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Synthesis and Pharmacological Evaluation of [(4-Arylpiperazin-1-yl)-alkyl]-carbamic Acid Ethyl Ester Derivatives as Potential Anxiolytic Agents

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On the basis of our earlier studies, a series of N-{4-[4-(aryl) piperazin-1-yl]-phenyl}-amine derivatives containing terminal carbamoyl fragment with alkyl spacer of different lengths (**15**-**20**) were synthesized as ligands, for 5-hydroxytryptamine-1A (5-HT_{1A}) receptor. Molecular modeling studies were undertaken to explain the influence of spacer length on ligands affinity towards 5-HT_{1A} receptor. Compound **19** showed all the specific interactions responsible for recognition. The protonated amine of the ligand forms an ionic hydrogen bond with the negatively charged Asp116 of transmembrane3 helix (TM3), while the carbamoyl moiety interacts with Asn386 and Tyr390 of TM7. The aryl group is involved in forming a CH-π interaction with Phe362. The strong interaction of compound **19** with 5-HT_{1A} receptor in docking studies was confirmed by radio ligand binding studies. Compound **19** showed high affinity for the receptor (Ki = 0.018 nM). *In vivo* pharmacological testing of compound **19** (3 mg/kg body weight) showed increased open arm entries, as well as time spent in Elevated plus Maze test. Toxicological analysis also revealed no significant biochemical or morphological alterations in the vital organs of experimental animals. Furthermore our results suggest that these compounds share some pharmacological effects with established anxiolytics and might prove to be effective compounds for the treatment of anxiety.

Key words: Serotonin receptor ligands, Anxiolytic activity, Molecular modeling, Ligand binding, Elevated plus Maze

INTRODUCTION

Psychiatric disorders such as anxiety and depression are closely connected with disturbances in the function of different neuromediator systems. Serotonin (5-hydroxytryptamine, 5-HT) is one of the important neurotransmitters, which mediates a wide variety of physiological responses in both the peripheral and central nervous systems. Alterations in serotonin neurotransmission have been implicated in a number of human disorders such as migraine, depression and anxiety, as well as in normal human functions such as sleep, sexual activity and appetite. 5-HT attains such variety of functions by acting on distinct receptor types (Buhot et al., 2000). Among the 14 different types of serotonin receptors belonging to G protein-coupled receptor (GPCR) family (Hoyer et al., 2002), 5-HT_{1A} subtype is a major target for neurobiological research and drug development. Its activation leads to a number of physiological changes. The development of drugs that alter 5-HT neurotransmission is thus an area of intense research.

The 5-HT_{1A} receptor has been the most extensively studied of all the serotonin receptors. The reason resides in the early availability of the selective agonist 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT) that has allowed extensive biochemical, physiological, and pharmacological characterization of the receptor (Gozlan et al., 1983). Several structural classes of agents are known

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to bind 5-HT_{1A} receptor sites, such as aminotetralins, indolylalkylamines, ergolines, aporphines, aryloxyalkylamines and arylpiperazines. Among these, the arylpiperazine derivatives represent one of the most important classes of 5-HT_{1A} receptor ligands (Obniska et al., 2006). Long chain aryl piperazines (LCAPs), having an alkyl chain attached to the N4 atom of the piperazine moiety and a terminal amide or an imide fragment, are the most thoroughly studied of all the aryl piperazine derivatives. The significance of the respective parts of LCAPs for 5-HT_{1A} receptor affinity, intrinsic activity, and selectivity has been the subject of many structure-activity relationship studies (Rodriguez et al., 2002).

Molecular modeling techniques provide useful information on structural and functional aspects of GPCRs and their interactions with ligands. GPCRs are membrane spanning receptors that mediate most of their intracellular actions through pathways involving activation of G-proteins. Until late 2007, the only high-resolution structures of GPCRs available were those of bovine rhodopsin (Okada et al., 2004), and GPCR homology models were necessarily based on this template (Kiss et al., 2008). Recently, several GPCR crystal structures have been reported, and the structures of human β_2-adrenergic receptor (β_2), (Rasmussen et al., 2007; Hanson et al., 2008) squid rhodopsin, (Murakami and Kouyama, 2008) turkey β_1-adrenergic receptor (β_1) (Warne et al., 2008) have been solved providing a wealth of information and revealing important structural differences between rhodopsin and other class A GPCRs, particularly in transmembrane helices and in the structure of loop regions (Michino et al., 2009). Very close evolutionary relationships between 5-HT receptors and beta adrenoreceptors provide a means of obtaining a better model for the serotonin receptors. Due to the absence of 3D structures of 5-HT receptors, a model of rat 5-HT_{1A} receptor was constructed from the X-ray structure of turkey beta 1 adrenergic receptor using molecular modeling.

We have previously concluded that aryl piperazine compounds with a unsubstituted phenyl piperazine fragment (**1**) or having an O-methoxy substituted phenyl piperazine fragment (**2**) along with carbamoyl functionality at their terminal N-4 fragment have the potential to be used as anxiolytic agents (Khatri et al., 2009). We have therefore synthesized a new series of (4-aryl-piperazin-1-yl) alkyl]carbamic acid ethyl ester derivatives (**15**-**20**). The structural modifications consist of the replacement of phenyl ring, which connects the aryl piperazine fragment to the carbamoyl terminal, with a different length alkyl spacer. The interactions of aryl piperazine derivatives with selective amino acid residues of 5-HT_{1A} receptor active site were studied using molecular docking analysis. *In vitro* radioligand binding study was carried out to evaluate the binding affinity. An elevated plus maze test was performed to assess potential antianxiety activity. Moreover the best anxiolytic derivatives were further analysed for their acute and subacute toxicity to evaluate the safety of these new compounds.

MATERIALS AND METHODS

Chemistry

All organic solvents and common reagents were procured from Merck India Ltd. All the arylpiperazines were procured from Aldrich Chemical Company, Inc. USA. TLC analysis was carried out on commercially available flexible TLC silica gel (silica gel 60 F254) plates (Merck). The purity of all organic compounds was confirmed by TLC, ^{1}H-NMR, IR, and Mass spectroscopy. ^{1}H-NMR spectra were recorded in Bruker Spectrospin Avance 300 instrument operating at 300 MHz in $CDCl_3$ or DMSO using TMS as an internal standard. The chemical shifts are reported in parts per million (δ) downfield from TMS and coupling constants are reported in hertz (Hz). IR spectra (KBr) were recorded on a Perkin-Elmer BX FT-IR instrument. Melting points were determined on a Buchi melting point B-450 instrument. Mass spectra were recorded on a Qstar (Applied biosystem) ESI-MS mass spectrometer. Elemental analysis was performed on Heraeus CHN rapid analyzer.

General procedure for synthesis of 3-8

Sodium carbonate (15 mM) was added to a stirred solution of aryl piperazine (10 mM) in ethanol (20 mL). Then bromo alkyl nitrile (10 mM) was added dropwise and the mixture was stirred for 2 h. The precipitated inorganic salts were then filtered off, washed with ethanol and the filtrate was concentrated on rotary evaporator. Then the compound was partitioned with ethyl acetate and water. The organic layer was dried over anhydrous sodium sulfate and concentrated on a rotary evaporator. Finally after evaporation of the solvent the residue was purified by column chromatography, using hexane/ethyl acetate (80:20) as eluent. The spectroscopic data for all these intermediate compounds (**3**-**8**) is given as supplementary material.

General procedure for synthesis of 9-14

A solution of (**3**-**8**, 5 mM) in tetrahydrofuran (15 mL) was added dropwise to a suspension of lithium aluminium hydride (380 mg, 10 mM) in dry tetrahydrofuran (20 mL) under nitrogen atmosphere at 0°C. The temperature of reaction mixture was then allowed to rise to room temperature and stirred for 2-3 h. The reac-

tion was then quenched by addition of water (0.47 mL), 15% aqueous NaOH (0.47 mL) and water (1.41 mL) sequentially. The resulting granular precipitate was passed through the pad of celite and washed with ethyl acetate. The combined filtrate was evaporated under reduced pressure, and the residue was chromatographed on silica gel. (Eluent, MeOH-CH_2Cl_2 30:70). The spectroscopic data for all these intermediate compounds (**9-14**) is given as supplementary material.

General procedure for synthesis of 15-20

A solution of ethyl chloro formate (540 µL, 5 mM) in dry DCM (5 mL) was added dropwise to a solution of **9-14** (5 mM) and triethylamine (2 mmol) in dry DCM (20 mL). The mixture was allowed to stir at room temperature for 3 h. The reaction mixture was poured into water (30 mL) and extracted with CH_2Cl_2 (2 × 30 mL). The organic layer was dried and the solvent was evaporated under reduced pressure to yield an oil, which was purified by silica gel column chromatography using a mixture of CH_2Cl_2 and MeOH (9.8:0.2) as eluent to yield (65-70%) of the desired product (Scheme 1).

[2-(4-Phenyl-piperazin-1-yl)-ethyl]-carbamic acid ethyl ester (15)

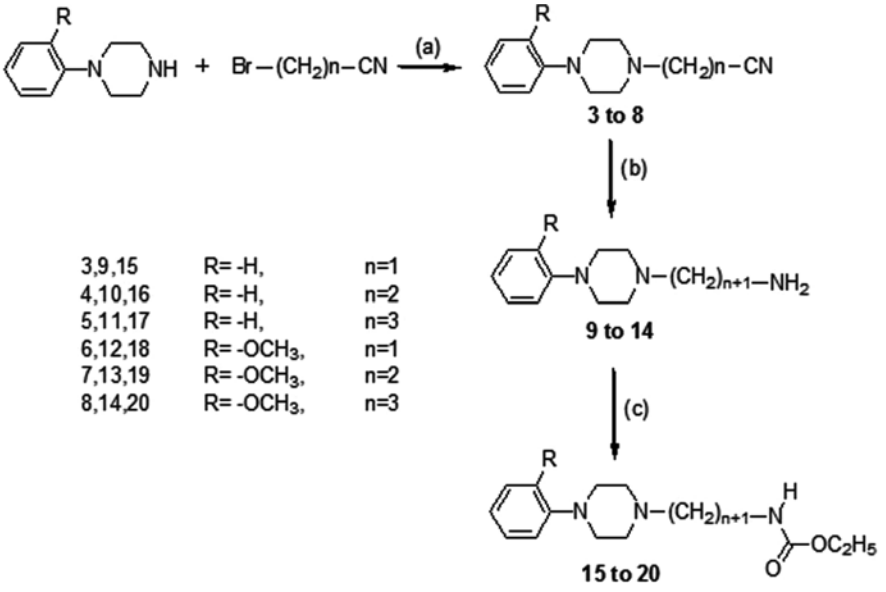
Yield: 85%; m.p. 175-178°C; IR(KBr) ν cm^{-1}: 1237.67 (C-N), 1448.57 (C=C), 2267.83 (C≡N), 2939.89 (C-H), 3346.45 (N-H); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.22-1.27 (t, 3H, -CH_3, *J* = 7.8 Hz), 2.51-2.55 (t, 2H, N-CH_2, *J* = 6.0 Hz), 2.60-2.63 (m, 4H, piperazine), 3.08-3.23 (m, 4H, piperazine), 3.29-3.33 (t, 2H, CH_2-N, *J* = 5.7 Hz), 4.08-4.15 (q, 2H, O-CH_2, *J* = 6.9 Hz), 5.18 (br, 1H, -NH), 6.83-7.2 (m, 5H, Ar-H); LC-MS *m/z* 278 (M^+ +1); Anal. Calcd. for $C_{15}H_{23}N_3O_2$: C, 64.95; H, 8.36; N, 15.15. Found: C, 64.82; H, 8.31; N, 15.09.

[3-(4-Phenyl-piperazin-1-yl)-propyl]-carbamic acid ethyl ester (16)

Yield: 83%; m.p. 189-191°C; IR(KBr) ν cm^{-1}: 1252.46 (C-N), 1442.40 (C=C), 2282.36 (C≡N), 2967.89 (C-H), 3339.45 (N-H); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.70-1.74 (t, 3H, -CH_3, *J* = 6.3 Hz), 1.9-2.04 (m, 2H, CH_2-**CH_2**-CH_2), 2.47-2.52 (t, 2H, CH_2-CN, *J* = 6.6 Hz), 2.61-2.63 (m, 4H, piperazine), 3.19-3.22 (m, 4H, piperazine), 3.27-3.31 (t, 2H, N-CH_2, *J* = 6 Hz), 4.10-4.17 (q, 2H, -O-CH_2, *J* = 7.2 Hz), 5.6 (br, 1H, -NH), 6.92-7.34 (m, 5H, Ar-H); LC-MS *m/z* 292 (M^+ +1); Anal. Calcd. for $C_{16}H_{25}N_3O_2$: C, 65.95; H, 8.65; N, 14.42. Found: C, 65.98; H, 8.72; N, 14.50.

[4-(4-Phenyl-piperazin-1-yl)-butyl]-carbamic acid ethyl ester (17)

Yield: 85%; m.p. 202-204°C; IR(KBr) ν cm^{-1}: 1239.46 (C-N), 1459.40 (C=C), 2248.78 (C≡N), 2972.9 (C-H), 3345.45 (N-H); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.18-1.24 (m, 2H, CH_2-**CH_2**-CH_2-CH_2), 1.57-1.61 (m, 5H, CH_2-CH_2-**CH_2**-CH_2, -CH_3), 2.39-2.44 (t, 2H, N-CH_2, *J* = 6.9 Hz), 2.60-2.63 (m, 4H, piperazine), 3.18-3.24 (m, 6H, piperazine, CH_2-NH), 4.04-4.11 (q, 2H, -OCH_2), 5.46 (br, 1H, -NH), 6.8-7.2 (m, 5H, Ar-H); LCMS ($C_{17}H_{27}N_3O_2$): *m/z* 306 (M^+ +1); Anal. Calcd. for $C_{17}H_{27}N_3O_2$: C, 65.95; H, 8.65; N, 14.42. Found: C, 65.98; H, 8.72; N, 14.50.



Scheme 1. General procedure for synthesis of compounds. Reagents and conditions: (a) EtOH, Na_2CO_3, rt, 2 h; (b) $LiAlH_4$, EtOAc, 0°C, 2 h; (c) $ClCOOC_2H_5$, DCM, Et_3N, 2 h.

{2-[4-(2-Methoxy-phenyl)-piperazine-1-yl]-ethyl}-carbamic acid ethyl ester (18)

Yield: 85%; m.p. 202-204°C; IR(KBr) ν cm^{-1}: 1267.46 (C-N), 1473.78 (C=C), 2238.91 (C≡N), 2948.92 (C-H), 3338.45 (N-H); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.22-1.27 (t, 3H, -CH_3, *J* = 7.2 Hz), 2.52-2.56 (t, 2H, N-CH_2, *J* = 6.0 Hz), 2.65 (s, 4H, piperazine), 3.07 (s, 4H, piperazine), 3.30-3.34 (t, 2H, CH_2-N, *J* = 5.4 Hz), 3.85 (s, 3H, -OCH_3), 4.08-4.15 (q, 2H, O-CH_2, *J* = 6.9 Hz), 5.24 (br, 1H, NH), 6.8-6.9 (m, 4H, Ar-H); LC-MS *m/z* 308 (M^+ +1); Anal. Calcd. for $C_{16}H_{25}N_3O_3$: C, 62.52; H, 8.20; N, 13.67. Found: C, 62.58; H, 8.12; N, 13.72.

{3-[4-(2-Methoxy-phenyl)-piperazine-1-yl]-propyl}-carbamic acid ethyl ester (19)

Yield: 80%; m.p. 216-218°C; IR(KBr) ν cm^{-1}: 1267.76 (C-N), 1448.89 (C=C), 2228.21 (C≡N), 2941.62 (C-H), 3348.85 (N-H); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.61-1.67 (s, 3H, -CH_3), 1.67-1.71 (m, 2H, CH_2-CH_2-CH_2), 2.48-2.53 (t, 2H, CH_2-NH, *J* = 6.6 Hz), 2.66 (s, 4H, piperazine), 3.10 (s, 4H, piperazine), 3.25-3.29 (t, 2H, N-CH_2, *J* = 5.4 Hz), 3.86 (s, 3H, -OCH_3), 4.06-4.09 (q, 2H, -OCH_2, *J* = 3.6 Hz), 5.24 (br, 1H, -NH), 6.89-7.19 (m, 4H, Ar-H); LC-MS *m/z* 322 (M^+ +1); Anal. Calcd. for $C_{17}H_{27}N_3O_3$: C, 63.53; H, 8.47; N, 13.07. Found: C, 63.62; H, 8.52; N, 13.14.

{4-[4-(2-Methoxy-phenyl)-piperazine-1-yl]-butyl}-carbamic acid ethyl ester (20)

Yield: 85%; m.p. 230-232°C; IR(KBr) ν cm^{-1}: 1252.87 (C-N), 1468.89 (C=C), 2238.10 (C≡N), 2945.72 (C-H), 3340.54 (N-H); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.19-1.34 (m, 5H, CH_2-CH_2-CH_2-CH_2, -CH_3), 1.57-1.59 (m, 2H, CH_2-CH_2-CH_2-CH_2), 2.41-2.46 (t, 2H, N-CH_2, *J* = 6.6 Hz), 2.67 (s, 4H, piperazine), 3.12-3.20 (m, 6H, piperazine, CH_2-CN), 3.86 (s, 3H, -OCH_3), 4.10-4.17 (q, 2H, -OCH_2), 5.54 (br, 1H, -NH), 6.8-6.99 (m, 4H, Ar-H); LC-MS *m/z* 336 (M^+ +1); Anal. Calcd. for $C_{18}H_{29}N_3O_3$: C, 64.45; H, 8.71; N, 12.53. Found: C, 64.52; H, 8.63; N, 12.48.

Molecular modeling

The comparative homology modeling of human 5-HT_{1A} receptor was carried out with MOE2009.10 (Chemical Computing Group) on Quad-Core intel Xeon workstation (2.33 GHz processor). The amino acid sequence of 5-HT_{1A} was obtained from SwissProt database. The 5-HT_{1A} receptor sequence (SP08908) has 422 amino acids. The X-ray structure of turkey beta1 adrenergic receptor bound to cyanopindolol (PDB code: 2VT4.pdb) (Berman et al., 2002) with 2.7 Å resolution was downloaded from Protein Data Bank and was used as a template for modeling the 3D structure of the $5HT_{1A}$ receptor.

The sequences of 5-HT_{1A} and 2VT4 were aligned using MOE-Align tool making sure that the gaps were inserted into the loop regions between the helices, preferably where there are no steric clashes with other parts of structure. 2VT4 has a longer loop than 5-HT_{1A} between helices IV and V (Cys192 and 198). This loop has Cys-Cys bond. To build the homology model for 5-HT_{1A}, the atoms for residues 192-201 in the 2VT4 template structure were deleted, and AMBER99 force field was used with solvation parameter set to R-field. The developed homology model was checked for quality using MOE suite. The criteria used in the analysis include bond lengths, bond angles, dihedrals, side chain contacts and chirality of alpha carbon atoms. The phi-psi map, Ramachandran plot, Chi plot and distance matrix plot of the model were constructed. Docking of ligands was done using MOE 2009.10 with MMFF94x force field. Force field bonded, Van der Waals and electrostatics energy was calculated with non-bonded cut-off switched on at 8 Å and switched off at 10 Å. Distance dependent dielectric (internal 1 and external 80) was used to model implicit solvation. Receptor was kept fixed during docking with pocket cut-off 6 Å.

Experimental animals

Male Wistar rats (6-8 weeks old), weighing 120-150 g each, were selected from the stock colony and maintained in our animal facility with free access to food and water. Animals were maintained in an air-conditioned room. The room was maintained at 25 ± 2°C with natural daytime and no light after 19:00 h, until morning. All the experiments were performed during the light phase according to the guidelines of the Institutional Animal Ethics committee.

5-HT_{1A} binding assay

Radioligand binding assay was performed following a published procedure (Schlegel and Peroutka, 1986). The cerebral cortex obtained from male Wistar rats was homogenized in 20 volumes of ice cold Tris-HCl buffer (50 mM, pH 7.7) and centrifuged at 50,000 g for 10 min at 0°C. The resulting pellet was resuspended, incubated at 37°C for 10 min and centrifuged again at 50,000 g for 10 min at 37°C. The final pellet was resuspended in Tris-HCl buffer containing 10 μM pargyline, 4 mM $CaCl_2$ and 0.1% ascorbate. 5-HT_{1A} binding was determined in the cortical preparation with or without 0.1 nM [^{3}H] 8-OH-DPAT (GE healthcare-TRK850) as a competitive binding agent. Specific binding was defined as the difference between binding in the absence and presence of 5-HT (10 μM).

Elevated plus Maze test

The elevated plus maze was comprised of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 30 cm). The arms extend from a central platform (5 cm × 5 cm) that was elevated to a height of 50 cm above floor level. The experiments were performed between 9:00 h and 15:00 h. Each rat was placed in the central area of the maze with its head facing an open arm. The following behavioural parameters were recorded, during 5 min exposure using FW: Camera O: Fire - iBBW 1.3 interface (Columbus Instruments): (1) time in open arms; (2) time in closed arms; (3) time in central area; (4) number of open arm entries; (5) number of closed arm entries; (6) number of central area entries. Any rat, which dropped off the plus maze, was excluded in the result (Walf and Frye, 2007).

The experimental animals were treated with Buspirone hydrochloride (Sigma-Aldrich Co.) (5 mg/kg, n = 8) or the compounds (3 mg/kg) intraperitoneally in a volume of 2 mL/kg body weight of rats, 60 min. before evaluation in the maze. The control group was given saline with 1% tween 80 and 1% DMSO.

Acute toxicity

As per the Organization of Economic Co-operation and Development (OECD) guidelines for testing of chemicals (OECD, 2001a), 12 rats were randomly chosen and divided into three groups. They were orally administered compounds at a dose of 375, 750 and 1000 mg/kg body weight and the control group received vehicle. Body weight, signs of toxicity and mortality were observed after the administration at the first, second, fourth and sixth hour and once daily for 14 days. On day 15, all rats were made to fast for 16-18 h, and then sacrificed for necropsy examination. The internal organs were excised and weighed. The gross pathological observations of the tissues were performed.

Subacute toxicity

The experiments were performed according to the OECD test guidelines with slightly modifications (OECD, 2001b). Male Wistar rats (n = 6) received water vehicle orally (control group) or compounds at the effective dose of 3 mg/kg/day for 15 consecutive days. The body weight was recorded weekly and their food and water intake was monitored daily. Animals were observed for signs of abnormalities during the treatment period. At the end of the treatment, the animals were made to fast overnight, but allowed access to water *ad libitum*. After the animals were anesthetized with ether, the blood samples were obtained by retro-orbital puncture (Waynforth, 1980) using capillary tubes for hematological and biochemical studies, with and without anticoagulant, respectively.

Haematological and biochemical analysis

Haematological analysis was performed using an automatic haematological analyzer (Coulter STKS, Beckman). The parameters included: red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb), haematocrit (HCt), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets count were determined (Costa-Silva et al., 2007). For biochemical analysis, blood was centrifuged at 1480 × *g* for 10 min to obtain serum, which was stored at 20°C until determination of the following parameters according to standard methods of Bergmeyer and Bernt (1974) using an assay kit: glucose, blood urea nitrogen (BUN), creatinine, Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, alkaline phosphatase (ALP), total and direct bilirubin, total protein.

RESULTS AND DISCUSSION

The synthetic methodology employed to develop target compounds is summarized in Scheme 1. The reaction of 1-aryl piperazine with bromoalkyl nitrile in ethanol in the presence of sodium carbonate afforded the corresponding nitrile derivatives (**3**-**8**). The nitrile group was reduced in tetrahydrofuran with lithium aluminium hydride to give (4-aryl-piperazin-1-yl)-alkylamines (**9**-**14**). Subseqent condensation of the resulting with ethyl chloroformate in methylene chloride in the presence of triethyl amine provided the target compounds [(4-aryl-piperazin-1-yl)-alkyl]-carbamic acid ethyl ester (**15**-**20**) in an approximate yield of 80%.

Molecular modeling

To explain the interaction of aryl piperazine derivatives with 5-HT_{1A} receptor molecular docking analysis of the compounds and natural ligand of serotonin receptor was carried out using a homology model based on a recently solved X-ray crystal structure of turkey beta 1 adrenergic receptor (PDB ID: 2VT4). The binding cavity is composed of TM2 (Ala93, Tyr96), TM3 (Ile113, Asp116, Cys120), TM5 (Cys187, Ile189, Ser199), TM6 (Phe361, Phe362) and TM7 (Asn386, Trp387, Tyr390) domains. Building a protein model requires several theoretical assumptions and methodological approximations. To validate the ligand receptor interactions, serotonin, the natural ligand, was docked in the developed homology model of the $5HT_{1A}$ receptor. The 5-HT was correctly positioned into the inner part of the seven helix bundle and the fundamental ligand-protein

interactions were detected. The available free space between the helices 3, 5 and 6 accommodate the phenolic oxygen of serotonin. The indole ring of serotonin is close to the backbone of helix V. The 5-OH group was found to form hydrogen bond with Ser199. The negatively charged Asp116 forms an ionic hydrogen bond with the protonated nitrogen of serotonin. The three dimensional model developed for serotonin binding was used to observe the binding mode of the synthesized compounds. The binding modes of these molecules were consistent with the binding mode of serotonin. Thus, this model can serve as a platform for designing of selective ligands for $5HT_{1A}$ receptor.

The 2-methoxyphenyl moiety of compounds **2** and **18** to **20** was found between transmembrane helices 3, 5 and 6 interacting with the aromatic cluster of helix 6, especially Phe362. In compound **2** this moiety was also involved in the formation of hydrogen bond with Ser199 (Fig. 3A). The carbamoyl domain interacted predominantly with the amino acids localized on helices 2, 3 and 7, forming both favourable Van der Waals contact with hydrophobic residues as well as hydrogen bonds with polar sites such as Tyr390 (Fig. 1). Analysis of the complexes between the ligands **15** to **20** and 5-HT_{1A} receptor model showed that introduction of an alkyl spacer allows the formation of a salt bridge between the protonated piperazine nitrogen of the compounds and Asp116 of the receptor which is generally considered crucial for all monoamine neurotransmitter receptors (Medina et al., 2009; Kowalski et al., 2011). Directed mutagenesis experiments also suggested that Asp116, Asn386 and Ser199 may be involved in the ligand binding (Liapakis et al., 2000). Introduction of a phenyl ring (**1** and **2**) instead of alkyl spacer makes the compound more rigid and causes unfavourable steric interactions with the receptor, leading to the loss of the essential anchoring interaction between the aromatic moiety and Asp116.

The length of the alkyl spacer was also optimized: compounds with piperazinyl propyl chain (**16** and **19**) showed good affinity for the 5-HT_{1A} receptor, (Fig. 2 and 3B) compared to compounds containing spacer with one atom shorter (**15** and **18**) or one atom longer (**17** and **20**). The formation of additional hydrogen bonds with Tyr390 and Asn386 might be attributed to the high affinity of these compounds for the receptor. On the other hand shortening of the spacer hindered the interaction with polar residues on TM7 making compounds with least affinity in the series at 5-HT_{1A} receptor site.

These results explained the influence of the spacer between the aryl piperazine and the carbamoyl fragment and that the length of the spacer is of great importance for 5-HT_{1A} affinity. Ligands with three carbon alkyl spacer (**16** and **19**) possess all the essential interactions when docked to the $5HT_{1A}$ receptor model, whereas compounds with two carbon alkyl spacer (**15** and **18**) are poorly active. Notably reduction of the spacer or replacement by the phenyl ring leads to rigid compounds **1** and **2**, maintaining the anxiolytic activity in the EPM model (Khatri et al., 2009) but their interactions within the 5-HT_{1A} receptor binding pocket were reduced explaining their lower activity as compared to compounds **16** and **19**.

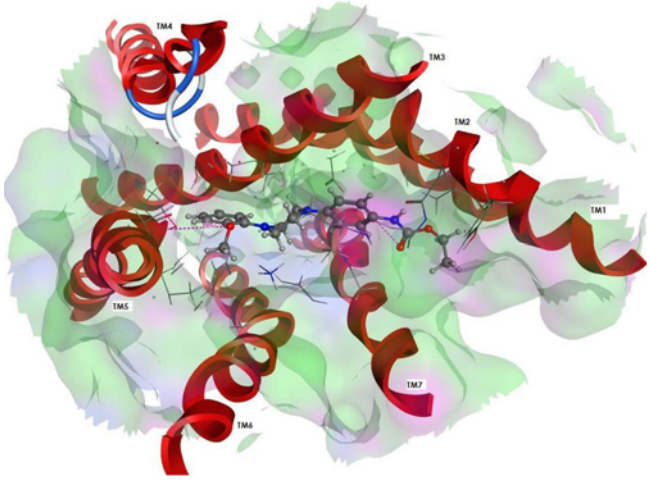


Fig. 1. Surface view of molecular model of the ligand receptor complex for compound **2** studied by means of ligand-receptor docking. Helical bundle is presented from the extracellular side. Sticks representation depicts residues used as "active site" in docking.

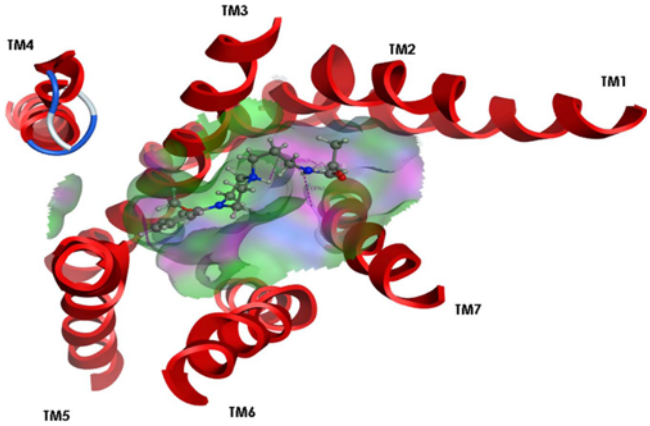


Fig. 2. Surface view of molecular model of the ligand receptor complex for compound **19** studied by means of ligand-receptor docking. Helical bundle is presented from the extracellular side. Sticks representation depicts residues used as "active site" in docking.

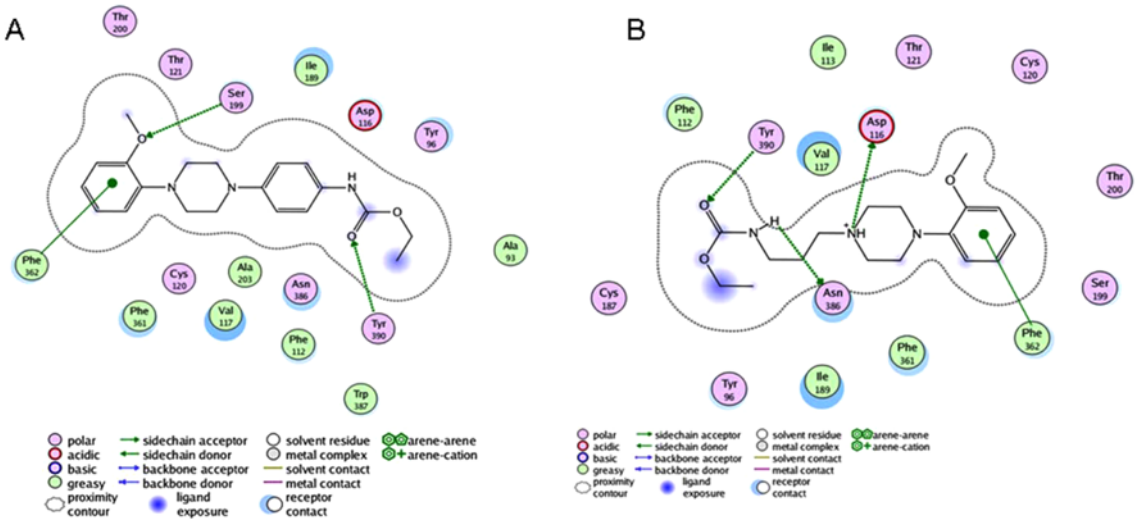
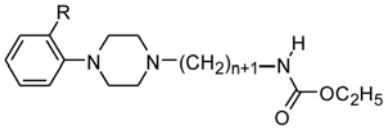


Fig. 3. Detailed view of molecular model of the ligand receptor complex studied by means of ligand-receptor docking. (**A**) for compound **2** (**B**) for compound **19**. In the detailed view for compound **2**, dashed green lines represent H-bonds with Ser199 and Tyr390. A solid green line shows a CH-π interaction with Phe362. In the detailed view for compound **19**, dashed green lines represent H-bonds with Asn386 and Tyr390 and a salt bridge with Asp116. A solid green line shows a CH-π interaction with Phe362.

5-HT$_{1A}$ receptor binding

The ability of aryl piperazine derivatives to displace [^{3}H]-8-OH DPAT, a 5-HT$_{1A}$ receptor agonist from the 5-HT$_{1A}$ receptor was determined and the results are summarized in Table I. All the compounds showed nanomolar or even subnanomolar affinities. All the compounds showed better results as compared to Buspirone (Ki = 12 nM) (Garrattini et al., 1988) except compound **15** (Ki = 28.2 nM) and **18** (Ki = 21.7 nM) having two carbon alkyl spacer. Besides the outstanding 5-HT$_{1A}$ receptor affinity of compound **19** (Ki = 0.018 nM) and compound **16** (Ki = 0.04 nM), Ki values were clustered in a relatively narrow range from 2.4 nM for compound **20** to 28.2 nM for compound **15**. Replacement of phenyl group with alkyl spacer had a significant effect on 5-HT$_{1A}$ binding, since its replacement caused a remarkable increase (compound **1**, 15.04 nM *vs* compound **19**, 0.018 nM). Moreover, regarding the influence of the alkyl spacer, compounds with piperazinyl propyl chain (**16** and **19**) had the highest affinity for the 5-HT$_{1A}$ receptor. Instead, the compounds with piperazinyl ethyl chain (**15** and **18**) seem to show a less favourable affinity profile as compared to **16** and **19**. Compounds having O-methoxy substituted phenyl piperazine fragment showed high affinity for the 5-HT$_{1A}$ receptor, relative to the compounds in which phenyl group attached to piperazine is unsubstituted. The binding profile observed for the derivatives, is in accordance with our molecular docking results.

Table I. *In vitro* binding affinities of compounds 1, 2 and 15 to 20 for 5-HT$_{1A}$ receptor



Compound	Substituents		Ki (5-HT$_{1A}$R) nM
	R	n	
1	H	Replaced with C_6H_4	15.04 ± 0.87
2	OCH_3	Replaced with C_6H_4	9.25 ± 1.1
15	H	1	28.2 ± 2.2
16	H	2	0.04 ± 0.001
17	H	3	8.37 ± 0.26
18	OCH_3	1	21.7 ± 1.6
19	OCH_3	2	0.018 ± 0.001
20	OCH_3	3	2.4 ± 0.21

For purpose of comparison, 8-OH-DPAT binds 5-HT$_{1A}$ receptor with Ki value of 0.80 nM, under same assay conditions. Ki values are mean ± S.D. of two to four experiments performed in triplicate.

Elevated plus Maze test

To validate the *in vitro* results, functional profile of all the derivatives was determined *in vivo*; to find out whether changes in the structure of the aryl fragment and the length of alkyl chain influence their anxiolytic activity. The Elevated plus Maze is one of the most widely used animal models for screening putative anxiolytics, in which rodents show an avoidance of exposed open areas of the maze, which are presumed to be the most aversive, and a preference for sections enclosed by protective walls (Dawson and Tricklebank,

1995). Conventional anxiety indices in the elevated plus-maze test comprise percent open arm entries and percent time spent in these areas in the maze, with anxiolytics generally increasing and anxiogenics decreasing these measures.

The effects of single administration of all the 6 aryl piperazine derivatives along with previously reported compound **1** and **2**, Diazepam, Buspirone and vehicle on spent time and the number of entries into the open arms of EPM are shown in Table II. One way analysis of variance revealed a significant increase in percentage time spent on the open arms after administration of both positive controls, Diazepam and Buspirone, as compared to control. The majority of tested compounds produced a significant anxiolytic action and displayed a considerable increase in the number of open arm entries. Among all the 6 compounds, **16** (3 mg/kg bwt) prolonged the percentage of entries in open arms with ~90 % ($p < 0.001$) and **19** (3 mg/kg bwt) with ~105% ($p < 0.001$) as compared to control. These results are found to be comparable with Buspirone. The total number of arm entries was not significantly different for all treatment groups.

The results presented in Table II have conclusively proven that the derivatives having alkyl chain with three carbons significantly increased the entry as well as time spent in open arms (**16** and **19**) compared to the corresponding derivatives having alkyl chain with two (**15** and **18**) and four carbons (**17** and **20**) and the previously reported compounds having hydrophobic phenyl ring instead of alkyl spacer (**1** and **2**). Within a set of respective structural analogs, phenyl piperazines with a methoxy group (-OCH_3) at the ortho position displayed good anxiolytic activity, compared to unsubstituted phenyl piperazines (**18** to **20**). From the results obtained compounds 19 turned out to be the most active as seen from the results of behavioural test. Our results showed that compounds **16** and **19** are the most anxiolytic and these compounds were further evaluated for their toxicity in rat model to establish their safety and efficacy.

Acute toxicity

Compound **16** and **19**, the most potent anxiolytics were selected for acute toxicity testing. Clinical symptoms were measured for 14 days after the single oral gavage administration of 1000 mg/kg. Acute toxicity describes the adverse effects of a substance which result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 h). The results showed that all the rats after administration of dose of 1000 mg/kg body weight/day did not show any mortality, and the autopsy of the animals at the end of the experimental period (14 days) revealed no apparent changes in any organs. The results indicated that the compounds were safe at dose of 1000 mg/kg or less in rats, which is according to the OECD guidelines categorised as unclassified and safe.

Subacute toxicity

In order to establish the safety and efficacy of a new drug, an essential first step is to evaluate the toxicity of the new molecular entity in experimental animals (Alam et al., 2006). Depending on the duration of exposure of the animal to the drug; toxicological studies may be classified as acute, subacute, or chronic. Rats in sub-acute toxicity study received repeated doses of both the compounds at their effective dose of 3 mg/kg (Khatri et al., 2009) intraperitoneally for 14 days. Changes in body weight and organ weight are used as

Table II. Effects of arylpiperazines (**1**, **2** and **15** to **20**) on the relevant anxiolytic parameters in the elevated plus maze test

Compound	Spent time into open arms	Number of entries into open arms	Number of entries into closed arms	Total number of entries	% Number of entries into open arms
1[a]	87.60 ± 21.40***	6.29 ± 2.40	9.19 ± 4.58	15.48 ± 5.28	40.64 ± 8.01***
2[a]	84.36 ± 23.68***	6.41 ± 2.12	11.80 ± 3.57	18.21 ± 4.80	35.23 ± 16.27***
15	60.30 ± 27.12*	3.56 ± 1.24	10.94 ± 4.62	14.50 ± 5.91	24.55 ± 13.21*
16	**89.92 ± 18.76*** **	6.72 ± 2.23	9.18 ± 2.65	15.90 ± 3.83	**42.26 ± 17.40*** **
17	74.47 ± 16.84**	5.12 ± 1.00	10.35 ± 3.91	15.47 ± 4.08	33.09 ± 12.43**
18	65.46 ± 23.05	3.97 ± 1.12	11.12 ± 3.51	15.09 ± 3.83	26.30 ± 11.23*
19	**98.23 ± 17.87*** **	7.47 ± 1.87	8.90 ± 4.08	16.37 ± 4.23	**45.63 ± 14.75*** **
20	73.78 ± 18.37	5.04 ± 2.52	11.20 ± 2.08	16.24 ± 4.46	31.03 ± 16.41**
Bus	85.56 ± 26.98***	5.35 ± 1.42	8.91 ± 4.20	14.26 ± 5.35	37.52 ± 12.38***
Dzp	105.36 ± 22.46***	8.34 ± 1.56	10.13 ± 3.76	18.47 ± 3.89	45.16 ± 14.7***
Veh	42.84 ± 17.51	3.03 ± 1.20	10.23 ± 3.40	13.26 ± 4.20	22.86 ± 10.28

Data represent the mean ± S.E.M.; n=8. Bus, Buspirone; Dzp, Diazepam; Veh, Tween 80 (1%), DMSO (1%). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared with vehicle. [a]Ref. (Khatri et al., 2009)

Table III. Effect of compounds **16** and **19** intraperitoneally on haematological and serum biochemical parameters in Wistar rats treated for 14 consecutive days

Parameters	Control (saline)	Compound **16** (3 mg/kg)	Compound **19** (3 mg/kg)
	Haematological Parameters		
RBC ($\times 10^6$ $(mL)^{-1}$)	8.0 ± 0.1	7.31 ± 0.36	8.23 ± 0.35
Hemoglobin (g/dL)	14.8 ± 0.2	14.20 ± 0.5	14.90 ± 0.60
Hematocrit (%)	42.7 ± 0.6	39.50 ± 1.34	44.50 ± 1.34
MCV (fL)	53.7 ± 1.34	53.90 ± 2.65	54.32 ± 2.10
MCH (rg)	18.6 ± 0.8	19.40 ± 0.65	18.23 ± 0.80
MCHC (g/dL)	34.4 ± 0.2	36.32 ± 1.7	33.80 ± 3.10
	Serum Biochemical Parameters		
ALT (U/L)	38.88 ± 3.3	40.21 ± 4.31	46.26 ± 3.84*
AST (U/L)	155.60 ± 14.85	148.41 ± 14.7	152.50 ± 17.8^{ns}
ALP (U/L)	145.65 ± 13.7	146.08 ± 16.7	140.21 ± 14.3
BUN (mg/dL)	5.38 ± 0.59	5.27 ± 0.460	5.43 ± 0.69
Creatinine (mg/dL)	0.74 ± 0.03	0.67 ± 0.041	0.97 ± 0.047*
Total Bilirubin (mg/dL)	0.28 ± 0.02	0.32 ± 0.024	0.35 ± 0.016^{ns}

The values are expressed as mean ± S.E.M. ($n = 6$ animals/group). *$p < 0.05$ as compared with vehicle, ns, nonsignificant. RBC: red blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration and WBC: white blood cell, ALT: alanin aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase and BUN: blood urea nitrogen

an indicator of adverse effect of drugs and chemicals (Raza et al., 2002). In the study, treatment resulted in small, and insignificant weight gains suggesting that these compounds are non-toxic in experimental rats. The status of bone marrow activity and intravascular effects were monitored by haematological examinations. There were no significant changes observed in various haematological parameters such as Hb, RBC, Hematocrit etc. compared to the control group, which indicates that compound **16** and **19** may not be toxic and does not affect circulating blood cells, haematopoiesis and leukopoeisis. The increase of serum transaminase enzymes (ALT and AST) levels is a good indicator of hepatocyte damage (Latha et al., 1998). Also, there were no significant changes observed in any liver function parameters, such as SGPT, SGOT and ALP compared to the control group (Table III). Increase in these parameters would have indicated hepatocyte damage. The normal levels of serum creatinine and urea indicate that these compounds did not interfere with renal function and that renal integrity was preserved.

In conclusion, compounds **16** and **19**, fitted well in the 5-HT_{1A} receptor site showed all the principle interactions in docking analysis and exhibited a high binding affinity (0.018 nM) in the radioligand binding assay. These compounds significantly increased number of entries as well as time spent in open arms, in the behavioural test EPM and revealed no toxicity in toxicological analysis. All these data suggest that these compounds might serve as candidates for the treatment of anxiety disorders and as lead compounds for further designing new potential anxiolytic agents. However, additional information related to the activity and receptor specificity of these compounds would be helpful in the elucidation of the mechanism of their anti anxiety activity, which will be reported at a later date.

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