Cell culture

The human cancer cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM), with 10% fetal bovine serum (FBS) and 1% penicillin and incubated in 37 °C incubators containing 5% CO_2 and 95% air.

NCI-60 sulphorhodamine B assay

Cancer cells (range of 2000 cell/well to 5000 cell/well) were inoculated into 96-well plates in 200 μL of media and incubated in 37 °C incubators containing 5% CO_2 and 95% air. After a 24 h incubation period, one plate for each cell line was fixed with 100 μL of 10% ice-cold trichloroacetic acid (TCA). This plate represents the behavior of the cells just prior to drug treatment and is accepted as the time-zero plate. The compounds to be tested were solubilized in dimethyl sulfoxide (DMSO) to a final concentration of 40 mM and stored at +4 °C. While treating the cells with the compounds, the corresponding volume of the compound was applied to the cell to achieve the desired drug concentration and diluted through serial dilution. After drug treatment, the cells were incubated in 37 °C incubators containing 5% CO_2 and 95% air for 72 h. Following the termination of the incubation period after drug treatment, the cells were fixed with 100 μL of 10% ice-cold TCA and incubated in the dark at +4 °C for 1 h. Then the TCA was washed away with ddH_2O five times, and the plates were left to air dry. For the final step, the plates were stained with 100 μL of 0.4% Sulphorhodamine B (SRB) (cat. no. 86183–5 g from Sigma) solution in 1% acetic acid solution. Following staining, the plates were incubated in dark for 10 min at room temperature. The unbound dye was washed away using 1% acetic acid, and the plates were left to air dry. To measure the absorbance results, the bound stain was then solubilized using 200 μL of 10 mM Tris-Base. The OD values were obtained at 515 nm.

Results and Discussion

The new piperazine substituted indole derivatives have been designed, according to the σ_1 receptorial model proposed by Glennon *et al.* (24,35,36), with the assumption that the indole moiety may interact with a secondary hydrophobic site corresponding to the hydrophobic ''A'' region, the basic piperazine N-atom linked by the alkylene chain to the indole moiety may interact with a receptorial proton donor site and the substituted N-phenyl/benzyl moiety may bind a primary hydrophobic region similar to the phenyl 'B' region of the σ_1 receptorial model and modulate the binding affinity of the compounds for σ_1 or σ_2 receptors. In Figure 1, the most potent σ_1 ligand is compared with Glennon model. The distance between the left basic N-atom and the terminal phenyl moiety is 5

atoms (6 bond lengths). This distance corresponds exactly with the most potent compounds of Glennon with a 5-phenylpentyl residue at the N-atom.

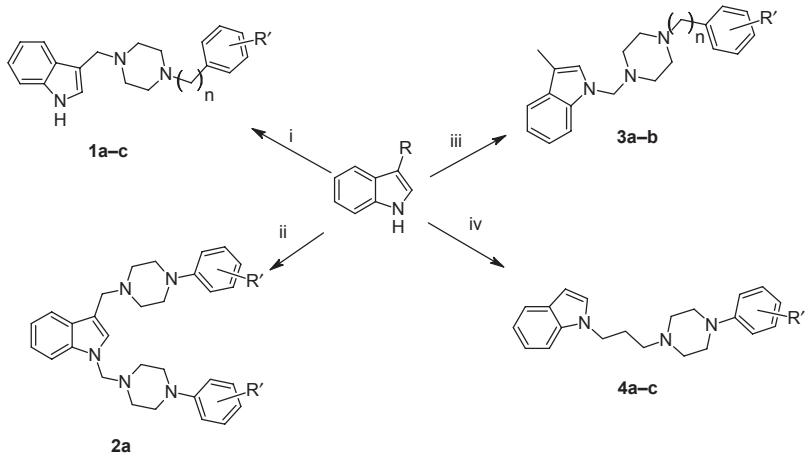
The preparation of the compounds is illustrated in Scheme 1. The groups of 3-{[4-(substitutedphenyl/benzyl)piperazin-1-yl]methyl}-1*H*-indole **1a**–**c**[a], 1,3-di-{[4-(4-fluorophenyl)piperazin-1-yl]methyl}-1*H*-indole **2a** and 1-{[4-(substitutedphenyl/phenylethyl)piperazin-1-yl]methyl}-3-methyl-1*H*-indole **3a**–**b** were prepared by Mannich reaction of substituted piperazine and formaldehyde with indole or 3-methylindole. The crude products were purified by recrystallization or column chromatography.

1-{3-[4-(substitutedphenyl)piperazin-1-yl]propyl}-1*H*-indole **4a**–**c**[b] were synthesized by the reaction of indole and 1-(3-chloropropyl)-4-(substitutedphenyl)piperazine in presence of potassium hydroxide. To obtain 1-(3-chloropropyl)-4-(substitutedphenyl)piperazine, substituted phenyl piperazine was reacted with 1-bromo-3-chloropropane. Compounds **4a**–**c** were purified by column chromatography on silica gel using ethyl acetate/n-hexane as a mobile phase system (Scheme 1).

The σ receptor affinity of the compounds was evaluated with receptor binding studies. The test compounds compete with tritium labeled ligands for a limited number of receptors. Homogenates of guinea-pig brain and rat liver were used as receptor material in the σ_1 assay and the σ_2 assay, respectively. In the σ_1 assay, [^{3}H]-(+)-pentazocine was employed as radioligand, and the non-specific binding was determined in the presence of a large excess of (+)-pentazocine. As a σ_2 selective radioligand is not commercially available, the non-selective radioligand [^{3}H]-ditolylguanidine was employed in the presence of a large excess of non-radiolabeled (+)-pentazocine (500 nM), which selectively occupies σ_1 receptors. Performing of the σ_2 assay in the presence of an excess of non-tritiated 1,3-di(o-tolyl)guanidine led to the non-specific binding of the radioligand (29–32).

Selectivity ratios between the σ_1 receptor and the σ_2 receptor were also calculated to determine relative specificity and are summarized in Table 1.

When structural modifications were examined (Table 1), the σ receptor affinities were low in 1-non-substitutedindol derivatives **1a**–**c**. Replacement of the phenyl ring by a benzyl moiety increased σ affinity of these compounds. Exchange of 2,5-difluoro for 3,4-dichloro substitution increased σ_1 and σ_2 affinity for the same group of compounds. Only low σ_1 and σ_2 receptor affinities were determined for 1,3-dipiperazinomethyl substituted indole **2a**. 1-{[4-(2-Phenylethyl)piperazin-1-yl]methyl}-3-methyl-1*H*-indole **3b** shows very high affinity for σ_1 (K_i = 14.2 nM) and σ_2 (K_i = 55.8 nM) receptors and, therefore, can be regarded as unselective σ ligand. Compound **3a** having 4-fluorophenyl subtitution showed 70 times lower affinity than **3b** for σ_1 receptor sites. A specific interaction of compound **3a** with σ_2 receptors could not be observed even at a concentration of 1 μM. A comparison of the σ receptor affinities of **3a** and **3b** showed that an increased distance between the piperazine ring and the phenyl moiety resulted in enhanced selectivity for the σ_1 subtype. Compounds **4a**–**c** displayed very high affinity and selectivity for σ_2 receptors *in vitro*. Remarkably, the σ_2 affinity of the compounds having a trimethylene spacer between indole and piperazine ring was 5–100 fold increased, whereas the σ_1 affinity was not changed. In this series of compounds, phenylpiperazin-1-ylpropyl derivatives **4a**–**c**, without a substituent in 3-position of the indole system, exhibited high affinity (K_i = 20, 9.9, and 75 nM) and selectivity for the σ_2 receptor. The highest σ_2/σ_1 selectivity was found for the 2-fluorophenyl derivative **4b** having the highest σ_2 receptor affinity.



	1a	1b	1c	2a	3a	3b	4a	4b	4c
R	H	H	H	H	CH_3	CH_3	H	H	H
n	0	1	1	-	0	2	-	-	-
R′	4-F	2,5-diF	3,4-diCl	4-F	4-F	H	4-F	2-F	H

Scheme 1: Synthesis of compounds **1a**–**c**, **2a**, **3a**–**b**, and **4a**–**c**. Reagent and conditions: (i) HCHO, substituted piperazine, EtOH, room temperature; (ii) HCHO, 4-F-phenylpiperazine, EtOH, reflux, 4 h; (iii) HCHO, substituted piperazine, EtOH, reflux, 4 h; and (iv) 87% KOH, DMSO, room temperature, 1 h; 1-(3-chloropropyl)-4-(substitutedphenyl)piperazine, DMSO, 0 °C, 20h.

Table 1: Binding affinities of compounds at σ_1 and σ_2 receptors

Compounds	K_i ± SEM (nM)		
	σ_1[a]	σ_2[b]	Selectivity ratio σ_1/σ_2
1a	3640	660	5.5
1b	216 ± 98	418 ± 55	0.5
1c	126 ± 23	130 ± 9	1
2a	2690	1000	2.7
3a	1000	0%[c]	–
3b	14.2 ± 4	55.8 ± 1.6	0.3
4a	456 ± 32	20 ± 3	22.8
4b	264 ± 130	9.9 ± 2.7	26.7
4c	1492	75 ± 7.7	19.9

Values are mean ± SEM of three experiments performed in duplicate.
[a]Displacement of [^{3}H](+)pentazocine.
[b]Displacement of [^{3}H]-ditolylguanidine in the presence of (+)pentazocine.
[c]Inhibition of radioligand [^{3}H]ditolylguanidine at concentration of 1 μM.

The compounds were tested for their effect on cellular viability against cancer cell lines from liver (HUH7), breast (MCF7), and colon (HCT-116) samples. The results are given in Table 2.

The cytotoxic activity of the synthesized compounds was investigated on liver (HUH7), breast (MCF7), and colon (HCT116) cancer cell lines, by means of sulphorhodamine B (SRB) assays in triplicate. Serial dilutions from 40 to 2.5 μM were used, and Camptothecin was the positive control for the cytotoxic effect (Table 2). As seen in Table 2, especially, **1c** and **1b** showed high cytotoxicity levels on the selected cancer cell lines. **1c** had lower IC_{50} values when compared with 5-FU. A 50% growth inhibition of the cancer cell lines was observed in micromolar concentrations. Among compounds, the best inhibitory activity against HUH7 (IC50 = 3.42 μM) was exhibited by compound **1c** (3-{[4-(3,4-dichlorobenzyl)piperazin-1-yl]methyl}-1*H*-indole) (Table 2). For MCF7 (IC50 = 2.92 μM), HCT116 (IC50 = 9.19 μM) cell lines, compound **1c** and compound **1b** showed the lowest IC50 values, respectively.

Conclusion and Future Directions

In conclusion, four different series of σ receptor ligands were synthesized and evaluated for their σ receptor affinities. To modulate the relative affinities and selectivities of ligand binding to σ_1 and σ_2 receptor subtypes, we synthesized several modifications of the indole derivatives. The findings from this study led to the conclusion that an increase in the linker length between the indole and piperazine rings to three methylene moieties results in compounds with high affinity and selectivity for σ_2 receptors. Additionally, introduction of two large substituents on indole ring was not tolerated by sigma receptors.

Table 2: IC50(μM) of the compounds for liver (HUH7), breast (MCF7), and colon (HCT116) carcinoma cell lines

Compounds	HUH7[a]	MCF7[a]	HCT116[a]
1a	14.63	9.32	11.65
1b	13.87	5.47	9.19
1c	3.42	2.92	9.33
2a	[b]	[b]	[b]
3a	NI	40.95	NI
3b	NI	26.23	23.86
4a	17.67	21.66	20.45
4b	NI	23.95	30.24
4c	NI	NI	NI
5-FU	30.66	3.5	18.7

[a]All the experiments were conducted in triplicate ($1 < R^2 < 0.8$). NI: no inhibition.
[b]Insoluble.

Currently, a large variety of chemotherapeutic drugs are used to treat cancer. However, many compounds have limited efficacy due to problems of delivery and penetration and a moderate degree of selectivity for cancer cells. In this study, our results demonstrate that some of the synthesized compounds exhibit a high cytotoxic effect on growing cancer cells *in vitro*. This study identifies this new series of agents for cancer therapy.

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Notes

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