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New alkylpiperazines as 5-HT$_7$R ligands

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Title: New alkylpiperazines as 5-HT_7R ligands

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Abstract

The 5-HT_7 receptor is the last member of the serotonin receptors family. This receptor, cloned and identified in 1993, belongs to the G protein-coupled receptor family and is positively coupled with adenylyl cyclase. Different isoforms, which differ only in the length and amino acid composition of their C-terminal tail, are generated by alternative splicing of the 5-HT_7 receptor gene and are namely: 5-$HT_{7(a),\ (b),\ (c)}$ in rat and 5-$HT_{7(a),\ (b),\ (d)}$ in human. These isoforms do not show significant differences in their pharmacological profile, signal transduction or tissue distribution and the 5-$HT_{7(a)}$ is the most abundant in human. Since its identification, 5-HT_7 receptor has been the subject of intense research efforts due to its presence in functionally relevant regions of the brain. For this reason, 5-HT_7 receptor has been suggested to have a role in a wide range of physiological functions such as nociception, sleep, locomotor activity regulation, learning and memory. Also, it seems to be involved in some pathologies like anxiety, depression, epilepsy, and Fragile X syndrome. After the cloning of 5-HT_7 receptor, a number of non-selective ligands, belonging to different chemical classes and showing high affinity toward this receptor, were identified; however, these compounds display multi-receptor affinity. In the last decade, there have been many efforts to discover selective agents for the 5-HT_7 receptor. Examples of such molecules are characterized as "long-chain" arylpiperazine compounds, which are categorized as 5-HT_7R ligands because they indicate high affinity and good selectivity for the receptor. Due to the high drug potential of long-chain arylpiperazines, with a number of successfully developed drugs or pharmacological tools, various structure-affinity relationships studies have been done. Furthermore, given the therapeutic potential of 5-HT_7 receptor agents in central nervous system disorders, we recently worked on the development of new selective 5-HT_7 receptor ligands to gain a comprehensive insight about their structure-affinity relationships and the functional properties. In this thesis, novel series of long-chain arylpiperazines were designed, synthesized, and tested to evaluate their affinity for the 5-HT_7 and 5-HT_{1A} receptors. Moreover, molecular modeling studies were performed in order to investigate these new ligands interactions with the 5-HT_7 receptor.

Keywords: Serotonin; 5-HT_{1A}R; 5-HT_7R; binding properties; structure–affinity relationship studies; homology models; molecular docking; *N*-long-chain arylpiperazine; *O*-long-chain arylpiperazine; bivalent ligand approach; dual ligands; selective 5-HT_7R ligands; bis-piperazines.

List of papers and manuscripts

The thesis is based on the following published paper and manuscripts:

I. Modica, M. N.; Intagliata, S.; Pittalà, V.; Salerno, L.; Siracusa, M. A.; Cagnotto, A.; Salmona, M.; Romeo, G. Synthesis and binding properties of new long-chain 4-substituted piperazine derivatives as 5-HT_{1A} and 5-HT_7 receptor ligands. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 1427. *Paper I*

II. Intagliata, S.; Modica, M. N.; Pittalà, V.; Salerno, L.; Siracusa, M. A.; Cagnotto, A.; Salmona, M.; Kurczab, R.; Bojarski, A. J.; Romeo, G. New *N*- and *O*-long-chain arylpiperazine derivatives as 5-HT_{1A} and 5-HT_7 receptor ligands: studies on quinazolin-4(3*H*)-one system. *Manuscript I*

III. Intagliata, S.; Modica, M. N.; Pittalà, V.; Salerno, L.; Siracusa, M. A.; Cagnotto, A.; Salmona, M.; Romeo, G. Bivalent ligand approach to the design of new 1-(4-aryl-1-piperazinyl)-3-[4-(phenylmethy)-1-piperazinyl]-1-propanone derivatives as highly selective ligands for 5-HT_7 over the 5-HT_{1A} receptor. *Manuscript II*

The following paper is related to the work described, but not included in the thesis:

1. Salerno, L.; Pittalà, V.; Modica, M.; Siracusa, M. A.; Intagliata, S.; Cagnotto, A.; Salmona, M.; Kurczab, R.; Bojarski, A. J.; Romeo, G. Structure-activity relationships and molecular modeling studies of novel arylpiperazinylalkyl 2-benzoxazolones and 2-benzothiazolones as 5-HT_7 and 5-HT_{1A} receptor ligands. *Eur. J. Med. Chem*. **2014**, *85*, 716.

Abbreviations and Acronyms

2-Br-LSD	2-Bromolysergic acid diethylamide
5-CT	5-Carboxamidotryptamine
5-HIAA	5-Hydroxyindole-3-acetic acid
5-HT	5-Hydroxytryptamine, serotonin
5-HT_{1A}R	5-HT_{1A} receptor
5-HT_{7}R	5-HT_{7} receptor
5-HTP	5-Hydroxytryptophan
5-MeOT	5-Methoxytryptamine
8-OH-DPAT	8-Hydroxy-2-(di-*n*-propylamino)tetraline
cAMP	3'-5' Cyclic Adenosine Monophosphate
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
FRET	Förster Resonance Energy Transfer
FXS	Fragile X Syndrome
GPCRs	G Protein-Coupled Receptors
HEK-293	Human Embryonic Kidney 293
HTS	High-Throughput Screening
IUPHAR	International Union of Pharmacology
KO	Knock-Out
LCAP	Long-Chain Aryl-Piperazine
LSD	Lysergic acid diethylamide
LTM	Long-Term Memory
MAO-A	Monoamine Oxidase-A
MECP2	Methyl CpG-binding protein 2
mGluR-LTD	Long-Term Depression mediated by metabotropic glutamate receptors
NMR	Nuclear Magnetic Resonance
NORT	Novel Object Recognition Task
PCP	Psychotogen phencyclidine
PDB	Protein Data Bank
PET	Positron Emission Tomography
PLC	Phospholipase C
PNS	Peripheral Nervous System
RTT	Rett syndrome
SAR	Structure–Activity Relationship
SERT	Serotonin reuptake transporter
SSRI	Selective Serotonin Reuptake Inhibitors
STM	Short-Term Memory
TMHs	Transmembrane helices

Contents

1. Introduction

1.1. Serotonin and its transmitter system

Serotonin (5-HT), is a monoamine neurotransmitter, characterized by a basic amino group connected to an indolic nucleus by a two carbon aliphatic linker.

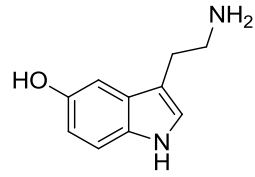


Figure 1. Structure of 5-HT

It was chemically identified in 1994 by organic chemist Maurice Rapport, who described the purification of serotonin from approximately "900 liters of serum collected from almost two tons of beef blood" over the course of his structure elucidation work.[1, 2] After that, 5-hydroxytryptamine (5-HT) became the preferred name within the pharmacological field (Fig. 1). 5-HT is one of the most ancient signaling molecules. It plays a variety of roles in physiology functions, including cardiovascular, gastrointestinal, and endocrine function, sensory perception, behaviors such as aggression, appetite, sex, sleep, mood, cognition, and memory.[3] 5-HT has two modes of actions, as a neurotransmitter within the central and peripheral nervous system (CNS and PNS), and as hormone in the gastrointestinal tract, cardiovascular system, and immune cells.

The majority of 5-HT in the body is found outside of the CNS and only 1% of the body's total 5-HT is detected in the brain;[4] despite this however, the 5-HT is involved in several neurotransmission pathway.[5] Therefore, it's not surprising that dysfunction in the 5-HT system has also been implicated in a variety of CNS disorders such as anxiety, depression, migraine, obsessive compulsive disorders, and schizophrenia.[6]

The neurons containing serotonin are concentrated in the raphe nuclei and their fibers project to the cerebral cortex, hippocampus, limbic system, and hypothalamus as well as down the spinal cord.[7, 8] The principal centers for serotonergic neurons are the rostral and caudal raphe nuclei. From the rostral raphe nuclei axons ascend to the cerebral cortex, limbic regions, and prominantly to the basal ganglia. Serotonergic nuclei in the brain stem give rise to descending axons, some of which terminate in the medulla; others descend along the spinal cord (Fig. 2).[9]

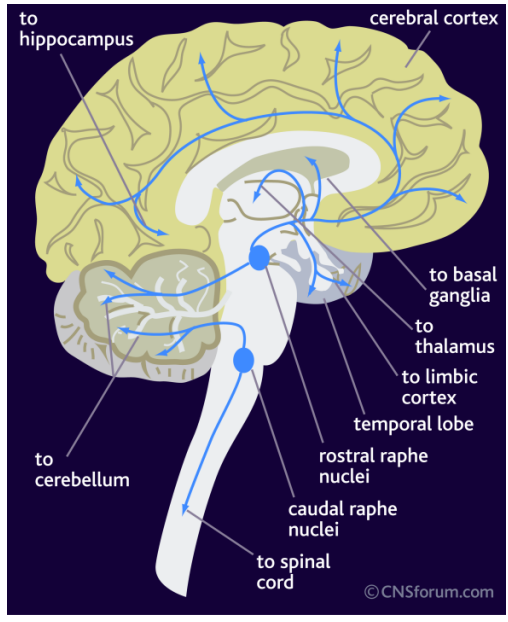


Figure 2. Serotonergic pathways in the human brain. http://www.cnsforum.com/educationalresources/imagebank/

5-HT is produced in a two-step process from the essential amino acid L-tryptophan (Fig. 3). First is the rate-limiting step where L-tryptophan hydroxylase acts on the benzoindole moiety and produces 5-hydroxytryptophan (5-HTP). In the second step, an aromatic amino acid decarboxylates the side chain to 5-HT. The main metabolic route of 5-HT is deamination by the monoamine oxidase-A (MAO-A) enzyme.

Following its biosynthesis, 5-HT is packaged into vesicles. When an axon potential reaches the terminal region, membrane depolarization leads to influx of calcium, which leads to fusion of the vesicle with the presynaptic membrane. This results in the release of 5-HT into the synaptic space, where it diffuses across to activate the postsynaptic receptors, thereby initiating the signaling cascades within the cell (Fig. 4).

5-HT is extracted from the synaptic cleft by specialized proteins in the presynaptic membrane, in this case the serotonin reuptake protein (SERT). The SERT pumps the free serotonin back into the neuron terminal, where it is repackaged into vesicles to repeat the cycle. Any 5-HT found in the cytoplasm and not stored in vesicles undergoes metabolism by MAO, enzymes bound to the outer membrane of mitochondria, to produce the biologically inert metabolite 5-hydroxyindole-3-acetic acid (5-HIAA).[10]

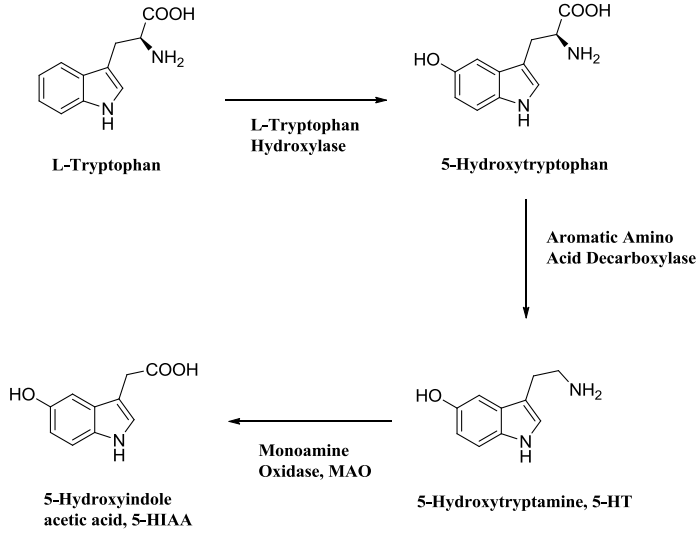


Figure 3. Biosynthesis and metabolism of serotonin.

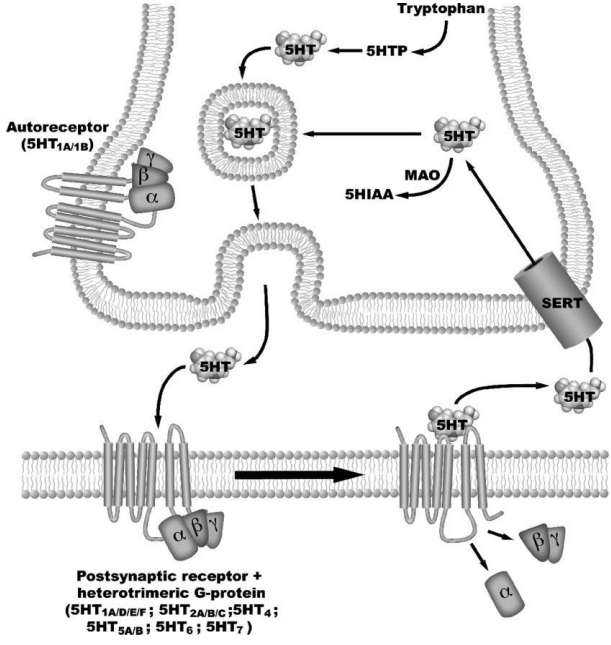


Figure 4. Model of a serotonergic synapse. Figure from Ref. 10.

1.1.1. The superfamily of G protein-coupled receptors

One of the largest and most studied gene families of mammalian genomes are the G protein-coupled receptors (GPCRs). All known GPCRs consist of an extracellular amino-terminus, seven membrane-spanning α-helices (for which reason they are often referred to as seven trans-membrane receptors), and an intracellular carboxyl-terminus.

The main role of GPCRs is to recognize a diversity of extracellular ligand such as hormones, proteins, lipids, and pheromones and to transduce their signals into the cell.[11] Hence, the mechanisms of sensing ligands and the transducing signals are highly variable. G-proteins transmit the signal to effector proteins, such as enzymes and ion channels, resulting in rapid changes in the concentration of intracellular signaling molecules such as cAMP, cGMP, inositol phosphates, diacylglycerol, arachidonic acid, and cytosolic ions.[12, 13]

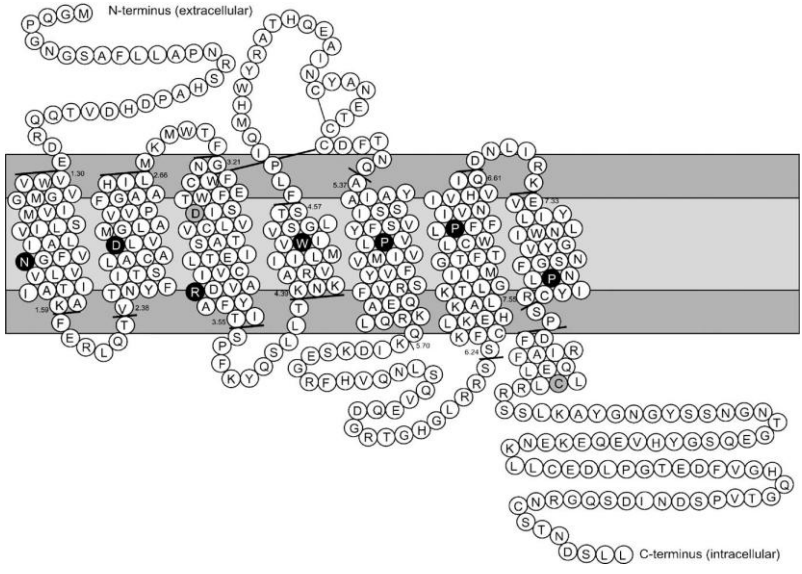


Figure 5. Schematic representation of the membrane topology of the human β_2 adrenergic receptor. The localizations of TMHs in the human β_2-adrenoceptor are indicated (black lines). The core and water-lipid interface regions of the lipid membrane are indicated with light gray and dark gray colors on the background. Figure from Ref. 15.

In mammalian species, GPCRs were sub-classified into seven families: A, B, large N-terminal family B-7 transmembrane helix, C, Frizzled/Smoothened, taste 2, and vomeronasal 1 receptors. All members

share a common membrane topology. Figure 5 shows a consensus membrane topology of family A GPCRs with an extracellular N-terminus, a cytoplasmic C-terminus, and seven transmembrane helices (TMHs) connected by loops.[14]

Each of the seven TMHs have one characteristic residue (Fig. 5, black circles with white text), which is found among the majority of family A receptors: (Asn51(1.50), Asp79(2.50), Arg131(3.50), Trp211(4.50), Pro288(5.50), Pro323(6.50), and Pro323(7.50). Disulfide bridges form between Cys106/Cys191 and Cys184/Cys190, a palmitoylation site (Cys341, gray color) in the C-terminus. Asp(3.32) residue (gray color) is the counterion for the binding of protonated amine agonists and antagonists to biogenic amine receptors.[15]

1.1.2. 5-HT receptors

After the first 5-HT receptor was cloned, it became clear that the mammalian family of serotonin receptors was large, and indeed it has proven to be much larger than that of any of the other GPCR-type neurotransmitter receptors, including those for dopamine, norepinephrine, glutamate or acetylcholine. Figure 6 shows the phylogenetic relationship of each receptor to the others.[10]

Serotoninergic receptors, according to the International Union of Pharmacology (IUPHAR), have been classified on the basis of structural, functional, and pharmacological criteria, into seven distinct classes (Fig. 7): 5-HT_1 (including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1e}, and 5-HT_{1F} subtypes), 5-HT_2 (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}), 5-HT_3, 5-HT_4, 5-ht_{5a}, 5-ht_{5b}, 5-HT_6, and 5-HT_7.[16] This classification does not include the multiple receptors generated by alternative splicing of single genes or editing of the receptor RNA.[17]

The 5-HT_3R differs from all known subtypes of serotonin because it is the only ionotropic receptor or ligand-gated ion channel. Functional channels may be comprised of five identical 5-HT_{3A}R subunits (homopentameric) or a mixture of 5-HT_{3A}R and one of the other four subunits, 5-HT_{3B}R, 5-HT_{3C}R, 5-HT_{3D}R, or 5-HT_{3E}R (heteropentameric). The 5-HT_3R is characterized by the presence of four transmembrane segments and a large extracellular N-terminal region. The functional receptor consists of five subunits that are arranged around a central ion conducting pore that is permeable to sodium, potassium, and calcium ions. Therefore, binding of the neurotransmitter 5-HT to these 5-HT_3R opens the channel, which in turn leads to an excitatory response in neurons.[18]

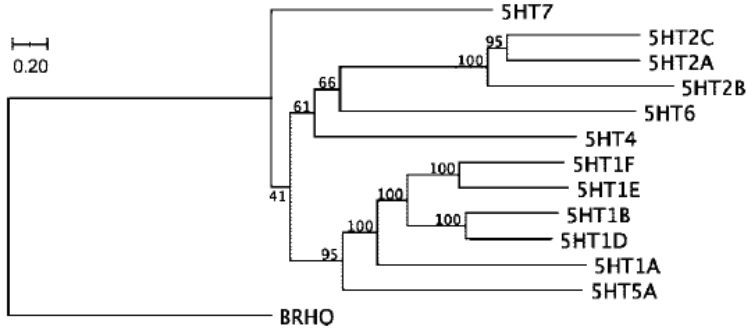


Figure 6. Scaled phylogenetic tree comparing all human serotonin receptors with bovine rhodopsin (BRHO). Results of bootstrap analysis with 100 replications are given above the branches. The scale bar corresponds to 0.2 substitutions per position for a unit branch length. The tree was constructed using the most current NIH Entrez sequence for each receptor with CLC Free Workbench software (CLC bio, Cambridge, MA). Figure from Ref. 10.

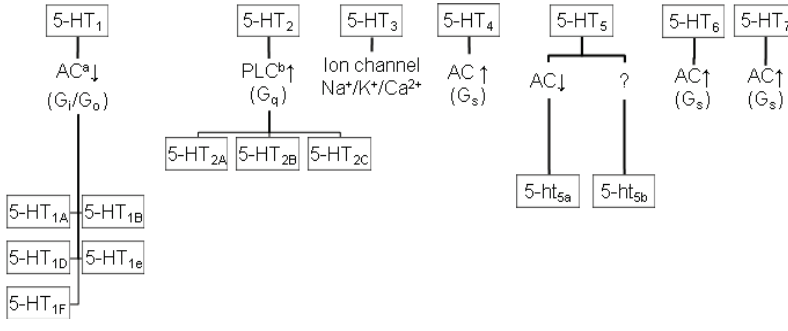


Figure 7. The seven classes of 5-HTRs, with their G-protein and signal pathways. [a]AC = adenylyl cyclase. [b]PLC = phospholipase C. Figure adapted from: http://uu.diva-portal.org/smash/get/diva2:165900/FULLTEXT01.pdf

1.2. 5-HT_{1A} receptors

1.2.1. Expression and distribution

The 5-HT_{1A} receptor (5-HT_{1A}R) was the first of the 5-HTRs to be cloned. The human receptor is located on chromosome 5q11.2-q13 and it is largely distributed throughout the CNS with some detectable presence in the PNS. In 1987, 5-HT_{1A}R was described as a genomic clone and identified as G-21.[19] One year later it was reported that the protein product of G-21, when transiently expressed in monkey kidney cells, had ligand-binding characteristics of the 5-HT_{1A}R.[20]

The distribution of 5-HT_{1A}R in the brain was mapped extensively by receptor autoradiography using a range of ligands including [^{3}H]-5-HT under appropriate conditions as well as subtype selective ligands like [^{3}H]-8-OH-DPAT, [^{3}H]-ipsapirone, [^{125}I]-BH-8-MeO-N-PAT, [^{125}I]-*p*-MPPI, and [^{3}H]-WAY 100635. Positron emission tomography (PET) studies have used [^{11}C]-WAY 100635 to image 5-HT_{1A}Rs in the living human brain.[21] The 5-HT_{1A} has higher binding density within the limbic brain areas, notably with the hippocampus, lateral septum, cortical areas (particularly cingulate and entorhinal cortex), and also the mesencephalic raphe nuclei.

Levels of 5-HT_{1A} binding sites in the basal ganglia and cerebellum are extremely low. The 5-HT_{1A}Rs are located both postsynaptic to 5-HT neurons (as in forebrain regions), and also on the 5-HT neurons themselves at the level of the soma and dendrites in the mesencephalic and medullary raphe nuclei (Fig. 8).

At the cellular level, in situ hybridization and immunocytochemical studies demonstrate the presence of 5-HT_{1A}Rs in cortical pyramidal neurons as well as in pyramidal and granular neurons of the hippocampus.[22]

5-HT_{1A}R is located both pre- and post-synaptically within the brain, and at either location, their activation leads to neuronal hyperpolarization and reduced firing rate. The presynaptic 5-HT_{1A}R expressed on raphe cells couple to $G\alpha_{i/o}$ proteins that activate inwardly rectifying potassium channels (GIRKs), causing neuronal membrane hyperpolarization,[23] which leads to a decreased rate of cell firing. Postsynaptic 5-HT_{1A}Rs are expressed at high density in limbic areas of the brain such as hippocampus and septum, and in the entorhinal cortex.[24] In the hippocampus, they are highly expressed in the CA1 and CA2 fields and dentate gyrus.[25] They also are expressed at high density in layers II and VI in the frontal cortex, with lesser expression in other layers. In the cortex, they are found on the axon hillock of pyramidal cells, where their activation hyperpolarizes the cell membrane.[10]

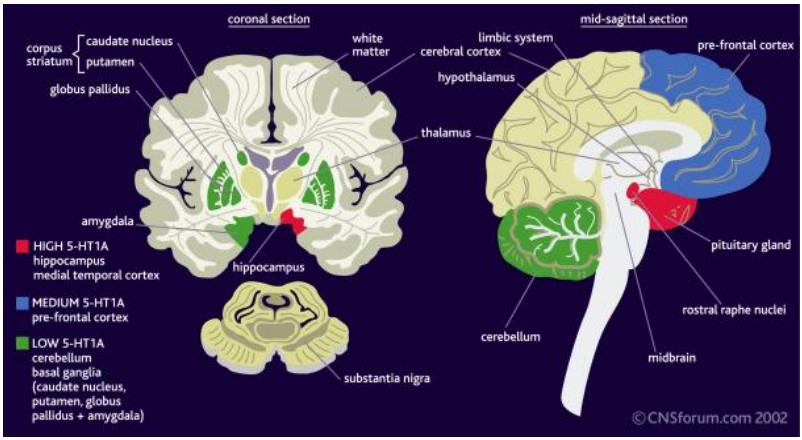


Figure 8. Distribution of 5-HT_{1A}R. http://www.cnsforum.com/educationalresources/imagebank/

1.2.2. 5-HT_{1A} ligand and pharmacology

The 5-HT_{1A}R subtype is one of the most studied and it is generally believed to be involved in anxiety and depression.[26, 27] With reference to the functional activeties of the ligands it has been suggested that 5-HT_{1A}R agonists have neuroprotective properties. Alternatively, 5-HT_{1A}R antagonists could be useful in the treatment of Alzheimer disease.[28-30] There are several examples of potent 5-HT_{1A}R ligands belonging to different chemical classes and the most important are: aminotetralines, indolylalkylamines, ergolines, arylpiperazines, aporphines, and aryloxyalkylamines.[31] Among them, the long-chain arylpiperazines (LCAPs) represent one of the most important class of 5-HT_{1A}R ligands.[32]

Unfortunately, arylpiperazine moiety lacks selectivity and is a good template for many different biological targets, especially in the CNS. For this reason, several compounds containing arylpiperazine portion have a high binding at 5-HT_{1A}R, but few of them show high selectivity for 5-HT_{1A}R over other receptors. Buspirone (Fig. 9) is one of the most known member of this class of ligands; it behaves as 5-HT_{1A} partial agonist, and it acts as an anxiolytic drug.[33] Furthermore, it shows high affinity for 5-HT_{1A}R, but poor selectivity over α_1-adrenergic receptor (α_1-AR). A highly selective compound for 5-HT_{1A}R is NAN-190, which stands out for its subnanomolar affinity (Fig. 9) and for the postsynaptic antagonist activity.[34]

Several agonists show selectivity for the 5-HT_{1A}R. An example agonist is 8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT, Fig. 9), which acts as a full agonist in experimental systems.[35]

Selective and high-affinity ligands for this receptor are WAY 100635 and NAD-299 (Fig. 9). The latter had affinity less than 1 μM for α_1 and β adrenoceptors with K_i values of 260 and 340 nM, respectively. Thus, the selectivity of NAD-299 for 5-HT_{1A} receptors was more than 400 fold. WAY 100635 had considerably higher affinity than NAD-299 for α_1 adrenoceptors (K_i = 45 nM) and dopamine D_2 and D_3 receptors (K_i = 79 and 67 nM, respectively). Like WAY 100635, NAD-299 competitively blocked 5-HT-induced inhibition of vasoactive intestinal peptide-stimulated cAMP production in GH_4ZD10 cells and without intrinsic activity. Both compounds were therefore 5-HT_{1A}R antagonists *in vitro* and also behaved as such in *in vivo* assays. Thus, both ligands competitively antagonized the 8-OH-DPAT induced 5-HT behavioral effects, hypothermia, corticosterone secretion and inhibition of passive avoidance behavior without causing any actions of their own.[36]

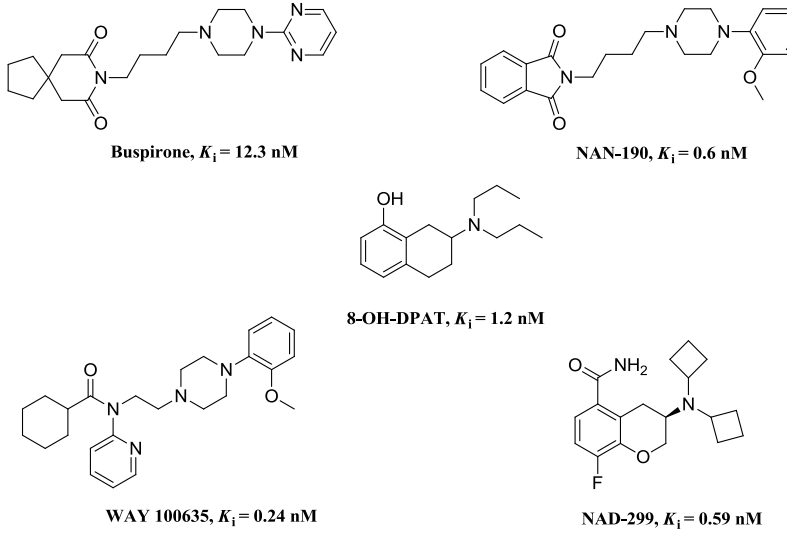


Figure 9. Chemical structures and binding constants (K_i) of some 5-HT_{1A}R ligands.

1.3. 5-HT$_7$ receptors

1.3.1. Expression and distribution

The 5-HT$_7$R possesses high sequence homology (90%) across different species (e.g. human, mouse, rat, guinea pig, and pig); whereas it possesses low (< 40%) overall homology with other 5-HT receptors.[37] The human receptor is located on chromosome 10q23.3-q24.4. The presence of introns in the 5-HT$_7$R gene is significant in that a number of functional splice variants of this receptor have been identified (Fig. 10).

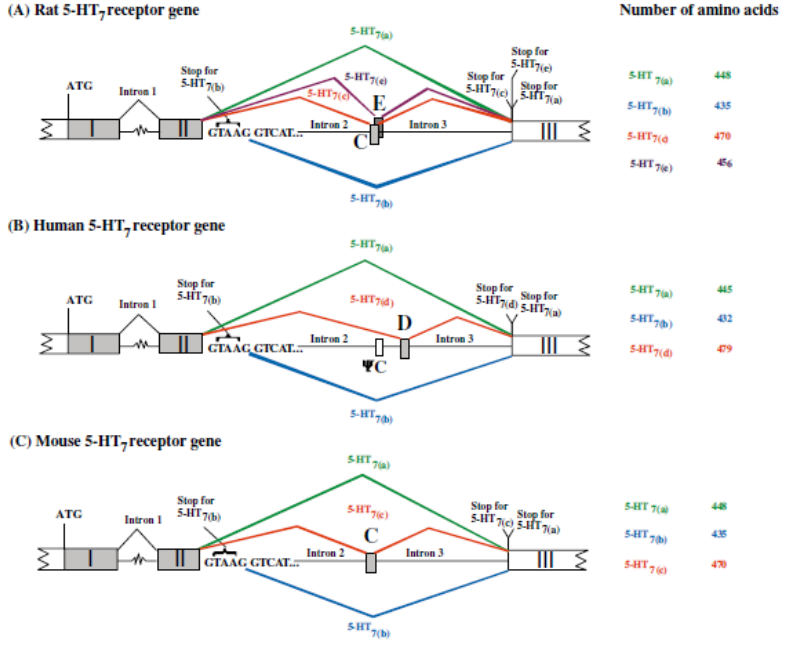


Figure 10. Schematic overview of the splicing process leading to different rat (**a**) human (**b**) and mouse (**c**) 5-HT$_7$R mRNA. Exons I, II, III, C, ψC, D, and E are indicated by boxes. Those that code for 5-HT$_7$R splice variants are shown in grey. Exon I consists of 549 bp, exon II of 755 bp, exon C of 97 bp, and exon III of 43 bp. The introns contain 86,902 bp (intron 1), 4,832 bp (intron 2), and 3,907 bp (intron 3). The rat and mouse 5-HT$_{7(a)}$R, 5-HT$_{7(b)}$R, 5-HT$_{7(c)}$R, and rat 5-HT$_{7(e)}$R isoforms are 448, 435, 470, and 456 amino acids in length, respectively, whereas the human 5-HT$_{7(a)}$R, 5-HT$_{7(b)}$R, and 5-HT$_{7(d)}$R isoforms are 445, 432, and 479 amino acids in length, respectively. Figure from Ref. 18.

Three different splice variants, namely 5-$HT_{7(a)}$, $_{(b)}$, $_{(d)}$, were found in human (Table 1).[38] The isoforms differ only in the length and amino acid composition of their carboxy-terminal tail; these isoforms do not show significant differences in their pharmacological profile, signal transduction, or tissue distribution.[39] Among these isoforms, the 5-$HT_{7(a)}$ is the most abundant in humans and it's positively coupled with adenylyl cyclase (AC) through the activation of Gs proteins.[39] In addition, it is coupled to the G_{12} protein to activate small GTPases of the Rho family (*i.e.*, Cdc42 and RhoA), leading to enhanced neurite outgrowth, synaptogenesis, and neuronal excitability.[40-42] Also, it's been demonstrated in cell lines that the 5-HT_7R can stimulate intracellular calcium release.[43]

5-HT_7R is defined pharmacologically by: *i*) its high affinity for 5-HT, 5-carboxytryptamine (5-CT), 5-methoxytryptamine (5-MeOT), and methiothepin; *ii*) its moderate affinity for mesulergine, 2-bromolysergic acid diethylamide (2-Br-LSD), methysergide, and spiperone; *iii*) its low affinity for tryptamine, 8-OH-DPAT, sumatriptan, and ketanserin (Table 2).

The 5-HT_7Rs are expressed in CNS and in peripheral tissues. With regards to CNS distribution, they are found in the thalamus, hypothalamus (including the suprachiasmatic nucleus), hippocampus, cerebral cortex, amygdala, the dorsal raphe, and in the Purkinje neurons of the cerebellum. On the other hand, in the peripheral tissues the 5-HT_7Rs are located in the smooth muscle cells of blood vessels and also in the gastrointestinal tract where they are involved in peristalsis. Different splice variants were present in most of the tissues examined; however, the relative expression level of each differed considerably (Table 1).[18]

Table 1. 5-HT_7R splice variants

Receptor	Receptor length (amino acids)	Distribution
5-HT_{7a}	445 (Human) 448 (Rat) 448 (Mouse) 446 (Guinea pig)	Thalamus, hypothalamus, hippocampus, brain stem, cortex, striatum, olfactory bulb, olfactory tubercle, spleen, kidney, heart, coronary artery.
5-HT_{7b}	432 (Human) 435 (Rat)	Caudate nucleus, hippocampus, spleen.
5-HT_{7c}	470 (Rat)	Cerebellum, hindbrain, spleen.
5-HT_{7d}	479 (Human)	Caudate nucleus, spleen.

Table 2. pK_i values of selected ligands at recombinat human 5-HT$_7$R expressed in Cos-7 cells using [^{3}H]5-HT as radioligand.

Ligand	5-HT$_{7h}$[a]	Affinity
5-CT	9.0	
Methiothepin	8.4	High
5-MeOT	8.3	
5-HT	8.1	
Mesulergine	7.7	
2-Br-LSD	7.5	Moderate
Methysergide	7.1	
Spiperone	7.0	
Tryptamine	6.8	
8-OH-DPAT	6.3	Low
Sumatriptan	6.0	
Ketanserin	5.9	

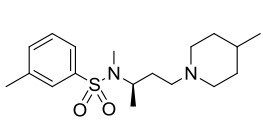
[a]Data from Ref. 39.

1.3.2. 5-HT$_7$R ligands

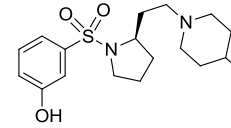
Nonselective 5-HT$_7$R ligands belong to different classes: ergolines, aporphine derivatives, tricyclic neuroleptics, piperidine analogues. However, due to the lack of selectivity, none was ever used as a lead compound, and the development of potent and selective 5-HT$_7$R ligands is still a key topic.

During a high-throughput screening (HTS) in 1998, GlaxoSmithKline identified the first selective 5-HT$_7$R antagonist called SB-258719 (Fig. 11, K_i = 32 nM), followed by SB-269970 (Fig. 11, K_i = 1 nM) that is still used as labelled standard selective 5-HT$_7$R antagonist, being > 100-fold selective over a broad range of CNS targets including other serotonergic, adrenergic, and dopaminergic receptors.[44]

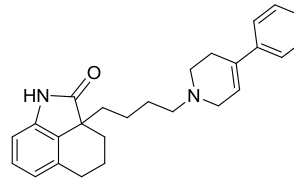
Another interesting class of selective 5-HT$_7$R antagonists are the tetrahydrobenzindole derivatives, and among them DR-4004 was patented by Meiji Seika Kaisha Co., Ltd. (Fig. 11, 5-HT$_7$ pK_i = 8.48; 5-HT$_2$ pK_i = 7.37).[44]



SB-258719



SB-269970



DR-4004

Figure 11. Chemical structures of 5-HT_7R antagonists.

Pfizer described one of the first 5-HT_7R agonist (Fig. 12, pK_i = 7.79), with a (4,5-dihydroimidazol-2-yl)biphenylamine structure. However, this derivative had dual affinity for α_1 and α_2 adrenoceptors (pK_i = 6.68 and 7.71, respectively).

Perrone and co-workers performed SAR studies on a novel class of 5-HT_7R agonists based on a 1-[6-(4-aryl-1-piperazinyl)alkyl]-1-arylketone moiety (Fig. 12). Some of which showed high affinity for 5-HT_7R and good selectivity over 5-HT_{1A}R, i.e. K_i = 2.93 (R = OH) and 0.90 nM (R = OCH_3). However, selectivity over 5-HT_{2A}, α_1, and D_4 receptors remained somewhat unsatisfactory.[44]

Leopoldo and co-worker, from the same group at the University of Bari, also reported a series of *N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-4-aryl-1-piperazinealkylamides as 5-HT_7R agents in 2004. Subsequent optimization studies in 2007 led to the synthesis of the agonist LP-44 (Fig. 12) endowed with high 5-HT_7R affinity (K_i = 0.22 nM), moderate 5-HT_{1A}R affinity (K_i = 52.7 nM), and very low affinity for 5-HT_{2A}R.[44]

Finally, worthy of mention is the 2-dimethylaminotetralin derivative AS-19 (Fig. 12) endowed with high 5-HT_7R affinity (K_i = 0.6) and > 80-fold selective over 5-HT_{1A}R (K_i = 89.7).[44]

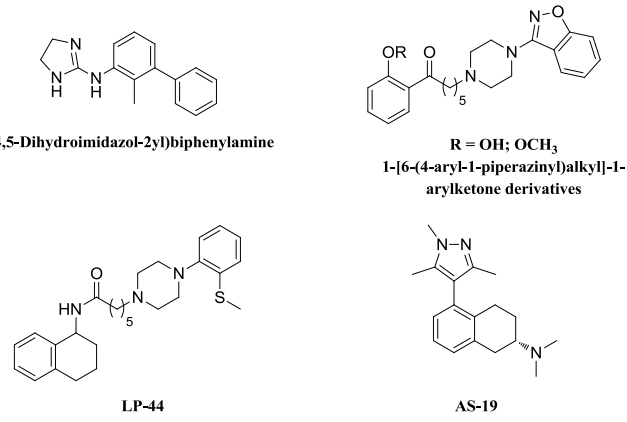


Figure 12. Chemical structures of 5-HT$_7$R agonists.

1.3.3. 5-HT$_7$R pharmacology

Synthesis and evaluation of selective ligands helped to define the pharmacological roles of the 5-HT$_7$R. Since its cloning, it was found to be linked to the regulation of circadian rhythm and thermoregulation.[45, 46] More recently, it was proposed that activation of 5-HT$_7$R expressed by γ-aminobutyric acid (GABA)-ergic interneurons decreases the activity of REM sleep-promoting cholinergic neurons in the laterodorsal and pedunculopontine tegmental (LDT/PPT) nuclei and reduces REM sleep.[47, 48] The use of 5-HT$_7$R knock-out (KO) mice suggests that this receptor plays a role in learning and memory, and this correlates to the 5-HT$_7$R involvement in hippocampal-dependent cognitive processes.[49] In general, 5-HT$_7$R agonists (AS-19 or LP-211) gave pro-cognitive actions, in particular AS-19 impaired short-term memory (STM), but improved long-term memory (LTM) (in an autoshaping Pavlovian/instrumental learning task). One the other hand, LP-211 did not affect STM, but it improved LTM.[50-52] Other studies suggested that selective 5-HT$_7$R ligands may have potential therapeutic applications for pain, although the role of 5-HT$_7$R seems to be quite complex.[53] Indeed, under sensitizing neuropathic conditions activation of 5-HT$_7$R exerts anti-nociceptive effects at the level of the spinal cord but pro-nociceptive effects in the periphery.[54] However, after systemic administration of 5-HT$_7$R agonists, the antinociceptive effect mediated by central 5-HT$_7$R predominates. In addition, further study demonstrated a novel implication for spinal 5-HT$_7$Rs in acute antinociception by systemic amitriptyline; spinal delivery of the selective 5-HT$_7$R antagonist (SB-269970), at a dose that was inactive alone, prevented the antinociceptive effects of systemic amitriptyline.[55]

The first evidence of 5-HT$_7$R possibly playing a role in schizophrenia

was suggested early on, because several antipsychotics showed high affinity for the 5-HT_7R, thus opening up the possibility that some atypical antipsychotics, such as clozapine and risperidone, may be mediated in their effect by this receptor.[56] Later, the prescription drug Amisulpride was identified to have dual antipsychotic and antidepressants properties and it showed high affinity for the 5-HT_7R.[57] Experimental data suggests that antagonism of 5-HT_7R show pro-cognitive effects. Co-treatment with the 5-HT_7R agonist AS-19 reversed the abilities of amisulpride and lurasidone to ameliorate the PCP-induced deficits in the NORT in rats,[58] as well as blocked the attenuating effects of lurasidone on the MK-801-induced deficits in the rat passive avoidance test.[59] In addition, it cannot be excluded that the 5-HT_7R may be involved in pro-cognitive effects of other antipsychotic drugs such as clozapine with high 5-HT_7R affinity too.[56] Further published preclinical data revealed that acute administration of SB-269970 (1 mg/kg) or amisulpride (3 mg/kg) ameliorated ketamine-induced cognitive inflexibility and novel object recognition deficit in rats. Both compounds were also effective in attenuating ketamine-evoked disruption of social interactions, thus they were confirmed as potential pharmacological target for treatment of schizophrenia.[60]

The 5-HT is able to induce cranial vasodilation under certain conditions so it was proposed to be one of the agent involved in migraine.[61] It is well known that the effect is most likely not mediated by a single receptor subtype, despite available evidence that clearly suggests 5-HT_7R plays a role. The believe that 5-HT_7R plays a role in migraine stems from the observation that several migraine prophylactic drugs showed moderate to high affinity for the 5-HT_7R. It was suggested that the 5-HT_7R mediates 5-HT induced dilation of the carotid artery following blockade of 5-$HT_{1B/1D}$ receptors in combination with low sympathetic tone; however, it is unclear how relevant such a mechanism might be for migraines. Selective antagonist ligands such as SB-269970 did block this vasodilation in vivo.[62] Despite the numerous studies that have been done, further efforts are still needed to determine if the 5-HT_7R can be targeted for the prophylaxis or treatment of migraine.

The involvement of the 5-HT_7Rs in the regulation of anxiety-like behaviours is less consistent than its well-established role in depression. Behavioral characterizations of mice lacking the 5-HT_7R −/− did not detect any differences compared to 5-HT_7R +/+ mice in two anxiety models; the mice were evaluated in a light-dark transfer test and both genotypes had an equal number of transitions between the light and dark compartments.[62, 63] However, selective 5-HT_7R antagonist SB-269970 exerted specific anti-anxiety-like effects in the Vogel conflict test and the elevated plus maze test in rats, as well as in the four-plate test in mice.[62, 64] In addition, the agonist LP-211 has been reported to reduce anxiety-like behaviour in the black and white box test and the dark/light test in mice.[65]

A role of 5-HT_7R was determined in fragile X syndrome (FXS), the

most common form of inherited mental retardation and the most common known cause of autism. In an FXS animal model, mice exhibited synapse malfunction in the hippocampus with abnormal enhancement of long-term depression mediated by metabotropic glutamate receptors (mGluR-LTD). The selective activation of 5-HT_7R reverses metabotropic glutamate receptor-induced AMPA receptor internalization and LTD, correcting excessive mGluR-LTD. On this basis, it was proposed that selective agonists of 5-HT_7R may be potential pharmacological tools for FXS therapy, as an alternative or concomitant therapy for chronic treatment using metabotropic glutamate receptor 5 antagonists.[66]

Recent findings indicate that pharmacological targeting of 5-HT_7R improves specific behavioral and molecular manifestations of Rett syndrome (RTT). RTT is a rare neurodevelopmental disorder characterized by severe behavioral and physiological symptoms. Mutations in the methyl CpG-binding protein 2 (MECP2) gene cause > 95% of classic cases of RTT and currently there is no cure for this devastating disorder. LP-211 did demonstrate an ability to improve Rett Syndrome-related defective performance including the anxiety-related profile, motor abilities and memory, in a mouse genetic model of the disease. Thus, representing a first step toward the validation of an innovative systemic treatment.[67]

The pharmacology and signal transductions of 5-HT_7Rs may be even more complicated than precieved. It has been recently shown that 5-HT_{1A}R and 5-HT_7R form homo- and heterodimers both *in vitro* and *in vivo* by a combination of computational protein–protein docking, site-directed mutagenesis, and Förster resonance energy transfer (FRET) based analysis.[68-71] From the functional point of view, heterodimerization has been shown to play an important role in the regulation of receptor-mediated signaling and internalization suggesting the possible role of heterodimerization between 5-HT_{1A}R and 5-HT_7R in anxiety and depression. Further study reveal that under physiological conditions, the amount of 5-HT_{1A}-5-HT_7 heterodimers in presynaptic 5-HT neurons is higher than in postsynaptic neurons thereby representing a mechanism responsible for the differential 5-HT or selective serotonin reuptake inhibitors (SSRI)-mediated internalization obtained for the 5-HT_{1A} auto- versus heteroreceptors. In depression disorders, the relationship between 5-HT_{1A}-5-HT_{1A} homodimers and 5-HT_{1A}-5-HT_7 heterodimers in presynaptic 5-HT neurons becomes shifted toward 5-HT_{1A}-5-HT_{1A} homodimers. This will result in decreased 5-HT or SSRI-mediated internalization of 5-HT_{1A} autoreceptors, which in turn will lead to 5-HT_{1A} receptor-mediated inhibition of 5-HT release. On the postsynaptic neurons, higher amount of heterodimers ([5-HT_{1A}-5-HT_7] > [5-HT_{1A}-5-HT_{1A}]) is expected during depression. Consequently, internalization of postsynaptic 5-HT_{1A}Rs will increase, leading to increase neuronal excitability.[72]

1.4. Long-chain arylpiperazine derivatives

LCAPs are a class of compounds extensively studied of 5-HT_{1A}R ligands.[31] LCAPs possess three main structural parts: the aryl group at N_1 of the piperazine ring, the aliphatic chain at N_4 position, and a terminal fragment (the most often having amide or imide moiety), (Fig. 13).

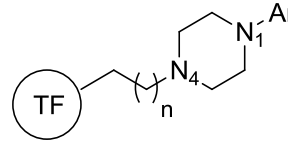


Figure 13. General structure of LCAPs. TF = terminal fragment.

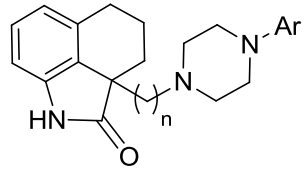
Due to the structural similarity between the 5-HT_7R and 5-HT_{1A}R, several research groups have modified the LCAPs template in order to identify selective ligands for one of these serotonin receptors.[73, 74]

Arylpiperazine derivatives **1**–**3** (Table 3) were identified by Meiji Seika Kaisha Co., Ltd. after a screening of a compound library against human cloned 5-HT_7R. Subsequently, between 1999 and 2002, Kikuchi *et al*. modified the framework of these compounds and obtained the following SARs: a butyl linker as in compound **3** was preferred; replacement of the phenyl with cyclohexyl removed all affinity (**3** *vs* **4**); introduction of methoxy group in different positions of the phenyl ring (**5**–**7**) gave affinity values ranked meta > ortho > para. Based on these results, a variety of 2-substituted derivatives with a butyl spacer were evaluated (compounds **8**–**14**). The pK_i values ranging from 7.13 to 8.82 were influenced by the nature of the substituent. The cyano (**9**) and acetyl (**11**) derivatives showed the highest affinity for the 5-HT_7R (pK_i 8.42 and 8.10, respectively).[75, 76, 77]

In 2003, Perrone and his co-workers focused their attention on 1-[ω-(4-aryl-1-piperazinyl)alkyl]-1-arylketone derivatives with a hydroxyl functional group on the aryl ketone moiety and a pentyl chain. The research led to 2-methoxyphenyl derivative **16** that demonstrated high 5-HT_7R affinity (K_i = 5.8 nM). Subsequently, the modification of **16** on the aromatic ring linked to the piperazine gave interesting results (Table 4). Generally, affinity data revealed that: *i*) the absence of an aryl group linked to the piperazine (derivatives **17** and **18**) led to a loss of affinity (K_i > 1000 nM), whereas aryl groups other than 2-methoxyphenyl (*i.e.* 2-Py, Ph, 3-CF_3-Ph, 2-benzoxazolyl, 2-benzimidazolyl) significantly reduced affinity (compounds **19**–**23**); *ii*) the 1,2-benzisoxazol-3-yl derivative **10** displayed selectivity marked by high 5-HT_7 affinity (K_i = 2.93 nM) and low 5-HT_{1A} affinity (K_i = 189 nM); *iii*) the

introduction of a methoxy group on the 1,2-benzisoxazolyl moiety (**25** *vs* **24**) gave a significant loss in 5-HT_7 affinity (K_i = 462 nM).

Table 3. Binding affinities of tetrahydrobenzindole derivatives (**1**-**15**) for 5-HT_7 and 5-HT_{2A} receptors. Table adapted from Ref. 75.



Comp.[a]	Ar	n	pK_i	
			5-HT_7[b]	5-HT_{2A}[c]
1	Ph	2	6.99	8.27
2	Ph	3	8.29	7.79
3	Ph	4	8.48	7.37
4	Cyclohexyl	4	<6	<6
5	2-MeO-Ph	4	8.29	6.95
6	3-MeO-Ph	4	8.63	7.19
7	4-MeO-Ph	4	7.76	6.69
8	2-Cl-Ph	4	7.91	7.01
9	2-CN-Ph	4	8.42	6.98
10	2-$CONH_2$-Ph	4	7.76	6.05
11	2-$COCH_3$-Ph	4	8.10	6.45
12	2-CF_3-Ph	4	7.13	5.86
13	2-NO_2-Ph	4	7.62	7.34
14	2-CH_3-Ph	4	7.98	7.35
15	2,6-diCH_3-Ph	4	6.83	5.85

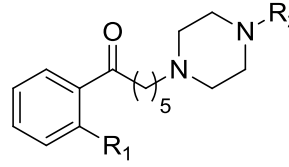
[a]Data from Ref. 76, 77.
[b]Determined at human 5-HT_7Rs in COS-7 cells using [^{3}H]-5-CT.
[c]Determined at 5-HT_{2A} receptors in rat cerebral cortex membranes using [^{3}H]ketanserin.

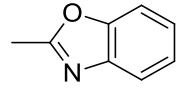
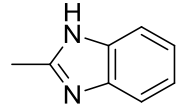
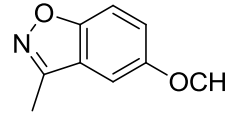
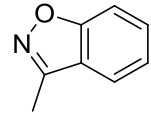
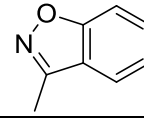
Consequently, compounds **24** and **25** were tested against the 5-HT_{2A}R, and although both **25** and **26** showed good selectivity over the 5-HT_{1A}R, they also showed equivalent selectivity for 5-HT_{2A}R. Moreover, derivatives **24** and **25** displayed agonist properties such as 5-CT when tested for 5-HT_7 receptor-mediated relaxation of substance P-induced guinea-pig ileum contraction.[75, 78]

Between 2004 and 2007, Leopoldo *et al.* also reported a series of *N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-4-aryl-1-piperazinealkylamides as 5-HT_7R agents. Initially, they focused their interest on exploring various alkyl length and functional groups liking the aryl rings to the piperazine moiety. The study of this class of compounds proceeded further by evaluating different substituents with a wide range of electronic, steric,

and polar properties at the 2-position of the aryl ring linked to the piperazine (Table 5).[75, 79, 80]

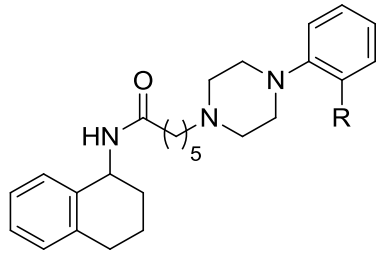
Table 4. Binding affinities of 1-[ω-(4-aryl-1-piperazinyl)alkyl]-1-arylketone derivatives for 5-HT_7 and 5-HT_{1A} receptors. Table adapted from Ref. 75.



Comp.[a]	R_1	R_2	K_i (nM)	
			5-HT_7[b]	5-HT_{1A}[c]
16	2-OH	2-CH_3O-Ph	5.8	5.8
17	2-OH	CH_3	>1000	>850
18	2-OH	Cycloexyl	>800	>850
19	2-OH	2-Py	105	56
20	2-OH	Ph	43	137
21	2-OH	3-CF_3-Ph	384	282
22	2-OH		148	1459
23	2-OH		682	>850
24	2-OH		462	389
25	2-OH		2.93	189
26	2-OCH_3		0.90	175

[a]Data from Ref. 78.
[b]Determined at rat 5-HT_7Rs in HEK-293 cells using [^{3}H]LSD.
[c]Determined at 5-HT_{1A}Rs in rat cerebral hippocampus membranes using [^{3}H]-8-OH-DPAT.

Table 5. K_i values of *N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-4-aryl-1-piperazinealkylamides derivatives for 5-HT_7 and 5-HT_{1A} receptors. Table adapted from Ref. 75.



Comp.[a]	R	K_i (nM)	
		5-HT_7[b]	5-HT_{1A}[c]
27 (LP-44)	SCH_3	0.22	52.7
28	CH_3	15.2	279
29	OH	11.4	24.0
30	H	65.6	128
31	NO_2	63.3	183
32	Cl	40.1	96.0
33	$CONH_2$	229	494
34	SO_2CH_3	298	3124
35	CH_2CH_3	7.10	79.2
36	$(CH_2)_2CH_3$	49.6	168
37	$(CH_2)_3CH_3$	2810	60.0
38	$CH(CH_3)_2$	1.10	167
39	$C(CH_3)_3$	538	1196
40 (LP-12)	Ph	0.13	60.9
41	$N(CH_3)_2$	0.90	112
42	$NHCH_3$	25.4	133
43	NH_2	8178	415
44	$NHCOCH_3$	338	2500
45	$NHSO_2CH_3$	4253	not tested
46	F	131	29.2

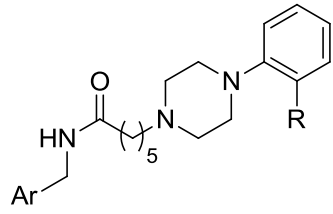
[a]Data from Ref. 80.
[b]Determined at rat 5-HT_7Rs in HEK-293 cells using [^{3}H]LSD.
[c]Determined at 5-HT_{1A}Rs in rat cerebral hippocampus membranes using [^{3}H]-8-OH-DPAT.

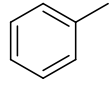
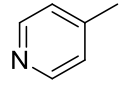
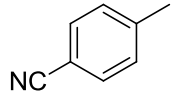
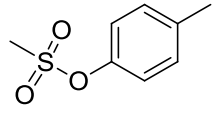
Binding data reported in table 5 suggested that the nature of the substituent markedly influenced the affinity: polar substituents were detrimental for affinity, whereas bulky apolar groups gave high affinity ligands. In addition to this, the intrinsic activities at 5-HT_7R were evaluated. The datum of compound **30** (40% Maximal Activity, EC_{50} = 8 μM) indicates that the *N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-4-phenyl-1-piperazinealkylamide framework was able to activate the 5-HT_7R without the presence of a substituent at the 2-position. In particular, compounds containing bulky lipophilic groups showed partial agonism (i.e. **38**, 83% Maximal Activity, EC_{50} = 0.90 μM and **40**, 74% Maximal Activity, EC_{50} = 1.77 μM). On the other hand, 2-OH and 2-$NHCH_3$ substituents switched intrinsic activity toward antagonism (compound **42**, 0% Maximal Activity, pA_2 = 7.7). [75, 79, 80]

Subsequently, structural simplification of the *N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-4-aryl-1-piperazinealkylamide was performed in order to obtain compounds with better physicochemical properties required for blood-brain barrier penetration. In this new series a benzyl moiety, with less impact on the lipophilicity, was considered (Tables 6). In general, the introduction of the benzyl group had a limited impact on 5-HT_7R affinity. The presence of 4-pyridinylmethyl, 4-aminophenylmethyl, and 4-methanesulfonylphenylmethyl as a terminal fragment was well tolerated as in the case of 2-phenyl derivatives **52**, **56**, and **60**, which showed high 5-HT_7R affinity (5.7 nM < K_i < 0.58 nM). In addition, compound **56** still retained nanomolar affinity at 5-HT_7R, and showed good selectivity over 5-HT_{1A}R (324-fold).

The authors evaluated the intrinsic activity at 5-HT_7R for compound **56** by measuring the 5-HT_7 agonist mediated relaxation of substance P-induced contraction in an isolated guinea-pig ileum assay. They did not observe changes in the intrinsic activity of **56** with respect to **40**, after modification of the terminal fragment (from 1,2,3,4-tetrahydronaphthalene to 1-arylpiperazine). So it was confirmed that the arylpiperazine moiety plays a predominant role on the intrinsic activity in this series of compounds.[75, 81]

Table 6. K_i values of 1-arylpiperazine derivatives for 5-HT_7 and 5-HT_{1A} receptors. Table adapted from Ref. 75.

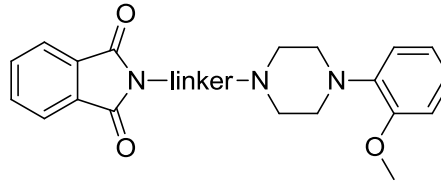


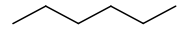
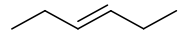
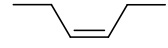
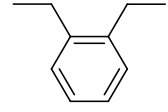
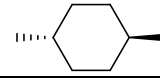
Comp.[a]	Ar	R	K_i (nM)	
			5-HT_7[b]	5-HT_{1A}[c]
47		SCH_3	22	12
48		Ph	n.t.[e]	161
49		$CH(CH_3)_2$	215	139
50		OCH_3	224	13
51		SCH_3	34.8	8.2
52		Ph	0.98	70
53		$CH(CH_3)_2$	5.1	325
54		OCH_3	389	19
55		SCH_3	9.0	94
56 (LP-211)		Ph	0.58	188
57		$CH(CH_3)_2$	8.6	53
58		OCH_3	296	43
59		SCH_3	148	10.3
60		Ph	5.7	106
61		$CH(CH_3)_2$	76	57
62		OCH_3	71	29

[a]Data from Ref. 81.
[b]Determined at rat 5-HT_7Rs in HEK-293 cells using [^{3}H]LSD.
[c]Determined at 5-HT_{1A}Rs in rat cerebral hippocampus membranes using [^{3}H]-8-OH-DPAT.
[e]Not tested.

In 2004, Bojarski *et al*. developed some constrained analogues of the 5-HT_{1A}R antagonist **63** (NAN-190) (Table 7) with the aim to investigate the role of the alkyl chains spacer of various "long-chain" arylpiperazines with respect to the affinity for 5-HT_7R. Comparing the affinity values the cis derivatives **65** and the bismethylbenzene derivative **66** were about 10-fold less active than the corresponding trans analogue **64**, instead the rigid compound **67**, containing a cyclohexane moiety, was devoid of 5-HT_7 activity. Thus, the authors suggested that the bent conformation of flexible "long-chain" arylpiperazines should be regarded as bioactive and that the partly constrained trans derivatives should be able to adopt a bent conformation during the interaction with this receptor.[75, 82]

Table 7. K_i values of 1-(2-methoxyphenyl)piperazine derivatives for 5-HT_7 and 5-HT_{1A} receptors. Table adapted from Ref. 75.



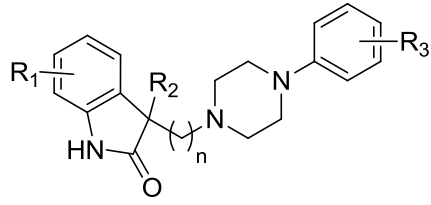
Comp.[a]	Linker	K_i (nM)	
		5-HT_7[b]	5-HT_{1A}[c]
63 (NAN-190)		87	0.6
64		36	5
65		353	77
66		367	405
67		2045	8

[a]Data from Ref. 82.
[b]Determined at 5-HT_7Rs in rat hypothalamus membranes using [^{3}H]-5-CT.
[c]Determined at 5-HT_{1A}Rs in rat cerebral hippocampus membranes using [^{3}H]-8-OH-DPAT.

A series of LCAPs bearing as terminal fragment an oxindole group (Table 8) was developed by researchers at EGIS Pharmaceuticals Plc. The position of the substituent was varied and structure-affinity studies were

done. The methoxy substituted isomers **68**–**70** showed the following behaviors for affinity values: in order meta > ortho > para, whereas for chloro derivatives **71**–**73** the order was para > meta > ortho. Regarding the alkyl chain, the best one was the butyl spacer. Inclusion of either chloro, fluoro, or methyl substituent at the 4-position of the aryl ring linked to the piperazine nucleus of **72** was well tolerated (derivatives **76**–**78**). Removal of the ethyl group on the oxindole ring of **72** was also well tolerated (**79**), whereas its replacement with an isobutyl (**80**) caused a 4-fold loss in affinity. The dihalo substitution on the oxindole ring of **72** led to compounds **81**–**84** with about 10-fold loss in affinity except for the fluoro derivative **82**.[75, 83]

Table 8. Binding affinities of (phenylpiperazinyl-butyl)oxindoles derivatives (**68**-**84**) for 5-HT_7R. Table adapted from Ref. 75.



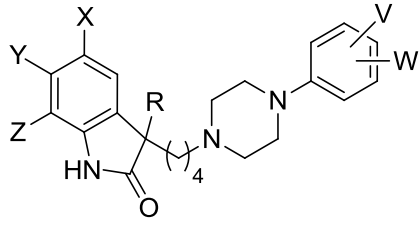
Comp.[a]	R_1	R_2	n	R_3	K_i (nM)[b]
68	H	Et	4	2-OCH_3	5.38
69	H	Et	4	3-OCH_3	2.55
70	H	Et	4	4-OCH_3	20.40
71	H	Et	4	2-Cl	5.11
72	H	Et	4	3-Cl	0.41
73	H	Et	4	4-Cl	0.38
74	H	Et	3	3-Cl	21
75	H	Et	5	3-Cl	1.45
76	H	Et	4	3-Cl, 4-F	0.60
77	H	Et	4	3-Cl, 4-CH_3	0.66
78	H	Et	4	4,4-diCl	0.63
79	H	H	4	3-Cl	0.49
80	H	*i*-But	4	3-Cl	1.80
81	5-Cl	Et	4	3-Cl	8.27
82	5-F	Et	4	3-Cl	0.67
83	5-F, 7-Cl	Et	4	3-Cl	4.75
84	5,7-diCl	Et	4	3-Cl	9.46

[a]Data from Ref. 83.
[b]Determined at human cloned 5-HT_7Rs in CHO cells using [^{3}H]LSD.

In 2011, the same group developed further series of (arylpiperazinylbutyl) oxindoles with highly potent 5-HT_7R antagonistic activity and selectivity toward the 5-HT_{1A}R and α_1-AR.[84] In this study, the effect of halogenation was studied both on the oxindole carbocycle and the aromatic ring of the piperazine moiety, in order to determine how electronegative halogen atoms affect the receptor binding affinities. For example, in the case of the fluoro derivatives a substitution at the 4-position (**108**) gave a more potent 5-HT_7R ligand than the 3-substituted analogue (**106**) (Table 9). This behavior contrasts the trend described in their previous paper. In fact, among methoxyphenyl analogues the position of the substituent changed the binding significantly, in an order of meta > ortho >> para. Concerning the chloro-substituted analogues, both 3- (**89**, **91**, **90**, **94**) and 4-chlorophenylpiperazines (**98**, **105**, **95**, **96**, **97**) show high affinity for the 5-HT_7R, and the 3,4-dichloro analogue (**111**) was similarly potent.[84] On the other hand, according to Na *et al*.,[85] the halogen substituent on the oxindole carbocycle did not substantially change the 5-HT_7R affinities with respect to the unsubstituted derivatives. However, in the di- (e.g., **93**, **103**) or especially tri-halogenated derivatives (**94**, **105**) a diminished 5-HT_7R affinity was observed.[84]

In 2008, Na *et al.* reported a new class of quinazolinone derivatives (Table 10). These compounds were divided into two groups: the first one characterized by a propyl spacer, and the second one by a butyl spacer. In both groups it was varied the nature and the position of the substituent on the aryl linked to the piperazine ring. Compounds with a butyl spacer showed binding affinities higher than those with a propyl chain (**112**–**116** *vs* **117**–**121**). Compounds with a substituent at the 2-position of the phenyl linked to the piperazine ring displayed higher 5-HT_7 affinities, whereas 4-substituted phenyl derivatives (**113**, **116**, **118**, **121**) were significantly less potent or devoid of affinity for the target receptor. In addition, 2-methoxy (**119**) and 2-ethoxy (**122**) substituents were preferred over 2-fluoro- or 2-chloro- (**123** and **124**, respectively). The authors also investigated fluorosubstituted quinazolinone derivatives, but they found no substantial differences when compared to the corresponding unsubstituted analogues (**125**, **126**, **127**). Finally, the selectivity of selected compounds was assessed over other receptors and the most potent compound **126** (IC_{50} = 12 nM) was 42-fold selective over 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and D_2 receptors.[75, 85]

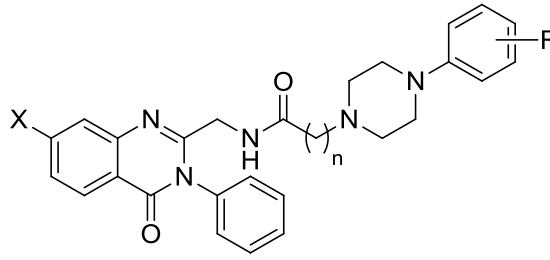
Table 9. Binding affinities of (phenylpiperazinyl-butyl)oxindoles derivatives (**85-111**) for 5-HT_7R.



Comp.[a]	V	W	X	Y	Z	R	K_i (nM)[b]
85	H	H	H	H	H	H	16
86	H	H	F	H	H	H	47
87	2-OCH_3	H	H	H	H	Et	5.4
88	2-Cl	H	H	H	H	Et	53
89	3-Cl	H	H	H	H	H	0.49
90	3-Cl	H	H	H	H	Et	0.40
91	3-Cl	H	H	F	H	H	6.7
92	3-Cl	H	F	H	H	Et	2.1
93	3-Cl	H	Cl	F	H	Et	9.07
94	3-Cl	H	Cl	F	Cl	Et	72
95	4-Cl	H	H	H	H	H	7.0
96	4-Cl	H	F	H	H	H	40
97	4-Cl	H	H	F	H	H	25
98	4-Cl	H	H	H	H	Et	0.38
99	4-Cl	H	F	H	H	Et	2.81
100	4-Cl	H	Cl	H	H	Et	1.1
101	4-Cl	H	H	F	H	Et	0.79
102	4-Cl	H	Cl	F	H	Et	10
103	4-Cl	H	Cl	H	Cl	Et	9.5
104	4-Cl	H	Cl	H	Cl	H	107
105	4-Cl	H	Cl	F	Cl	Et	96
106	3-F	H	H	H	H	Et	11
107	4-F	H	H	H	H	H	5.0
108	4-F	H	H	H	H	Et	0.43
109	3-CF_3	H	H	H	H	Et	5.1
110	2-Cl	4-Cl	H	H	H	Et	139
111	3-Cl	4-Cl	H	H	H	Et	0.60

[a]Data from Ref. 84.
[b]Determined at human cloned 5-HT_7Rs in CHO cells using [^{3}H]-CT.

Table 10. Binding affinities of quinazolinone derivatives (**112-127**) for 5-HT_7R. Table adapted from Ref. 75.

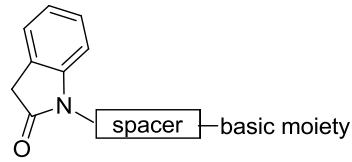


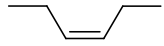
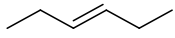
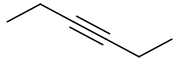
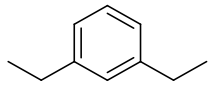
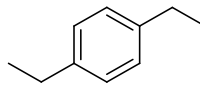
Comp.[a]	X	n	R_3	IC_{50} (nM)[b]
112	H	3	3-Cl	510
113	H	3	4-Cl	370
114	H	3	2-OCH_3	80
115	H	3	3-OCH_3	1400
116	H	3	4-OCH_3	710
117	H	4	3-Cl	110
118	H	4	4-Cl	450
119	H	4	2-OCH_3	21
120	H	4	3-OCH_3	>10000
121	H	4	4-OCH_3	2000
122	H	4	2-OC_2H_5	26
123	H	4	2-F	400
124	H	4	2-Cl	130
125	F	4	2-OCH_3	120
126	F	4	2-OC_2H_5	12
127	F	4	2-Cl	200

[a]Data from Ref. 85.
[b]Determined at human cloned 5-HT_7Rs in HEK-293 cells using [^{3}H]LSD.

In 2009, Medina *et al*. designed new 1,3-dihydro-2*H*-indol-2-one derivatives (Table 11) on the basis of their previous pharmacophore model for 5-HT_7R antagonists.[86, 87] The study confirmed previous findings about spacer characteristics and the nature of the aryl linked to the piperazine ring. In particular, the most potent compound was **139**, which contains a 1,2,3,4-tetrahydroisoquinoline nucleus as basic moiety instead of piperazine. It showed the best 5-HT_7/5-HT_{1A} receptor selectivity (31-fold) in this series; furthermore, it was pharmacologically characterized as a partial agonist.

Table 11. Binding affinities of 1,3-dihydro-2*H*-indol-2-one derivatives for 5-HT_7 and 5-HT_{1A} receptors. Table adapted from Ref. 75.



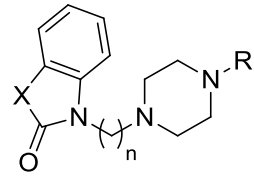
Comp.[a]	Spacer	Basic moiety	K_i (nM)	
			5-HT_7[b]	5-HT_{1A}[c]
128	$(CH_2)_4$	Ph-piperazine	74	124
129	$(CH_2)_4$	2-OCH_3-Ph-piperazine	32	>1000
130	$(CH_2)_4$	1-naphthalenyl-piperazine	47	22
131	$(CH_2)_5$	Ph-piperazine	63	>1000
132	$(CH_2)_5$	2-OCH_3-Ph-piperazine	63	>1000
133	$(CH_2)_5$	1-naphthalenyl-piperazine	62	16
134		1-naphthalenyl-piperazine	250	11.5
135		1-naphthalenyl-piperazine	69	39
136		1-naphthalenyl-piperazine	>1000	26
137		1-naphthalenyl-piperazine	>1000	>1000
138		1-naphthalenyl-piperazine	>1000	181
139	$(CH_2)_4$	1,2,3,4-tetrahydroisoquinoline	7	219
140	$(CH_2)_3$	1,2,3,4-tetrahydroisoquinoline	105	>1000
141	$(CH_2)_2$	1,2,3,4-tetrahydroisoquinoline	350	>1000

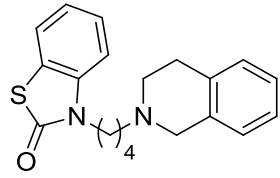
[a]Data from Ref. 86.
[b]Determined at human cloned 5-HT_7Rs in HEK-293 cells using [^{3}H]LSD.
[c]Determined at human cloned 5-HT_{1A}Rs in CHO cells using [^{3}H]-8-OH-DPAT.

A novel series of arylpiperazinylalkyl-2-benzoxazolones and 2-benzothiazolones **142-162** was designed (Table 12), synthesized and tested to evaluate their affinity for the 5-HT_7 and 5-HT_{1A} receptors by Salerno *et al.* in 2014.[88] Generally, the 2-benzothiazolone derivatives had affinity values higher than the corresponding 2-benzoxazolone compounds. In particular, derivatives possessing a six or seven carbon chain linker between 2-benzothiazolone and arylpiperazine had K_i values in the subnanomolar range for the 5-HT_{1A}R and in the low nanomolar range for the 5-HT_7R. The authors synthesized and tested compounds **146-150** bearing various aryl substituents at the 1-position of the piperazine moiety. With the exception of the benzyl and 4-chlorophenyl derivatives **147** and **148**, the other examined compounds had a higher 5-HT_7R affinity compared with the corresponding phenyl analogue **143**. Because increased linker length did not have an effect on 5-HT_{1A}/5-HT_7 selectivity, they subsequently synthesized compounds **142-145**, which are characterized by a four carbon linker, these last compounds displayed the best selectivity for 5-HT_7R over 5-HT_{1A}R (e.g., compound **143** with K_i 5-HT_{1A}/K_i 5-HT_7 ratio = 25).[88]

Canale *et al.*, very recently (2014 and 2015), reported a library of novel LCAPs which contained primary and tertiary amides of cyclic amino acids such as proline (Pro) and 1,2,3,4-tetrahydroisoquinoline-3-carboxamide (Tic) as terminal fragment (Table 13).[89, 90] The nature of the amino acid fragment only influenced the affinity for 5-HT_7R. In particular, derivatives containing Tic-amides displayed lower K_i values than Pro-amide analogues (**163** *vs* **168** and **166** *vs* **172**); on the other hand, different amino acid fragments did not influence the affinity for 5-HT_{1A}R (**167** *vs* **175**, and **179** *vs* **186**). Within compounds **176-188** the introduction of the piperidinyl and morpholinyl moieties did not change the affinity for 5-HT_7R. Among the primary amides (**163-175**) the elongation of an alkyl spacer from a four to a five or a six carbon chain decreased the affinity for 5-HT_7R. Regarding the substituent in the phenylpiperazine portion, in contrast to the result reported by Leopoldo *et al.* in 2007,[80] the 2-methylthio derivative in this series possessed more affinity for 5-HT_{1A}R. Finally, as a general trend for both primary and tertiary amides, the phenyl-substituted derivatives displayed higher affinity than their 2-isopropyl analogues for 5-HT_7R (*i.e.* **180** > **176**, **167** > **166**, and **175** > **172**).[90]

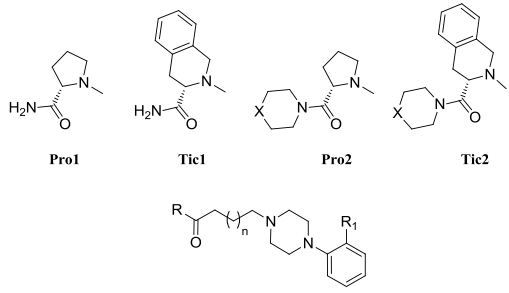
Table 12. Binding affinities of 2-benzoxazolone and 2-benzothiazolone derivatives for 5-HT_7 and 5-HT_{1A} receptors.



Comp.[a]	X	n	R	K_i (nM)	
				5-HT_7[b]	5-HT_{1A}[c]
142	O	4	C_6H_5	26.33	90.33
143	S	4	C_6H_5	36.48	913
144	O	4	2-$OCH_3C_6H_4$	10.35	19.22
145	S	4	2-$OCH_3C_6H_4$	7.77	24.58
146	S	4	2-$OCH_3C_6H_4$	0.99	10.36
147	S	4	$CH_2C_6H_5$	41.93	695.11
148	S	4	4-ClC_6H_4	46.83	83.33
149	S	4	3-ClC_6H_4	1.72	6.13
150				2.84	74.92
151	O	5	C_6H_5	57.39	19.22
152	S	5	C_6H_5	20.00	95.66
153	O	5	2-$OCH_3C_6H_4$	5.90	11.86
154	S	5	2-$OCH_3C_6H_4$	5.16	7.13
155	O	6	C_6H_5	14.10	3.96
156	S	6	C_6H_5	2.96	0.91
157	O	6	2-$OCH_3C_6H_4$	11.68	1.30
158	S	6	2-$OCH_3C_6H_4$	2.90	0.43
159	O	7	C_6H_5	45.57	29.78
160	S	7	C_6H_5	7.58	0.27
161	O	7	2-$OCH_3C_6H_4$	7.73	0.19
162	S	7	2-$OCH_3C_6H_4$	8.49	0.21

[a]Data from Ref. 88.
[b]Determined at human cloned 5-HT_7Rs in CHO cells using [^{3}H]5-HT.
[c]Determined at human cloned 5-HT_{1A}Rs in CHO cells using [^{3}H]-8-OH-DPAT.

Table 13. Binding affinities of 2-benzoxazolone and 2-benzothiazolone derivatives for 5-HT_7 and 5-HT_{1A} receptors.



Comp.	R	X	n	R_1	K_i (nM)	
					5-HT_7[c]	5-HT_{1A}[d]
163[a]	Pro1	-	2	SCH_3	152	47
164[a]	Pro1	-	3	SCH_3	163	14
165[a]	Pro1	-	4	SCH_3	490	23
166[b]	Pro1	-	4	isopropyl	116	16
167[b]	Pro1	-	4	phenyl	18	53
168[a]	Tic1	-	2	SCH_3	51	18
169[a]	Tic1	-	3	SCH_3	78	<1
170[a]	Tic1	-	4	SCH_3	111	2
171[b]	Tic1	-	2	isopropyl	30	34
172[b]	Tic1	-	4	isopropyl	43	20
173[b]	Tic1	-	2	phenyl	12	66
174[b]	Tic1	-	3	phenyl	10	26
175[b]	Tic1	-	4	phenyl	11	61
176[b]	Pro2	CH_2	4	isopropyl	77	1
177[b]	Pro2	O	4	isopropyl	135	17
178[b]	Pro2	CH_2	2	phenyl	5	35
179[b]	Pro2	CH_2	3	phenyl	16	18
180[b]	Pro2	CH_2	4	phenyl	15	9
181[b]	Pro2	O	4	phenyl	30	35
182[b]	Tic2	CH_2	3	isopropyl	40	16
183[b]	Tic2	CH_2	4	isopropyl	22	14
184[b]	Tic2	CH_2	2	phenyl	10	47
185[b]	Tic2	O	2	phenyl	9	36
186[b]	Tic2	CH_2	3	phenyl	9	19
187[b]	Tic2	CH_2	4	phenyl	12	40
188[b]	Tic2	O	4	phenyl	19	16

[a]Data from Ref. 89.

[b]Data from Ref. 90.

[c]Determined at human cloned 5-HT_{7B} receptors in HEK-293 cells using [^{3}H]-5-CT.

[d]Determined at human cloned 5-HT_{1A}Rs in HEK-293 cells using [^{3}H]-8-OH-DPAT.

1.5. Pharmacophore models for 5-HT$_7$R

1.5.1. Pharmacophores for 5-HT$_7$R antagonists

Two different pharmacophore models for 5-HT$_7$R antagonists were proposed by different research groups with the aim of investigating the major structural features necessary to obtain high 5-HT$_7$R affinity, and define the selectivity for this receptor subtype.

The first model was proposed by López-Rodríguez *et al.* in 2000, subsequently optimized in 2003.[91, 87] The final model consists of five features: *i*) a positive ionizable atom (PI); *ii*) an H-bond accepting group (HBA); *iii*) and three hydrophobic regions (HYD1-3). All of these features were fixed in a well-established disposition with appropriate distance between them (*i.e.* PI-HYD3 = 5.4–6.4 Å; PI-HYD1 = 5.2–6.2 Å; PI-HBA = 5.6–6.6 Å; HYD1-HBA = 3.6–4.8 Å; HYD1-HYD3 = 9.3–10.3 Å), (Fig. 14).

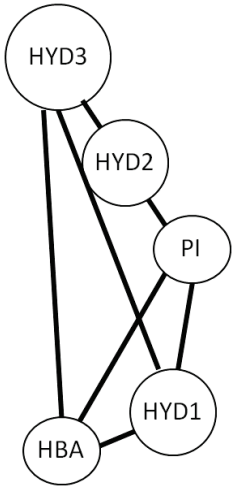
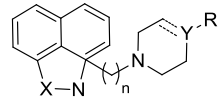


Figure 14. Optimized pharmacophore model for 5-HT$_7$R antagonists proposed by López-Rodríguez *et al*., 2003. Figure adapted from Ref. 75.

The model has been supported by the synthesis of new naphtholactam and naphthosultam derivatives (general structure Fig. 15).

The authors provided a remarkable conclusion about the pharmacofores, in particular: *i*) the HBA binds Ser5.42 and Thr5.43; *ii*) the HYD1 interacts with Phe6.52; *iii*) the PI forms an ionic interaction with Asp3.32; and *iv*) the HYD3/AR interacts with aromatic residues Phe3.28 and Tyr7.43.



X = CO, SO2
n = 1-6
Y = N, C, CH
R = Ph, 2-MeO-Phe, H, i-Pr, Cyclohexyl

Figure 15. General structure of naphtholactam and naphthosultam derivatives used to support the pharmacophore model.

The second model was proposed by Kołaczkowski *et al.* in 2006, and it was the first receptor-based pharmacophore for the 5-HT_7R. Authors have constructed the model by evaluating the binding mode interaction with the receptor binding site through docking studies of selective and nonselective antagonists. The authors suggested that selective and nonselective antagonists might have different binding modes with the receptor, thus they have hypothesized two distinct submodels: the first one for the "affinity" pharmacophore and the second one for the "selectivity" pharmacophore.[92]

The "affinity" pharmacophore was characterized by six features: *i*) a protonated nitrogen (PI), *ii*) three hydrophobic/aromatic regions (HYD/ AR1-3), *iii*) two H-bond acceptors (HB1 and HB2). It designateed an "essential triplet" that must be present in order to reach the affinity for the receptor; in particular PI and one of ARs (capable of specific CH–π or π-π interaction) are fixed and necessary, while the third may be variable (*i.e.* HBA or another HYD/AR region) (Fig. 16). This paper identified the specific amino acids that interact with the pharmacophores: PI is involved in the salt bridge formation with Asp3.32; AR1 interacts with Phe3.28 (CH–π or π-π) and/or Arg7.36 (ion-π), AR2 and AR3 have CH–π interaction with Phe6.52 and Phe6.51, HBA1 and HBA2 form H-bonds with Tyr7.43 and Ser5.42.[92]

The "selectivity" pharmacophore was based on the docked poses of selective 5-HT_7R antagonists (including, DR4004, SB-656104, SB-258719, and SB-269970), consisting of three crucial features. Two of these features are common for all selective antagonists: PI and AR1, which form strong interactions with specific residues, the amino acid in the TMHs 7-3 (especially Phe3.28 and Arg7.36). The third feature is necessary for selectivity and can be either: *a*) HBA1, an H-bond acceptor situated near Tyr7.43; or *b*) HYD/AR2, a hydrophobic or an aromatic moiety penetrating the pocket between TMHs 4-6, but its interactions should not dominate that of the AR1 (Fig. 16). Essentially, the geometry of the terminal portion containing AR1 (aromatic imide/amide/sulfonamide) should enable the formation of π-π stacking with Phe3.28, ion-π interaction with Arg7.36, or, optimally, both. [92]

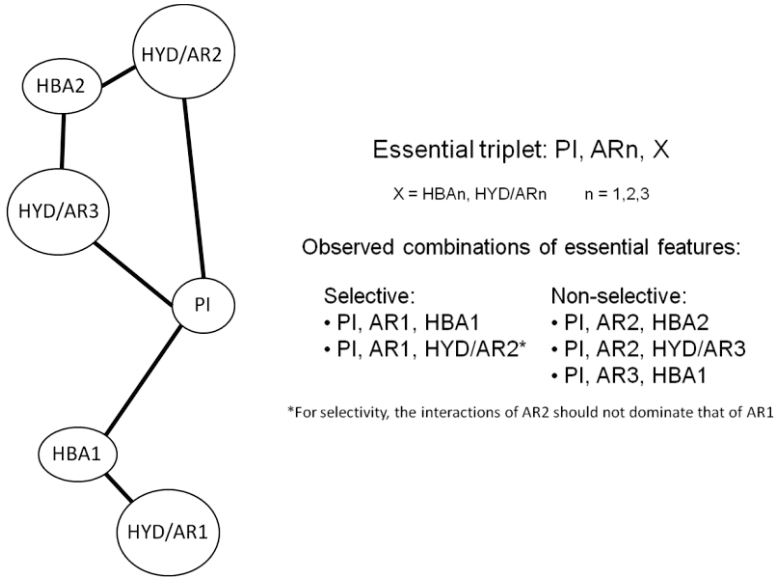


Figure 16. Receptor based pharmacophore model for 5-HT_7 antagonists proposed by Kołaczkowski *et al.*, 2006, and pharmacophoric features that providing affinity and selectivity toward 5-HT_7R. Figure adapted from Ref. 75 and 92.

1.5.2. Pharmacophore for 5-HT_7R agonists

On the basis of a set of twenty different 5-HT_7R agonists (including 5-HT, 5-CT, 8-OH-DPAT, 1-(1-naphthalenyl)piperazine, 1-(2-methoxyphenyl)piperazine, LSD, and AS-19), the pharmacophore for 5-HT_7R agonism was determined by Vermeulen *et al.* in 2003. Full conformational analysis of the set of compounds in their protonated form was performed with the MacroModel molecular modeling software package and followed by a pharmacophore-identifying procedure through ligand overlap using the Automated PharmacOphore Location through Ligand Overlap (APOLLO) procedure. Ultimately, they defined the distances between the four pharmacophoric features: PI-HYD1 = 5.7 Å; PI-HBA = 6.2 Å; HYD1-HBA = 3.0 Å; HYD1-HYD2 = 4.2 Å (Fig. 17).[93]

The CoMFA analysis used to map the agonist binding site of the model of the 5-HT_7R shows an important role in ligand binding that was attributed to Asp162 of TM3 (interaction with a protonated nitrogen), and Thr244 of TM5 (interaction with a substituent at an aromatic moiety). In addition, agonists that have lost a hydrogen-bond-accepting moiety, but possess an aromatic substituent, could bind to the receptor with high affinity as well by occupying a lipophilic pocket hosted by residues of TM5 and TM6.[93]

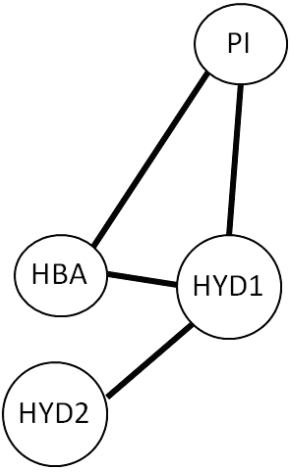
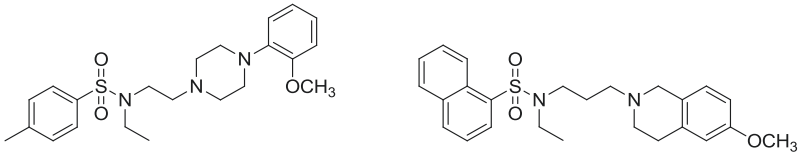


Figure 17. Pharmacophore model for 5-HT$_7$R agonists proposed by Vermeulen *et al.*, 2003. Figure adapted from Ref. 75.

1.5.3. Pharmacophore for 5-HT$_7$R inverse agonists

The pharmacophore model for 5-HT$_7$R inverse agonists was also proposed by Vermeulen *et al*. a year later. They synthesized and evaluated a series of arylpiperazine- and 1,2,3,4-tetrahydroisoquinoline-based arylsulfonamides (exemplified in Fig. 18). Effects on basal adenylyl cyclase (AC) activity were measured using HEK-293 cells expressing the rat 5-HT$_7$R and indicated that all ligands produced a decrease of AC level, thus acting as inverse agonists.[94]



K_i = 19 nM K_i = 19 nM

Figure 18. 5-HT$_7$R inverse agonists developed by Vermeulen *et al*., 2004. Figure from Ref. 75.

Additionally, computational studies (CoMFA model) showed a good correlation between experimental and predicted pK_i values, and a pharmacophore model that shows similarity with the model proposed by López-Rodríguez for selective antagonists. In particular, the pharmacophoric features for this model and the distances between them were: PI-HYD1 = 4.4 Å; PI-HBA = 5.8 Å; HYD1-HBA = 3.3 Å; HYD1-HYD4 = 4.4 Å; HYD4-HBA = 3.8 Å; HY3-HBA = 8.7 Å (Fig. 19), the main difference in López-Rodríguez's model was the additional HYD4 region. Furthermore, slightly shorter distances between HYD3-HBA and HYD2-HYD3 were also hypothesized. Interestingly, the model of inverse agonists revealed a close similarity to that of agonists, the main difference between the models is the presence of both HYD2 and HYD3 regions.[94]

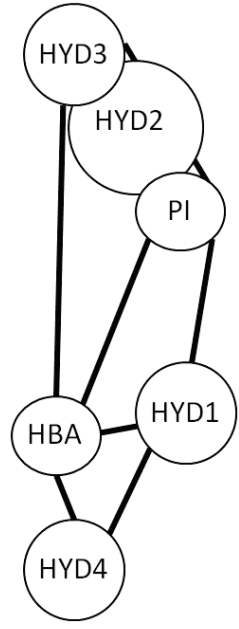


Figure 19. Pharmacophore model for 5-HT$_7$R inverse agonists proposed by Vermeulen *et al*., 2004. Figure adapted from Ref. 75.

1.6. Homology models of 5-HT_{1A}R and 5-HT_7R

Molecular modeling encompasses all theoretical methods and computational techniques used to model or mimic the behaviour of molecules. In particular, homology modeling refers to constructing an atomic-resolution model of the "*target*" protein from its amino acid sequence and an experimental three-dimensional structure of a related homologous protein (the "*template*").

This would allow users to safely use rapidly generated in silico protein models in all fields where it is useful to provide a solid basis, such as in the structure-based drug design, analysis of protein function, interactions, antigenic behavior and in generating receptor-based 3D pharmacophore models. On the other hand, sometimes many proteins are simply too large for nuclear magnetic resonance (NMR) analysis and cannot be crystallized for an X-ray crystallography, so the development of homology modeling is the only way to obtain structural information if experimental techniques fail.

The homology modeling method is based on two major rules. The first, is that the structure of a protein is uniquely determined by its amino acid sequence.[95] The second, is that during evolution the structure is more stable and changes much slower than the associated sequence. Similar sequences adopt practically identical structures, and distantly related sequences still fold into similar structures as reported by Chothia and Lesk (1986) and later quantified by Sander and Schneider (1991).[96, 97] After, thanks to the rapid growth of the Protein Data Bank (PDB), Rost (1999) could derive a precise limit for this rule, shown in Fig. 20.[98] As long as the length of the two sequences and the percentage of identical residues fall in the region marked as “safe,” the two sequences are practically guaranteed to adopt a similar structure.

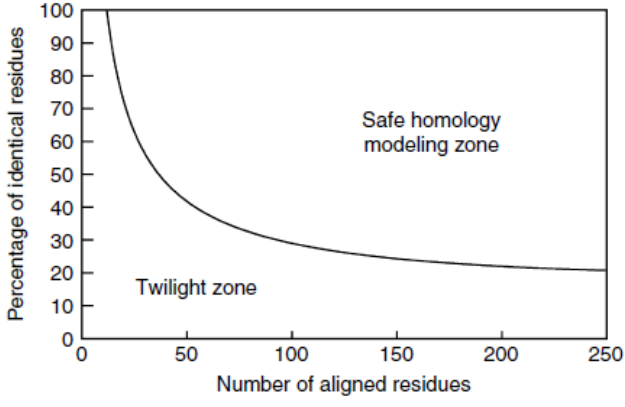


Figure 20. The two zones of sequence alignments. Figure from Ref. 99.

The homology modeling process is composed by seven steps as listed below (Fig. 21):

1. Template recognition and initial alignment
2. Alignment correction
3. Backbone generation
4. Loop modeling
5. Side-chain modeling
6. Model optimization
7. Model validation

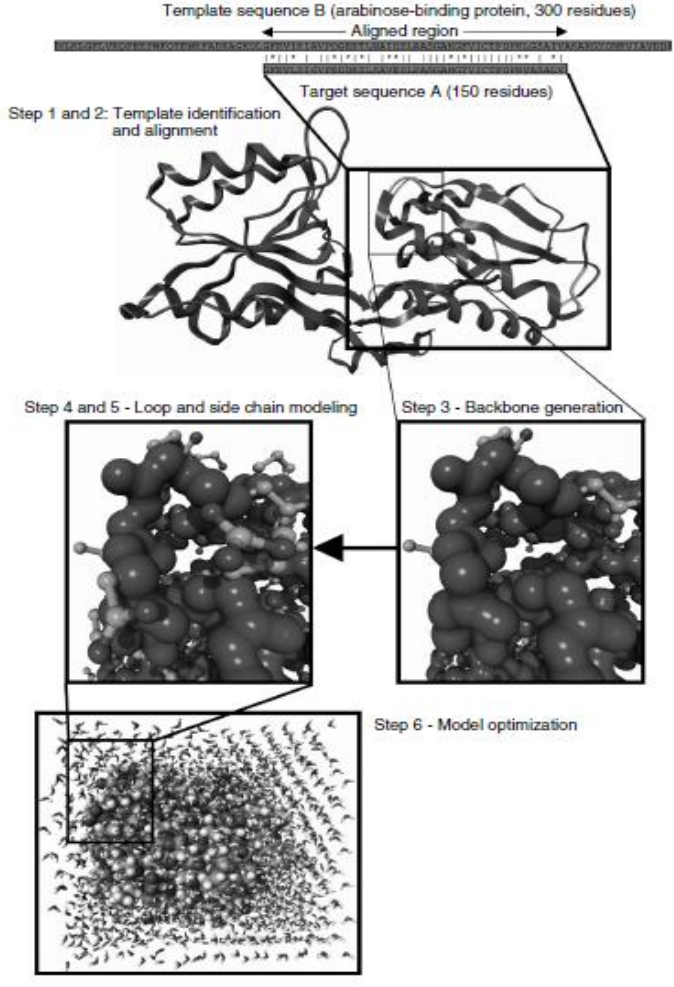


Figure 21. The multistep process to homology modeling. Figure from Ref. 99.

The modeler does not know with absolute certainty the best choices in each step, and thus a large part of the modeling process consists of serious thought about how to decide between multiple seemingly similar choices. A lot of effort has been made on programming the computer to know how to make these decisions, so that homology models can be fully built automatically. Currently, this allows modelers to construct models for about 25% of the amino acids in a genome. Although this average value of 25% differs significantly between individual genomes, for example ranging from 16% for the Mycoplasma pneumoniae and to 30% in the case of the Haemophilus influenza.[99] The quality of the homology model is dependent on the quality of the sequence alignment and template structure. The approach can be complicated by the presence of alignment gaps (commonly called indels) that indicate a structural region present in the target but not in the template, and by structure gaps in the template that arise from poor resolution in the experimental procedure (usually X-ray crystallography) used to solve the structure. Model quality declines with decreasing sequence identity; a typical model has ~1–2 Å root mean square deviation between the matched Cα atoms at 70% sequence identity but only 2–4 Å agreement at 25% sequence identity. However, the errors are significantly higher in the loop regions, where the amino acid sequences of the target and template proteins may be completely different.[99] Herein, further details about the multistep process for the construction of the protein homology model are not given.

In 2014, our research group worked on the modification of the structure of benzoxazole- and benzothiazole-based 5-HT_{1A} ligands (Fig. 22) in order to identify novel 5-HT_7R ligands (described before in the paragraph 1.4., pag 30, Table 12).[88]

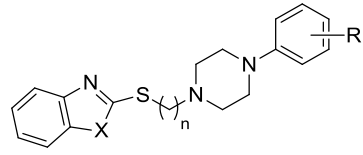


Figure 22. General formula of the benzoxazole- and benzothiazole 5-HT_{1A}R ligands.

Additionally, to further characterize the potential interactions of the newly developed ligands with 5-HT_{1A}R and 5-HT_7R, an intensive modeling study was performed. Using homology models of both proteins, the specific binding mode for LCAPs was explored and the structural interaction fingerprints method was applied to support the analysis of docking results. Despite the successful application of the previous rhodopsin-based models of both targets,[73] new models were created due to the availability of more homologous templates.[88] Using a similar approach and nine crystal structures of family A GPCRs, a set of 1800 models with extracellular loops (200 per template) for each receptor was generated in Modeller v. 9.8.[100] Interestingly, none of the 5-HT_{1A}R models based on the crystal structure of the evolutionarily closest 5-HT_{1B} and 5-HT_{2B} templates passed the first selection step, *i.e.*, at least 15 of 30 diversified ligands docked with a score ≤ 3. The 5-HT_{1B} template was also excluded from further modeling of 5-HT_7R for this reason. The rejection of crystal structure of proteins from the same receptor family from the procedure of homology model selection may seem unexpected, nevertheless the protein molecule exists in multiple conformational states and the crystal structure represents only one adopted during the interaction with a particular ligand. In the case of 5-HT_{1B} and 5-HT_{2B} receptors they were co-crystallized with ergotamine and dihydroergotamine, thus showing specific contacts for one class of 5-HTR ligand-ergoline derivatives. Moreover, we have found no correlation between sequence identity and homology model quality leading to the conclusion that the closest phylogenetic relative is not always the best template for homology modeling.[101]

Subsequently, based on docking the extended set of ligands, two models per template were selected, thus 14 conformations of 5-HT_{1A}R and 16 of 5-HT_7R entered the ligand-directed optimization of binding site procedure. Finally, induced-fit docking (IFD) of five arylpiperazines (Fig. 25) returned their coherent binding mode only in models generated on the D_3 template. Structural alignment of the optimized 5-HT_{1A}R and 5-HT_7R homology models with the crystal structure of the D_3 receptor displayed only minor conformational changes of the backbone of TM helices and extracellular loops (Fig. 26). The positions and spatial orientation of the conserved residues were similar; however the overall shape of the binding sites was different. This difference was particularly noticeable in the cavity between TMHs 2, 3, and 7 and EL1, which was smaller in 5-HT_7R due to more voluminous amino acids in position 2.61, 7.36, and 7.39 (Val, Arg, and Leu *vs* Ala, Ala, and Asn in 5-HT_{1A}R).[88]

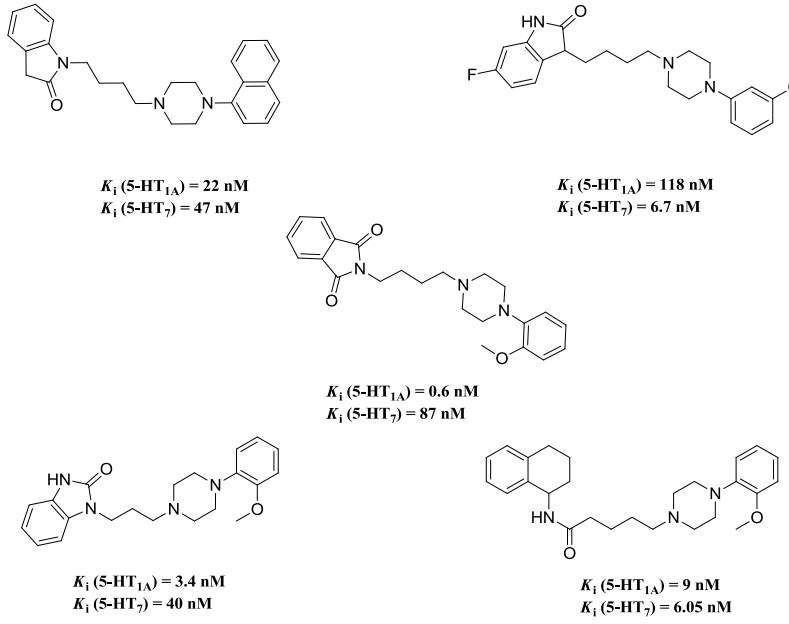


Figure 25. The training set of ligands used in induced-fit docking refinement of the 5-HT$_{1A}$R and 5-HT$_7$R models. Figure from Ref. 88.

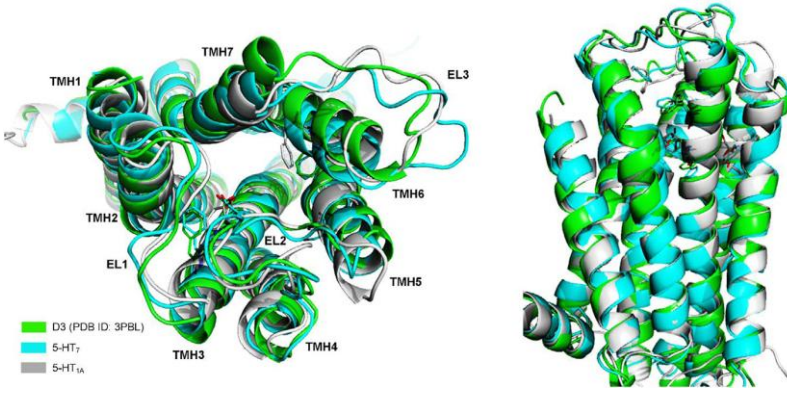


Figure 26. Alignment of the D$_3$ crystal structure (green) with 5-HT$_{1A}$R (gray) and 5-HT$_7$R (cyan), selected based on the IFD protocol. Figure from Ref. 88.

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2. Scope of this thesis

As previously mentioned in the paragraph *1.3.3.* (5-HT_7R pharmacology, pag. 14), the high potential of the 5-HT_7R as a target led to successful development of pharmacological tools for this serotonin receptor. Unfortunately, the high homology of transmembrane domains of 5-HT_7R with those for 5-HT_{1A}R gene (about 40%) is relatively determinative because several ligands posses both the 5-HT_{1A} and 5-HT_7 receptors affinity. Thus, a selectivity issue is encountered for these two receptor subtypes.

The aim of this thesis is:

1. To analyze the binding properties of new thienopyrimidinone and quinazolinone derivatives on human cloned 5-HT_{1A}R and 5-HT_7R with the purpose of exploring how some structural changes in the terminal fragment, in the chain length, and in the aryl substituents could influence affinity and selectivity for 5-HT_{1A}R and 5-HT_7R (*Paper I*, pag. 49).

2. To synthesize novel series of LCAPs, in the interest of thoroughly researching the quinazolinone as a terminal fragment. In this study the 6-phenylpyrimidine nucleus was used as a versatile building block for the preparation of new 5-HT_{1A} and 5-HT_7 receptor ligands. In addition, a molecular modeling study has been done on our previous receptor homology models to fully investigate the binding mode of the new and previous reported ligands (*Manuscript I*, pag. 75).

3. To identify and synthesize new classes of selective 5-HT_7R ligands based on the bis-piperazine skeleton in order to elucidate the SARs concerning those differently substituted in the arylpiperazine moiety (*Manuscript II*, pag. 101).

3. Paper I

Synthesis and binding properties of new long-chain 4-substituted piperazine derivatives as 5-HT_{1A} and 5-HT_7 receptor ligands

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Abstract

New long-chain 4-substituted piperazines linked to a thienopyrimidine or a quinazoline system were synthesized and tested for their binding properties on human cloned 5-HT_{1A} and 5-HT_7 serotonin receptors. Some structural modifications, concerning three main portions *i.e.* terminal fragment, chain length, and aryl substituents, were examined. The 2- and 3-substituted thienopyrimidinone and quinazolinone systems were selected as terminal fragment and a chain length of four or five methylene units was set. Explored aryl substituents were phenyl, phenylmethyl, 3- or 4-chlorophenyl, and 2-ethoxyphenyl. Title compounds showed affinity for 5-HT_{1A} and 5-HT_7 receptors. In particular, 2-ethoxyphenyl derivatives **40** and **45** displayed K_i values in the nanomolar range on both receptors, acting as dual ligands.

Keywords: Serotonin; 5-HT_{1A}; 5-HT_7; ligands; binding properties.

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Serotonin 5-HT_{1A}R and 5-HT_7R are members of the superfamily of seven-transmembrane GPCRs; 5-HT_{1A}R and 5-HT_7R have been identified in several species, including in humans, and are coupled to adenylyl cyclase via G_i and G_s proteins, respectively. Both receptors are widely distributed in human body and are located peripherally and in the CNS. In some tissues, such as hippocampus, thalamus, amygdala, kidney, and heart, they are both present. They are involved in physiological functions such as nociception, sleep, and locomotor activity regulation. Moreover, both exert a role in learning and memory and are involved in pathologies such as anxiety and epilepsy.[1-6]

Medicinal chemistry research had successfully obtained high-affinity and selective ligands for each of these receptors with the aim to clarify their role in the above mentioned physiological and pathological processes.[3-5] Recently, part of the research efforts have been directed towards the development of ligands for both receptors. Particularly, a number of studies suggest that ligands with partial agonist at 5-HT_{1A}R and antagonist properties on 5-HT_7R can display antidepressant-like effects.[7-9]

One of the most promising groups of serotonergic ligands are constituted by LCAPs with a number of pharmacological tools [3-5] and successfully developed drugs, such as buspirone, gepirone, tandospirone, and aripiprazole.[6, 10]

The drug potential of LCAPs had led to various SAR studies focused on the three main structural features of these agents (Fig. 1): the aryl substituent at the piperazine moiety, the aliphatic chain linker, and the terminal fragment (often an heterocyclic scaffold having an amide or imide moiety).

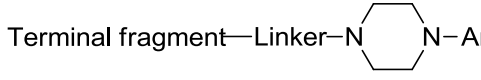


Figure 1. General structure of LCAPs.

Many research groups have focused their studies on the influence of these essential structural elements on 5-HT_{1A}R and 5-HT_7R affinity. Particularly, a large number of papers have been devoted to explore, through several structural modifications, the role of the terminal fragment and the aryl substituent in ligand-receptor interaction.

Within the framework of our studies on serotonin receptor ligands,[11] this paper reports the binding properties on human cloned 5-HT_{1A}R and 5-HT_7R of new derivatives (**14**, **16-29**, and **35-45**) bearing, with the exception of compound **14**, a thienopyrimidinone or quinazolinone as terminal fragment (Fig. 2).[12]

The purpose of this research was to explore how some structural

changes in this kind of molecules could influence affinity and selectivity for 5-HT_{1A}R and 5-HT_7R. These compounds, following the general structure of LCAPs, possess a variable alkyl chain linker and a substituted basic side portion (i.e. piperazine or piperidine).

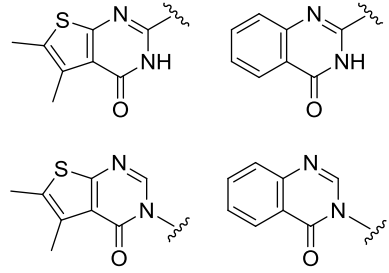
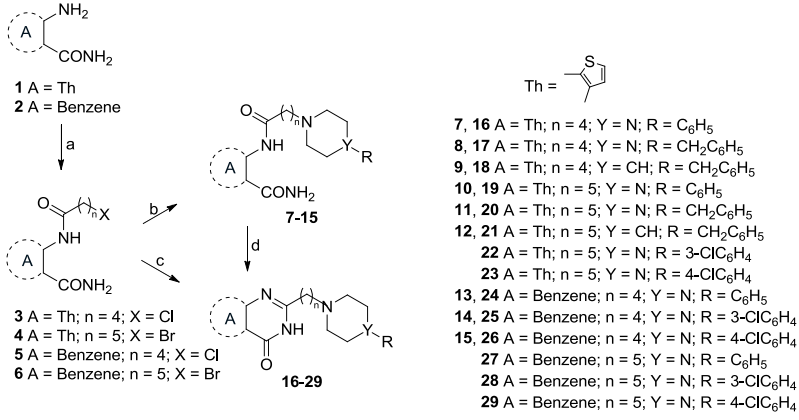


Figure 2. Structures of terminal fragment present in title compounds

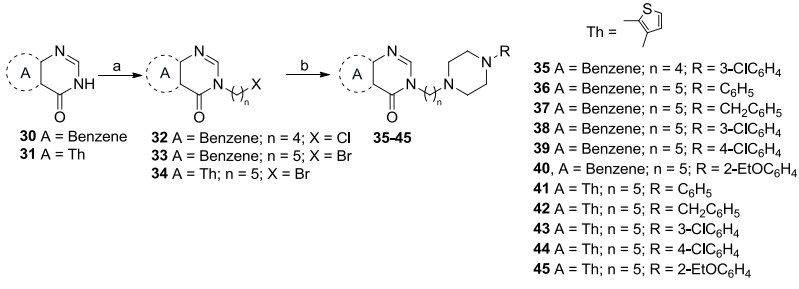
Moreover, the choice of substituents at the piperazine or piperidine nucleus, such as 3- and 4-chlorophenyl or 2-ethoxyphenyl, was inspired by literature reports[13-17] in which their introduction in LCAP derivatives increased affinity for 5-HT_7R or shifted the selectivity to the 5-HT_7R with respect to 5-HT_{1A}R.

The synthetic procedures adopted for new 2- and 3-substituted thieno[2,3-*d*]pyrimidinones and quinazolinones (**16-29**, **35-45**) are outlined in Schemes 1 and 2. The 2-amino-4,5-dimethyl-3-thiophenecarboxamide (**1**) or anthranilamide (**2**), both commercially available, reacted with 5-chloropentanoyl chloride or 6-bromohexanoyl chloride in dichloromethane to give halo derivatives **3-6**. Compounds **7-15** were prepared from halo derivatives **3-5** by reaction with an excess of the properly substituted piperidine or piperazine. Final compounds, **16-21** and **24-26**, were obtained by cyclization of intermediates **7-15** with a NaOH 10% aqueous solution. Final compounds **22**, **23**, and **27-29** were obtained, through a one step reaction, from bromo derivatives **4** and **6** and substituted piperazine hydrochlorides, in the presence of potassium carbonate and a catalytic amount of potassium iodide (Scheme 1).



Scheme 1. Reagents and conditions: (a) $XCO(CH_2)_nX$, CH_2Cl_2, reflux; (b) substituted piperidine or piperazine, 100 °C or reflux; (c) substituted piperazine hydrochlorides, K_2CO_3, KI catalytic amount, EtOH, reflux; (d) EtOH, NaOH 10% aqueous solution, room temperature or 100 °C.

Quinazolinone (**30**) and 5,6-dimethyl-thieno[2,3-*d*]pyrimidinone (**31**), commercially available, were alkylated, by reaction with 1-chloro-4-bromobutane or 1,5-dibromopentane under microwave irradiation, to obtain halo derivatives **32-34**, which finally were reacted with substituted piperazines to afford compounds **35-45** (Scheme 2).



Scheme 2. Reagents and conditions: (a) $Br(CH_2)_nX$, K_2CO_3, KI catalytic amount, CH_3CN, mw, 90 °C; (b) substituted piperazines, K_2CO_3, KI catalytic amount, EtOH, reflux.

Binding assays were carried on new derivatives **14**, **16-29**, and **35-45**, following a previously reported procedure,[11] on human cloned 5-HT_{1A} and 5-HT_7 serotonin receptors, expressed in CHO-K1 cells. [^{3}H]-5-HT (5 nM)

and [^{3}H]-8-OH-DPAT (1 nM) as radioligands, serotonin 10 μM and 1 μM for nonspecific binding at 5-HT_{1A}R and 5-HT_7R, have been used, respectively.

The first step of the study was the analysis of the main SARs of 2-substituted thienopyrimidinones **16-23**. Phenylpiperazine derivative **16** exhibits low nanomolar affinity for 5-HT_{1A}R and about 10-fold selectivity over the 5-HT_7R, the elongation of alkyl chain (derivative **19**) increases affinity for both receptors, particularly for the 5-HT_7R. The substitution of the phenylpiperazine with a phenylmethylpiperazine or phenylmethylpiperidine moiety is detrimental for affinity on both receptors for compounds with a four and five methylene chain linker (**17**, **18**, **20**, and **21**). The preparation of piperidine derivatives **18** and **21** has been done taking into account their bioisosterism with the piperazine analogues **17** and **20**. The aim was to determine the influence on affinity of the second nitrogen atom of piperazine ring in **17** and **20**. Unfortunately, derivatives having a four methylene linker (**17** and **18**) showed no appreciable affinity on 5-HT_7R and, for them, screening on 5-HT_{1A}R was not performed. On the other hand, both of derivatives having a five methylene linker (**20** and **21**) exhibit moderate and similar affinity on the two receptors; this suggests that substitution of the nitrogen with a carbon atom is tolerated on both receptors but it do not determine any really improvement in affinity or selectivity. The 3-chlorophenylpiperazine derivative **22** demonstrates moderate affinity for 5-HT_7R and 2-fold selectivity over 5-HT_{1A}R. Noteworthy, shift of the chloro atom to the 4-position (**23**) decreases the affinity for 5-HT_7R and makes **23** a selective compound for 5-HT_{1A}R. This result is unexpected, taking into account that, generally, the introduction of a chloro atom at the 4-position in other LCAPs enhanced the selectivity for the 5-HT_7R versus the 5-HT_{1A}R.[13, 14, 16, 17]

The next step of the work was focused on the modification of the terminal fragment with the isosteric substitution with a benzene in place of the dimethylthiophene ring to obtain a quinazolinone system. In quinazoline phenylpiperazines, the elongation of the chain linker from four (**24**) to five methylenes (**27**) determines an opposite trend in affinity values for both receptors with respect to the corresponding thienopyrimidine phenylpiperazines **16** and **19**. In fact, compound **27** exhibits lower affinity than **24** for both 5-HT_{1A} and 5-HT_7 receptors. The 3-chlorophenyl derivatives **25** and **28** display the highest affinity values for 5-HT_{1A}R and 5-HT_7R, acting as dual ligands, whereas the corresponding 4-chlorophenyl derivatives **26** and **29** show a noteworthy decrease of affinity for both receptors. Compound **14**, the corresponding open analogue of **25**, revels a 13- and 8-fold decrease in affinity for 5-HT_{1A}R and 5-HT_7R, respectively.

The subsequent step was the synthesis of a new set of compounds in which the anchoring point of the alkyl linker was the 3-position of the

quinazoline and thienopyrimidine systems (**35-45**). Generally, the two series of 3-substituted quinazoline and thienopyrimidine derivatives display a similar behavior. Phenylpiperazines **36** and **41** exhibit good affinity for 5-HT_{1A}R comparable to that of the corresponding 2-substituted compound **27**. Moreover, **36** and **41** display a higher affinity for 5-HT_{1A}R with respect to 5-HT_7R. Phenylmethyl derivatives **37** and **42** show not interesting results, confirming a trend similar to that observed for 2-substituted derivatives **20** and **21**. 3-Chlorophenyl derivatives **35**, **38**, and **43** display high-affinity values and selectivity for 5-HT_{1A}R with respect to 5-HT_7R; in particular, compound **43** shows 27-fold higher selectivity for 5-HT_{1A}R with respect to 5-HT_7R. 3-Chlorophenyl derivative **35** had been already reported by Bojarski *et al.*[18] and tested for 5-HT_{1A}R affinity using rat hippocampus as receptor source (Table 1; K_i = 50 nM). This compound when tested on human cloned 5-HT_{1A}R shows a higher affinity value (Table 1; K_i = 5.30 nM). This discrepancy could be attributable to the nature of 5-HT_{1A}R (native *vs* cloned; rat *vs* human) used in binding assays.

A slight decrease of affinity for 5-HT_{1A}R is displayed by 4-chlorophenyl derivatives **39** and **44** in comparison to 3-chlorophenyl analogues **38** and **43**. Noteworthy, compound **44** retains selectivity for 5-HT_{1A}R with respect to 5-HT_7R, whereas compound **39** shows an inversion of selectivity.

The introduction of a 2-ethoxy group on the phenylpiperazine moiety (**40** and **45**) lead to the highest affinity values in the series for 5-HT_7R, but unexpectedly, these compounds have also the highest affinity values for 5-HT_{1A}R; therefore **40** and **45** act as potent dual ligands.

In addition, cLogP of compounds **14**, **16-29**, **35-45** have been calculated and listed in Table 1.[19] Although title compounds show relatively high lipophilicy, with the exclusion of **18** and **21**, all them possess cLogP values ≤ 5, thus complying with Lipinski's rule of five. As a general trend, benzylpiperidines have cLogP higher than benzylpiperazine and phenylpiperazine analogues (**18** *vs* **17** and **16**; **21** *vs* **20** and **19**) and the same could be observed for the 3-substituted derivatives with respect to the corresponding 2-substituted compounds (e.g.: **36** *vs* **27** and **41** *vs* **19**).

Table 1. Binding properties of derivatives **14**, **16-29**, **35-45**, reference compounds SB 269970, 8-OH-DPAT, and 5-HT and cLogP of the new compounds.

Comp.	K_i[a] (nM)		cLogP
	5-HT_{1A}	5-HT_7	
14	39.6 ± 9.8	168 ± 21	2.88 ± 0.49
16	37.4 ± 5.3	357 ± 46	3.50 ± 0.92
17	NT[b]	NA[c]	3.54 ± 0.92
18	NT[b]	NA[c]	5.27 ± 0.87
19	15.2 ± 0.90	30.1 ± 6.4	3.92 ± 0.92
20	363 ± 16	147 ± 7.2	3.96 ± 0.92
21	148 ± 17	185 ± 40	5.69 ± 0.87
22	114 ± 35	68.4 ± 11	4.59 ± 0.92
23	53.9 ± 6.0	1464 ± 422	4.78 ± 0.94
24	36.6 ± 5.9	145 ± 29	3.34 ± 0.56
25	2.96 ± 0.19	21.2 ± 2.0	4.00 ± 0.57
26	75.3 ± 13	1135 ± 294	4.20 ± 0.57
27	43.5 ± 5.4	228 ± 12	3.76 ± 0.56
28	7.33 ± 0.77	11.9 ± 3.2	4.42 ± 0.57
29	116 ± 20	101 ± 26	4.62 ± 0.57
35	5.30 ± 0.35 (50 ± 9[d])	502 ± 108	4.36 ± 0.45
36	28.9 ± 4.6	307 ± 57	3.97 ± 0.43
37	268 ± 25	1082 ± 100	4.01 ± 0.43
38	6.28 ± 0.86	35.8 ± 7.0	4.64 ± 0.44
39	51.5 ± 11	12.9 ± 0.85	4.83 ± 0.47
40	1.04 ± 0.13	6.88 ± 0.66	4.64 ± 0.46
41	16.1 ± 1.2	143 ± 14	4.14 ± 0.84
42	211 ± 13	1650 ± 223	4.17 ± 0.84
43	3.23 ± 0.37	88.5 ± 22	4.80 ± 0.85
44	26.4 ± 3.1	213 ± 24	5.00 ± 0.86
45	1.03 ± 0.07	2.99 ± 0.60	4.81 ± 0.86
SB-269970	9024 ± 181	0.71 ± 0.06	
8-OH-DPAT	2.65 ± 0.10	388 ± 58	
5-HT	0.91 ± 0.10	2.12 ± 0.41	

[a]Each value is the mean ± SD of the data from three separate experiments.
[b]NT = not tested.
[c]NA = < 50% inhibition at 10^{-5} M.
[d]Binding test on rat hippocampus as receptor source, data from Ref. 18.

Moreover, the functional properties of compounds **40** and **45** on 5-HT_7R were evaluated using their ability to inhibit cAMP production induced by 5-CT, a 5-HT_7R agonist, in a HEK293 cells overexpressing 5-HT_7R. Both compounds were tested in a concentration of 1 μM and show antagonistic properties (42 and 38% of inhibition of control agonist response for **40** and **45**, respectively), which were weaker than for

reference antagonist SB-269970 (90% at 1 μM; K_b = 1 nM). It should be mentioned however, that low solubility of both compounds in the buffer solution significantly limited the range of concentrations which could be examined and determination of a full dose-response curve was not feasible.

In conclusion, we report the synthesis of new long-chain piperazines with structural modifications in the terminal fragment, in the alkyl chain length and in the substituents on the piperazine fragment. New derivatives have been evaluated for binding affinities at human cloned 5-HT_{1A}R and 5-HT_7R and the main structure-affinity relationships were outlined. Generally, elongation of the alkyl chain spacer improves affinity for 5-HT_7R. However, this structural modification is not enough to induce 5-HT_7R selectivity over 5-HT_{1A}R. Analysis of binding data reveals that affinity for both receptors are greatly influenced by the arylpiperazine mojety rather than by the alkyl chain length or by the nature of the terminal amide fragment. The 2-ethoxy derivatives **40** and **45** were the best ligands in the series, showing high affinity for both receptors, but, conversely to what was expected, and along with 3- and 4-chloro compounds, they do not display any 5-HT_7R selectivity over the 5-HT_{1A}R. Preliminary data on functional activity indicate that compounds **40** and **45** act as antagonists at 5-HT_7R. Further functional studies along with the synthesis of new long-chain 4-substituted piperazines are currently being developed.

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19. ACD/LogP DB, version 11.01.

3.2. Supplementary material

3.2.1. Experimental protocols

Melting points were determined in an Electrothermal IA9200 apparatus in glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin Elmer series FTIR 1600 spectrometer in KBr disks. Elemental analyses for C, H, N, and S were within ± 0.4% of theoretical values and were performed on a Carlo Erba Elemental Analyzer Mod. 1108 apparatus. ^{1}H NMR spectra were recorded on a Varian Inova Unity 200 spectrometer (200 MHz) in DMSO-d_6 or $CDCl_3$ solution. Chemical shifts are given in δ values (ppm), using tetramethylsilane as the internal standard; coupling constants (*J*) are given in Hz. Signal multiplicities are characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). Microwave irradiation experiments were carried out with a CEM Discover instrument using closed Pyrex glass tubes (ca. 10 mL) with Teflon-coated septa. All the synthesized compounds were tested for purity on TLC (aluminium sheet coated with silica gel 60 F_{254}, Merck) and visualized by UV light (λ = 254 and 366 nm). Purification of synthesized compounds by flash column chromatography was performed using Merck silica gel (0.040-0.063 mm). All chemicals and solvents were reagent grade and were purchased from commercial vendors. The known compounds **32**[1] and **35**[2] were synthesized using slightly modified procedures.

2-[(5-Chloro-1-oxopentyl)amino]-4,5-dimethyl-3-thiophenecarboxamide (3). To a suspension of amide **1** (0.20 g, 1.41 mmol) in dichloromethane (3 mL) 5-chloropentanoyl chloride (0.18 mL, 1.39 mmol) was added and the mixture was refluxed under stirring for 6 hours. After being cooled, the solvent was removed under reduced pressure. The solid obtained was collected by filtration, washed with water, and dried. Recrystallization from ethanol gave compound **3** (0.24 g, 70%), mp 136.0-139.0 °C. IR (KBr, selected lines) cm^{-1} 3513, 3163, 1681, 1647, 1583, 1555, 1515, 1458, 1397, 1317, 1267. ^{1}H NMR ($CDCl_3$) δ 1.72-2.00 (m, 2H + 2H, CH_2CH_2), 2.29 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 2.46 (t, *J* = 6.2 Hz, 2H, $COCH_2$), 3.57 (t, *J* = 6.2 Hz, 2H, CH_2Cl), 5.87 (br s, 2H, NH_2), 11.98 (br s, 1H, CONH). Anal. ($C_{12}H_{17}ClN_2O_2S$) C, H, N, S.

2-[(6-Bromo-1-oxohexyl)amino]-4,5-dimethyl-3-thiophenecarboxamide (4). The title compound was obtained by using the same procedure for the preparation of compound **3** starting from amide **1** and 6-bromohexanoyl chloride. The crude product was purified by flash

chromatography using a mixture of cyclohexane/ethyl acetate (5:5, v/v) as eluent, obtaining compound **4** as a pure solid (29%), mp 123.0 °C. IR (KBr, selected lines) cm^{-1} 3479, 3425, 3175, 1645, 1514, 1454, 1401, 1336, 1272, 993, 804. ^1H NMR (DMSO-d_6) δ 1.39-1.49 (m, 2H, CH_2), 1.49-1.68 (m, 2H, CH_2), 1.74-1.90 (m, 2H, CH_2), 2.15 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.40 (t, J = 7.0 Hz, 2H, CH_2CO), 3.52 (t, J = 6.6 Hz, 2H, CH_2Br), 7.36 (br s, 2H, NH_2), 11.15 (br s, 1H, CONH). Anal. ($C_{13}H_{19}BrN_2O_2S$) C, H, N, S.

2-[(5-Chloro-1-oxopentyl)amino]benzamide (5). To a suspension of antranilamide **2** (1.00 g, 7.34 mmol) in dichloromethane (16 mL) 5-chloropentanoyl chloride (1.04 mL, 8.09 mmol) and sodium carbonate were added (0.78 g, 7.36 mmol). The mixture was refluxed under stirring for 4 hours. After being cooled, the solid was removed under reduced pressure and the filtrate was concentrated to dryness. The crude product was purified by flash chromatography using ethyl acetate 100% as eluent, obtaining compound **5** as a pure solid (1.27 g, 68%), mp 112.5-114.5 °C. IR (KBr, selected lines) cm^{-1} 3400, 3181, 2956, 2926, 1673, 1519, 1455, 1393, 1311, 959, 759, 641. ^1H NMR (DMSO-d_6) δ 1.62-1.90 (m, 2H + 2H, CH_2CH_2), 2.38 (t, J = 6.8 Hz, 2H, $COCH_2$), 3.67 (t, J = 6.2 Hz, 2H, CH_2Cl), 7.02-7.19 (m, 1H, aromatic), 7.42-7.58 (m, 1H, aromatic), 7.73 (br s, 1H, NH), 7.79 (dd, J = 7.8 and 1.4 Hz, 1H, aromatic), 8.27 (br s, 1H, NH), 8.46 (dd, J = 8.2 and 1.0 Hz, 1H, aromatic), 11.67 (s, 1H, CONH). Anal. ($C_{12}H_{15}ClN_2O_2$) C, H, N.

2-[(6-Bromo-1-oxohexyl)amino]benzamide (6). The title compound was obtained by using the same procedure for the preparation of compound **5** starting from amide **2** and 6-bromohexanoyl chloride. The crude product was purified by flash chromatography using a mixture of cyclohexane/ethyl acetate (5:5, v/v) as eluent, obtaining compound **6** as a pure solid (30%), mp 92.5 °C. IR (KBr, selected lines) cm^{-1} 3333, 3166, 2926, 2847, 1662, 1621, 1587, 1522, 1397, 1360, 1245, 770, 638. ^1H NMR (DMSO-d_6) δ 1.38-1.47 (m, 2H, CH_2), 1.47-1.67 (m, 2H, CH_2), 1.67-1.95 (m, 2H, CH_2), 2.35 (t, J = 6.6 Hz, 2H, $COCH_2$), 3.54 (t, J = 6.6 Hz, 2H, CH_2Br), 7.05-7.20 (m, 1H, aromatic), 7.41-7.59 (m, 1H, aromatic), 7.74 (br s, 1H, NH), 7.79 (d, J = 8.0 Hz, 1H, aromatic), 8.28 (br s, 1H, NH), 8.47 (d, J = 8.0 Hz, 1H, aromatic), 11.70 (br s, 1H, CONH). Anal. ($C_{13}H_{17}BrN_2O_2$) C, H, N.

4,5-Dimethyl-2-[5-(4-phenyl-1-piperazinyl)-1-oxopentylamino]-3-thiophenecarboxamide (7). A mixture of chloro derivative **3** (0.40 g, 1.38 mmol) and 1-phenylpiperazine (0.43 mL, 2.81 mmol) was heated at 100 °C with stirring for 2 hours. After being cooled, the sticky residue was collected by filtration, washed with water, and dried. Recrystallization from ethanol gave **7** (0.25 g, 44%), mp 127.0-131.0 °C (dec). IR (KBr,

selected lines) cm^{-1} 3510, 3321, 3171, 2939, 2821, 1670, 1650, 1592, 1555, 1519, 1450, 1396, 1323. ^1H NMR ($CDCl_3$) δ 1.50-1.90 (m, 2H + 2H, CH_2CH_2), 2.26 (s, 3H, CH_3), 2.28 (s, 3H, CH_3), 2.37-2.53 (m, 2H + 2H, $COCH_2$ + CH_2N), 2.51-2.65 (m, 4H, piperazine), 3.10-3.25 (m, 4H, piperazine), 5.72 (br s, 2H, NH_2), 6.79-7.00 (m, 3H, aromatic), 7.19-7.38 (m, 2H, aromatic), 11.93 (br s, 1H, CONH). Anal. ($C_{22}H_{30}N_4O_2S$) C, H, N, S.

4,5-Dimethyl-2-[5-[4-(phenylmethyl)-1-piperazinyl]-1-oxopentylamino]-3-thiophenecarboxamide (8). The title compound was obtained from chloro derivative **3** and 1-(phenylmethyl)piperazine following the same procedure for the preparation of **7**. Recrystallization from ethanol gave **8** (50%), mp 139.0-142.0 °C. IR (KBr, selected lines) cm^{-1} 3506, 3140, 2940, 2815, 1667, 1644, 1560, 1522, 1396, 1333, 1266, 1134, 1000, 750. ^1H NMR (DMSO-d_6) δ 1.36-1.64 (m, 2H + 2H, CH_2CH_2), 2.16 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.25-2.46 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.42 (s, 2H, $CH_2C_6H_5$), 7.15-7.40 (m, 5H, aromatic), 11.17 (br s, 1H, CONH). Anal. ($C_{23}H_{32}N_4O_2S$) C, H, N, S.

4,5-Dimethyl-2-[5-[4-(phenylmethyl)-1-piperidinyl]-1-oxopentylamino]-3-thiophenecarboxamide (9). The title compound was obtained from chloro derivative **3** and 4-(phenylmethyl)piperidine following the same procedure for the preparation of **7** prolonging the reflux to 5 hours. Recrystallization from ethanol yielded **9** (95%), mp 109.0 °C. IR (KBr, selected lines) cm^{-1} 3498, 3345, 3175, 3024, 2920, 2768, 1679, 1639, 1555, 1524, 1452, 1400, 1320. ^1H NMR (DMSO-d_6) δ 1.04-1.29 (m, 2H, piperidine), 1.33-1.67 (m, 2H + 2H + 2H + 1H, CH_2CH_2 + piperidine), 1.70-1.90 (m, 2H, piperidine), 2.16 (s, 3H, CH_3), 2.21 (s, 3H, CH_3), 2.25 (t, J = 7.0 Hz, 2H, $COCH_2$), 2.34-2.55 (m, 2H + 2H, CH_2N + piperidine), 2.81 (d, J = 11.4 Hz, 2H, $CH_2C_6H_5$), 7.08-7.35 (m, 5H, aromatic), 11.17 (br s, 1H, CONH). Anal. ($C_{24}H_{33}N_3O_2S$) C, H, N, S.

4,5-Dimethyl-2-[6-(4-phenyl-1-piperazinyl)-1-oxohexylamino]-3-thiophenecarboxamide (10). A mixture of bromo derivative **4** (0.20 g, 0.57 mmol) and 1-phenylpiperazine (0.18 mL, 1.18 mmol) was heated at 100 °C under stirring for 1 hour. After being cooled, ethyl acetate was added to the mixture and the obtained solid was removed by filtration. The filtrate was washed with water (20 mL × 2), dried over anhydrous sodium sulfate, and concentrated to dryness. The obtained solid was collected with diethyl ether and dried. Compound **10** was obtained as a pure solid (0.061 g, 25%), mp 138.0-141.0 °C. IR (KBr, selected lines) cm^{-1} 3446, 3183, 2944, 1645, 1557, 1522, 1502, 1457, 1400, 1235, 756. ^1H NMR (DMSO-d_6) δ 1.41-1.70 (m, 2H + 2H + 2H, $CH_2CH_2CH_2$), 2.16 (s, 3H, CH_3), 2.21 (s, 3H, CH_3), 2.30-2.48 (m, 2H + 2H, $COCH_2$ + CH_2N), 2.48-2.61 (m, 4H, piperazine), 3.08-3.19 (m, 4H, piperazine), 6.72-6.84 (m, 1H, aromatic),

6.85-6.96 (m, 2H, aromatic), 7.17-7.30 (m, 2H, aromatic), 7.42 (br s, 2H, NH_2), 11.22 (br s, 1H, CONH). Anal. ($C_{23}H_{32}N_4O_2S$) C, H, N, S.

4,5-Dimethyl-2-[6-[4-(phenylmethyl)-1-piperazinyl)-1-oxohexylamino]-3-thiophenecarboxamide (11). A mixture of bromo derivative **4** (0.51 g, 1.47 mmol) and 1-(phenylmethyl)piperazine (0.47 mL, 2.70 mmol) was heated at 100 °C under stirring for 1.30 hours. Successively, ethanol (20 mL) was added to the mixture, which was refluxed under stirring for 4 hours. After being cooled, the solvent was removed under reduced pressure and ethyl acetate was added. The solution was washed with water (20 mL × 2), dried over anhydrous sodium sulfate, and concentrated to dryness. The crude product was purified by flash chromatography using ethyl acetate/methanol (5:5, v/v) as eluent, obtaining compound **11** as a pure solid (0.27 g, 41%), mp 121.0-123.0 °C (dec). IR (KBr, selected lines) cm^{-1} 3508, 3152, 2936, 2815, 1684, 1652, 1560, 1526, 1398, 1325, 1276, 1153, 743. ^1H NMR (DMSO-d_6) δ 1.53-1.70 (m, 2H + 2H + 2H, $CH_2CH_2CH_2$), 2.16 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.24-2.45 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.42 (s, 2H, $CH_2C_6H_5$), 7.18-7.38 (m, 5H, aromatic), 11.20 (br s, 1H, CONH). Anal. ($C_{24}H_{34}N_4O_2S$) C, H, N, S.

2-[4-(4-Phenyl-1-piperazinyl)-1-oxopentylamino]benzamide (13). A mixture of chloro derivative **5** (0.84 g, 3.29 mmol) and 1-phenylpiperazine (0.75 mL, 4.91 mmol) in ethanol (20 mL) was refluxed with stirring for 2 hours. After being cooled, the volume of the solution was reduced under vacuum and from the solution after 12 hours was obtained a solid, collected by filtration, washed with water, and dried. Recrystallization from ethanol gave compound **13** (0.23 g, 18%), mp 193.0-194.7 °C. IR (KBr, selected lines) cm^{-1} 3357, 3167, 2931, 2821, 1664, 1522, 1502, 1452, 1399, 1246, 1236, 1131. ^1H NMR (DMSO-d_6) δ 1.40-1.80 (m, 2H + 2H, CH_2CH_2), 2.24-2.42 (m, 2H + 2H, $COCH_2$ + CH_2N), 2.42-2.56 (m, 4H, piperazine), 3.02-3.18 (m, 4H, piperazine), 6.70-6.82 (m, 1H, aromatic), 6.84-6.96 (m, 2H, aromatic), 7.04-7.26 (m, 3H, aromatic), 7.42-7.54 (m, 1H, aromatic), 7.75 (br s, 1H, NH), 7.79 (dd, J = 8.2 and 1.6 Hz, 1H, aromatic), 8.28 (br s, 1H, NH), 8.48 (dd, J = 8.4 and 0.8 Hz, 1H, aromatic), 11.70 (br s, 1H, CONH). Anal. ($C_{22}H_{28}N_4O_2$) C, H, N.

2-[4-[4-(3-Chlorophenyl)-1-piperazinyl]-1-oxopentylamino]benzamide (14). A mixture of chloro derivative **5** (0.75 g, 2.94 mmol) and 1-(3-chlorophenyl)piperazine (1.16 g, 5.90 mmol) in ethanol (10 mL) was refluxed under stirring for 3 hours. After being cooled, water was added to the solution and the obtained suspension was stirred at room temperature for 30 min. Successively, the solid was filtered under reduced pressure and dried. Recrystallization from ethanol gave compound **14** (0.18 g, 15%), mp 168.8-169.7 °C. IR (KBr, selected lines)

cm^{-1} 3373, 2949, 2831, 1681, 1595, 1514, 1446, 1383, 1288, 1247, 1228. ^1H NMR (DMSO-d_6) δ 1.40-1.74 (m, 2H + 2H, CH_2CH_2), 2.24-2.42 (m, 2H + 2H, $COCH_2$ + CH_2N), 2.42-2.56 (m, 4H, piperazine), 3.04-3.22 (m, 4H, piperazine), 6.72-6.82 (m, 1H, aromatic), 6.82-6.96 (m, 2H, aromatic), 7.02-7.26 (m, 2H, aromatic), 7.40-7.54 (m, 1H, aromatic), 7.73 (br s, 1H, NH), 7.79 (dd, *J* = 7.8 and 1.4 Hz, 1H, aromatic), 8.27 (br s, 1H, NH), 8.47 (dd, *J* = 8.2 and 0.6 Hz, 1H, aromatic), 11.69 (br s, 1H, CONH). Anal. ($C_{22}H_{27}ClN_4O_2$) C, H, N.

2-[4-[4-(4-Chlorophenyl)-1-piperazinyl]-1-oxopentylamino]benzamide (15). A mixture of chloro derivative **5** (0.44 g, 1.73 mmol) and 1-(4-chlorophenyl)piperazine (0.69 g, 3.50 mmol) was suspended in ethanol (10 mL). The mixture was refluxed under stirring for 3 hours and after being cooled the solvent was removed to dryness. The crude product was purified by flash chromatography using a mixture of ethyl acetate/methanol (8:2, v/v) as eluent, obtaining compound **15** as a pure solid (0.20 g, 28%), mp 139.1-141.1 °C. IR (KBr, selected lines) cm^{-1} 3502, 3163, 2940, 2836, 1667, 1516, 1496, 1456, 1395, 1303, 1235. ^1H NMR (DMSO-d_6) δ 1.40-1.80 (m, 2H + 2H, CH_2CH_2), 2.25-2.45 (m, 2H + 2H, $COCH_2$ + CH_2N), 2.45-2.60 (m, 4H, piperazine), 2.98-3.20 (m, 4H, piperazine), 6.92 (d, *J* = 9.0 Hz, 2H, aromatic), 7.04-7.16 (m, 1H, aromatic), 7.21 (d, *J* = 9.2 Hz, 2H, aromatic), 7.42-7.54 (m, 1H, aromatic), 7.72 (br s, 1H, NH), 7.79 (dd, *J* = 7.8 and 1.0 Hz, 1H, aromatic), 8.27 (br s, 1H, NH), 8.47 (dd, *J* = 8.4 and 1.0 Hz, 1H, aromatic), 11.69 (br s, 1H, CONH). Anal. ($C_{22}H_{27}ClN_4O_2$) C, H, N.

5,6-Dimethyl-2-[4-(4-phenyl-1-piperazinyl)butyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (16). A solution of compound **7** (0.091 g, 0.22 mmol) in a sodium hydroxide 10% water solution (2 mL) and in ethanol (2 mL) was stirred at room temperature for 18 hours. Then the solution was neutralized with hydrochloric acid 1N and the suspension was stirred for 2 hours at room temperature. The solid obtained was collected by filtration, washed with water, and dried. Recrystallization from ethanol gave compound **16** (0.075 g, 86%), mp 187.0-190.0 °C (dec). IR (KBr, selected lines) cm^{-1} 2935, 2819, 1662, 1593, 1500, 1237, 1209, 1138, 919, 756. ^1H NMR ($CDCl_3$) δ 1.56-1.80 (m, 2H, CH_2), 1.80-1.98 (m, 2H, CH_2), 2.39 (s, 3H, CH_3), 2.43-2.56 (m, 2H + 3H, CH_2N + CH_3), 2.56-2.68 (m, 4H, piperazine), 2.77 (t, *J* = 7.4 Hz, 2H, CCH_2), 3.18-3.30 (m, 4H, piperazine), 6.80-7.00 (m, 3H, aromatic), 7.20-7.35 (m, 2H, aromatic), 11.88 (br s, 1H, NH). Anal. ($C_{22}H_{28}N_4OS$), C, H, N, S.

5,6-Dimethyl-2-[4-[4-(phenylmethyl)-1-piperazinyl]butyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (17). The title compound was prepared from derivative **8** following the same procedure for the preparation of **16**. From the neutralized solution was obtained a

solid, collected by filtration, washed with water and dried. Recrystallization from ethanol gave **17** (59%), mp 132.0-134.0 °C. IR (KBr, selected lines) cm^{-1} 3446, 2938, 2811, 1659, 1591, 1444, 1319, 1209, 1157, 925. ^1H NMR (DMSO-d_6) δ 1.32-1.50 (m, 2H, CH_2), 1.55-1.75 (m, 2H, CH_2), 2.15-2.40 (m, 2H + 8H + 3H + 3H, CH_2N + piperazine + CH_3 + CH_3), 2.56 (t, J = 7.4 Hz, 2H, CCH_2), 3.42 (s, 2H, $CH_2C_6H_5$), 7.18-7.39 (m, 5H, aromatic), 12.16 (br s, 1H, NH). Anal. ($C_{23}H_{30}N_4OS$), C, H, N, S.

5,6-Dimethyl-2-[4-[4-(phenylmethyl)-1-piperidyl]butyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (18). The title compound was prepared from derivative **9** following the same procedure for the preparation of **16**. The neutralized solution was stirred another 30 min at room temperature to obtain a solid, collected by filtration, washed with water, and dried. Recrystallization from ethanol gave **18** (87%), mp 126.0-129.0 °C. IR (KBr, selected lines) cm^{-1} 3448, 2938, 2874, 2814, 2769, 1772, 1659, 1592, 1559, 1506, 1442. ^1H NMR (DMSO-d_6) δ 1.06-1.32 (m, 2H, piperidine), 1.36-1.61 (m, 2H + 2H + 1H, CH_2 + piperidine), 1.61-1.86 (m, 2H + 2H, CH_2 + piperidine), 2.25 (t, J = 6.7 Hz, 2H, CH_2N), 2.37 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 2.46-2.58 (m, 2H, piperidine), 2.61 (t, J = 7.2 Hz, 2H, CCH_2), 2.81 (d, J = 11.2 Hz, 2H, $CH_2C_6H_5$), 7.10-7.39 (m, 5H, aromatic), 12.21 (br s, 1H, NH). Anal. ($C_{24}H_{31}N_3OS$) C, H, N, S.

5,6-Dimethyl-2-[5-(4-phenyl-1-piperazinyl)pentyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (19). The title compound was prepared from derivative **10** following the same procedure for the preparation of **16**. The neutralized solution was stirred another 10 min at room temperature to obtain a solid, collected by filtration, washed with water, and dried. Recrystallization from ethanol gave **19** (47%), mp 154.0-157.0 °C. IR (KBr, selected lines) cm^{-1} 3443, 2937, 2819, 1665, 1596, 1500, 1454, 1381, 1315, 1234, 1208, 1140. ^1H NMR (DMSO-d_6) δ 1.22-1.56 (m, 2H + 2H, CH_2CH_2), 1.58-1.80 (m, 2H, CH_2), 2.29 (t, J = 7.4 Hz, 2H, CH_2N), 2.31 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.40-2.53 (m, 4H, piperazine), 2.58 (t, J = 7.4 Hz, 2H, CCH_2), 3.00-3.14 (m, 4H, piperazine), 6.74-6.80 (m, 1H, aromatic), 6.80-6.98 (m, 2H, aromatic), 7.17-7.24 (m, 2H, aromatic), 12.11 (br s, 1H, NH). Anal. ($C_{23}H_{30}N_4OS$) C, H, N, S.

5,6-Dimethyl-2-[5-[4-(phenylmethyl)-1-piperazinyl]pentyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (20). The title compound was prepared from derivative **11** following the same procedure for the preparation of **16**. The neutralized solution was stirred another 5 hours at room temperature to obtain a solid, collected by filtration, washed with water, and dried. Recrystallization from ethanol gave **20** (38%), mp 120.0-122.0 °C (dec). IR (KBr, selected lines) cm^{-1} 3445, 2934, 2856, 2810, 1669, 1593, 1499, 1457, 1318, 1207, 1154. ^1H NMR (DMSO-d_6) δ

1.20-1.50 (m, 2H + 2H, CH_2CH_2), 1.54-1.76 (m, 2H, CH_2), 2.21 (t, J = 7.0 Hz, 2H, CH_2N), 2.26-2.34 (m, 3H + 8H, CH_3 + piperazine), 2.35 (s, 3H, CH_3), 2.55 (t, J = 7.2 Hz, 2H, CCH_2), 3.40 (s, 2H, $CH_2C_6H_5$), 7.20-7.38 (m, 5H, aromatic), 12.15 (br s, 1H, NH). Anal. ($C_{24}H_{32}N_4OS$) C, H, N, S.

5,6-Dimethyl-2-[5-[4-(phenylmethyl)-1-piperidyl]pentyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (21). A mixture of bromo derivative **4** (0.51 g, 1.47 mmol) and 4-(phenylmethyl)piperidine (0.47 mL, 2.70 mmol) was heated at 100 °C under stirring for 2 hours. Successively, ethanol (20 mL) was added to the mixture, which was refluxed under stirring for 4 hours. After being cooled, the solvent was removed under reduced pressure and ethyl acetate was added. The organic phase was washed with water (20 mL × 2), dried over anhydrous sodium sulfate, and concentrated to dryness. The crude product was purified by flash chromatography using a mixture of ethyl acetate/methanol (5:5, v/v) as eluent, obtaining compound **12** (0.12 g, 19%), which was used in the next step without further purification. A solution of compound **12** (0.091 g, 0.22 mmol) in a sodium hydroxide 10% water solution (2 mL) and in ethanol (2 mL) was stirred at room temperature for 20 hours. Then the solution was neutralized with hydrochloric acid 1N. The solid obtained was collected by filtration, washed with water, and dried. Recrystallization from ethanol gave compound **21** as a pure solid (0.022 g, 23%), mp 134.0-136.0 °C (dec). IR (KBr, selected lines) cm^{-1} 3443, 2927, 1670, 1596, 1450, 1319, 1207, 918, 747, 700, 629. ^{1}H NMR (DMSO-d_6) δ 1.00-1.58 (m, 2H + 2H + 2H + 2H + 1H, CH_2CH_2 + piperidine), 1.58-1.82 (m, 2H + 2H, CH_2 + piperidine), 2.17 (t, J = 7.0 Hz, 2H, CH_2N), 2.32 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 2.40-2.55 (m, 2H, piperidine), 2.55 (t, J = 7.4 Hz, 2H, CCH_2), 2.76 (d, J = 11.0 Hz, 2H, $CH_2C_6H_5$), 7.13-7.32 (m, 5H, aromatic), 12.17 (br s, 1H, NH). Anal. ($C_{25}H_{33}N_3OS$) C, H, N, S.

2-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentyl]-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (22). A mixture of bromo derivative **4** (0.40 g, 1.15 mmol), 1-(3-chlorophenyl)piperazine hydrochloride (0.56 g, 2.40 mmol) and potassium carbonate (0.90 g, 6.51 mmol) in ethanol (10 mL) was refluxed under stirring for 9 hours. After being cooled, water was added to the mixture. After 24 hours, the obtained solid was collected by filtration, washed with water, and dried. The crude product was purified by flash chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluent, obtaining compound **22** as a pure solid (0.30 g, 62%), mp 163.0-165.0 °C. IR (KBr, selected lines) cm^{-1} 3854, 3447, 2925, 2827, 1665, 1593, 1488, 1446, 1246, 946. ^{1}H NMR (DMSO-d_6) δ 1.21-1.57 (m, 2H + 2H, CH_2CH_2), 1.57-1.78 (m, 2H, CH_2), 2.28 (t, J = 7.4 Hz, 2H, CH_2N), 2.32 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.39-2.48 (m, 4H, piperazine), 2.58 (t, J = 7.4 Hz, 2H, CCH_2), 3.04-3.18 (m, 4H, piperazine), 6.78-6.98 (m, 3H, aromatic), 7.18-7.28 (m, 1H, aromatic),

12.23 (br s, 1H, NH). Anal. ($C_{23}H_{29}ClN_4OS$) C, H, N, S.

2-[5-[4-(4-Chlorophenyl)-1-piperazinyl]pentyl]-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (23). The title compound was obtained from bromo derivative **4** and 1-(4-chlorophenyl)piperazine dihydrochloride following the same procedure for the preparation of **22**. The crude product was purified by flash chromatography using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (9:1, v/v) as eluents, obtaining compound **23** as a pure solid (84%), mp 190.0-192.0 °C. IR (KBr, selected lines) cm^{-1} 3158, 3097, 2930, 2853, 1668 (broad), 1591, 4096, 1381, 1315, 1207, 1039, 918. ^1H NMR (DMSO-d_6) δ 1.20-1.56 (m, 2H + 2H, CH_2CH_2), 1.56-1.80 (m, 2H, CH_2), 2.26 (t, J = 7.2 Hz, 2H, CH_2N), 2.32 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.38-2.48 (m, 4H, piperazine), 2.58 (t, J = 7.2 Hz, 2H, CCH_2), 2.98-3.15 (m, 4H, piperazine), 6.91 (d, J = 9.0 Hz, 2H, aromatic), 7.21 (d, J = 9.0 Hz, 2H, aromatic), 12.22 (br s, 1H, NH). Anal. ($C_{23}H_{29}ClN_4OS$) C, H, N, S.

2-[4-(4-Phenyl-1-piperazinyl)butyl]-4(3*H*)-quinazolinone (24). A solution of derivative **13** (0.15 g, 0.39 mmol) in a sodium hydroxyde 10% water solution (3 mL) and in ethanol (3 mL) was heated at 100 °C and stirred for 6 hours. After being cooled, the solid was removed by filtration. Then, the solution was acidified with hydrochloric acid 1N and, successively, a solution of sodium hydrogen carbonate 5% was added. The obtained suspension was stirred for 3 hours at room temperature. Finally, the solid obtained was filtered off, washed with water, and dried. Recrystallization from ethanol gave compound **24** (0.11 g, 80%), mp 193.0-194.7 °C. IR (KBr, selected lines) cm^{-1} 3437, 2940, 2820, 1678, 1619, 1601, 1496, 1469, 1334, 1238, 1135, 1006, 769. ^1H NMR (DMSO-d_6) δ 1.42-1.60 (m, 2H, CH_2), 1.65-1.85 (m, 2H, CH_2), 2.35 (t, J = 6.8 Hz, 2H, CH_2N), 2.40-2.55 (m, 4H, piperazine), 2.63 (t, J = 7.4 Hz, 2H, CCH_2), 3.02-3.20 (m, 4H, piperazine), 6.70-6.82 (m, 1H, aromatic), 6.82-6.98 (m, 2H, aromatic), 7.10-7.25 (m, 2H, aromatic), 7.39-7.50 (m, 1H, aromatic), 7.59 (dd, J = 8.2 and 0.6 Hz, 1H, aromatic), 7.70-7.82 (m, 1H, aromatic), 8.08 (dd, J = 7.8 and 1.6 Hz, 1H, aromatic). Anal. ($C_{22}H_{26}N_4O$) C, H, N.

2-[4-[4-(3-Chlorophenyl)-1-piperazinyl]butyl]-4(3*H*)-quinazolinone (25). The title compound was obtained from derivative **14** following the same procedure for the preparation of **24**. Recrystallization from ethanol gave compound **25** as a pure solid (53%), mp 168.4 °C (dec). IR (KBr, selected lines) cm^{-1} 3421, 2933, 2819, 1656, 1613, 1593, 1469, 1241, 1151, 1136, 987, 945, 779. ^1H NMR (DMSO-d_6) δ 1.40-1.62 (m, 2H, CH_2), 1.62-1.85 (m, 2H, CH_2), 2.34 (t, J = 7.0 Hz, 2H, CH_2N), 2.38-2.49 (m, 4H, piperazine), 2.62 (t, J = 7.4 Hz, 2H, CCH_2), 3.08-3.20 (m, 4H, piperazine), 6.70-6.80 (m, 1H, aromatic), 6.80-6.95 (m, 2H, aromatic), 7.10-7.30 (m, 1H, aromatic), 7.38-7.50 (m, 1H, aromatic), 7.59 (d, J = 7.6

Hz, 1H, aromatic), 7.70-7.82 (m, 1H, aromatic), 8.07 (dd, *J* = 8.0 and 1.4 Hz, 1H, aromatic). Anal. ($C_{22}H_{25}ClN_4O$) C, H, N.

2-[4-[4-(4-Chlorophenyl)-1-piperazinyl]butyl]-4(3*H*)-quinazolinone (26). The title compound was obtained from derivative **15** following the same procedure for the preparation of **24**. Recrystallization from ethanol gave compound **26** as a pure solid (24%), mp 223.0-225.0 °C. IR (KBr, selected lines) cm^{-1} 2921, 2816, 1674, 1615, 1497, 1471, 1450, 1243, 1135, 819, 771. ^1H NMR (DMSO-d_6) δ 1.40-1.60 (m, 2H, CH_2), 1.62-1.85 (m, 2H, CH_2), 2.34 (t, *J* = 7.2 Hz, 2H, CH_2N), 2.45-2.56 (m, 4H, piperazine), 2.61 (t, *J* = 7.2 Hz, 2H, CCH_2), 3.00-3.18 (m, 4H, piperazine), 6.92 (d, *J* = 9.0 Hz, 2H, aromatic), 7.21 (d, *J* = 9.0 Hz, 2H, aromatic), 7.38-7.50 (m, 1H, aromatic), 7.57 (d, *J* = 7.6 Hz, 1H, aromatic), 7.65-7.80 (m, 1H, aromatic), 8.06 (dd, *J* = 8.0 and 1.4 Hz, 1H, aromatic). Anal. ($C_{22}H_{25}ClN_4O$) C, H, N.

2-[5-(4-Phenyl-1-piperazinyl)pentyl]-4(3*H*)-quinazolinone (27). To a solution of bromo derivative **6** (1.56 g, 4.98 mmol) in ethanol (20 mL) were added 1-phenylpiperazine (1.47 mL, 9.62 mmol), potassium carbonate (1.33 g, 9.62 mmol), and the mixture was refluxed and stirred for 2 hours. After being cooled, the solid was removed by filtration and the filtrate was concentrated to dryness. The residue was dissolved in dichloromethane and washed with water (20 mL × 2). The organic layer was collected, dried over anhydrous sodium sulfate, and concentrated to dryness. The crude product was purified by flash chromatography using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (9:1, v/v) as eluents, obtaining compound **27** as a pure solid (0.19 g, 10%), mp 185.6-187.8 °C. IR (KBr, selected lines) cm^{-1} 2927, 2826, 1675, 1615, 1503, 1469, 1238, 1151, 1134, 770, 758, 688. ^1H NMR (DMSO-d_6) δ 1.23-1.60 (m, 2H + 2H, CH_2CH_2), 1.66-1.86 (m, 2H, CH_2), 2.29 (t, *J* = 6.8 Hz, 2H, CH_2N), 2.40-2.54 (m, 4H, piperazine), 2.61 (t, *J* = 7.4 Hz, 2H, CCH_2), 3.00-3.16 (m, 4H, piperazine), 6.71-6.82 (m, 1H, aromatic), 6.84-6.98 (m, 2H, aromatic), 7.12-7.26 (m, 2H, aromatic), 7.40-7.52 (m, 1H, aromatic), 7.60 (d, *J* = 7.8 Hz, 1H, aromatic), 7.72-7.84 (m, 1H, aromatic), 8.08 (dd, *J* = 8.0 and 1.2 Hz, 1H, aromatic), 12.19 (br s, 1H, NH). Anal. ($C_{23}H_{28}N_4O$) C, H, N.

2-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentyl]-4(3*H*)-quinazolinone (28). The title compound was obtained from bromo derivative **6** following the same procedure for the preparation of **27** by using 8 hours of reflux. After being cooled, water was added to the mixture and the solid obtained was collected by filtration, washed with water, and dried. Recrystallization from ethanol gave compound **28** (12%), mp 142.0-142.5 °C. IR (KBr, selected lines) cm^{-1} 2927, 2821, 1675, 1618, 1598, 1563, 1470, 1239, 1149, 948, 769. ^1H NMR (DMSO-d_6) δ 1.22-1.59

(m, 2H + 2H, CH_2CH_2), 1.60-1.85 (m, 2H, CH_2), 2.29 (t, *J* = 7.0 Hz, 2H, CH_2N), 2.38-2.45 (m, 4H, piperazine), 2.61 (t, *J* = 7.4 Hz, 2H, CCH_2), 3.00-3.18 (m, 4H, piperazine), 6.72-6.94 (m, 3H, aromatic), 7.12-7.25 (m, 1H, aromatic), 7.40-7.50 (m, 1H, aromatic), 7.60 (d, *J* = 8.0 Hz, 1H, aromatic), 7.70-7.83 (m, 1H, aromatic), 8.08 (dd, *J* = 8.0 and 1.4 Hz, 1H, aromatic), 12.20 (br s, 1H, NH). Anal. ($C_{23}H_{27}ClN_4O$) C, H, N.

2-[5-[4-(4-Clorophenyl)-1-piperazinyl]pentyl]-3*H*-quinazolinone-4-one (29). To a solution of bromo derivative **6** (0.78 g, 2.49 mmol) in ethanol (20 mL) 1-(4-chlorophenyl)piperazine dihydrochloride (1.34 g, 4.97 mmol) and potassium carbonate (2.06 g, 14.9 mmol) were added. The suspension was refluxed under stirring for 8 hours. After being cooled, the suspension was diluted with water and the obtained solid was collected by filtration. The solid was resuspended in water, the suspension was acidified with HCl 1N and then was added a solution of sodium hydrogen carbonate 5% to pH 8.5. Successively, the solid was collected by filtration and dried. The crude product was purified by flash chromatography using a mixture of methanol/ethyl acetate (5:5, v/v) as eluent, obtaining compound **29** as a pure solid (0.15 g, 15%), mp 187.0 °C (dec). IR (KBr, selected lines) cm^{-1} 2931, 2838, 1689, 1612, 1498, 1469, 1244, 1139, 997, 818, 769. ^1H NMR (DMSO-d_6) δ 1.20-1.60 (m, 2H + 2H, CH_2CH_2), 1.62-1.82 (m, 2H, CH_2), 2.30 (t, *J* = 7.0 Hz, 2H, CH_2N), 2.40-2.51 (m, 4H, piperazine), 2.61 (t, *J* = 7.2 Hz, 2H, CCH_2), 3.00-3.18 (m, 4H, piperazine), 6.91 (d, *J* = 9.0 Hz, 2H, aromatic), 7.21 (d, *J* = 8.8 Hz, 2H, aromatic), 7.40-7.50 (m, 1H, aromatic), 7.59 (d, *J* = 7.6 Hz, 1H, aromatic), 7.65-7.82 (m, 1H, aromatic), 8.07 (dd, *J* = 7.6 and 1.2 Hz, 1H, aromatic), 12.18 (s, 1H, NH). Anal. ($C_{23}H_{27}ClN_4O$) C, H, N.

3-(4-Chlorobutyl)-4(3*H*)-quinazolinone (32). To a suspension of quinazolinone **30** (0.21 g, 1.44 mmol) in acetonitrile (3 mL) were added 1-bromo-4-chlorobutane (0.33 mL, 2.88 mmol), potassium carbonate (0.30 g, 2.17 mmol), and a catalytic amount of potassium iodide. The mixture and a magnetic bar was sealed in a Pyrex tube and was heated at 90 °C by microwave irradiation for 40 min (run time 2 min, microwave max power 150 W and max pressure 150 Psi). After being cooled, the solid was removed by filtration and the solution was concentrated to dryness. The crude product was purified by flash chromatography using a mixture of cyclohexane/ethyl acetate/NH_4OH (5:5:0.05, v/v/v) as eluent, obtaining compound **32** as a pure solid (0.17 g, 50%), mp 78.7-79.7 °C. IR (KBr, selected lines) cm^{-1} 1658, 1613, 1473, 1372, 1325, 771, 697. ^1H NMR (DMSO-d_6) δ 1.65-1.98 (m, 2H + 2H, CH_2CH_2), 3.68 (t, *J* = 6.2 Hz, 2H, CH_2Cl), 4.02 (t, *J* = 6.6 Hz, 2H, $CONCH_2$), 7.50-7.61 (m, 1H, aromatic), 7.68 (dd, *J* = 8.2 and 0.6 Hz, 1H, aromatic), 7.78-7.90 (m, 1H, aromatic), 0 Hz, 1H, aromatic), 8.42 (s, 1H, NCH). Anal. ($C_{12}H_{13}ClN_2O$) C, H, N.

3-(5-Bromopentyl)-4(3*H*)-quinazolinone (33). The title compound was prepared from quinazolinone **30** (1.37 mmol) in acetonitrile (3 mL), 1,5-dibromopentane (1.91 mmol), potassium carbonate (2.10 mmol), and a catalytic amount of potassium iodide following the same procedure for the preparation of **32**. The crude product was purified by flash chromatography using ethyl acetate 100% as eluent, obtaining compound **33** as a pure solid (26%), mp 78.5-79.4 °C. IR (KBr, selected lines) cm^{-1} 1657, 1613, 1471, 1367, 1231, 768, 696. ^1H NMR (DMSO-d_6) δ 1.30-1.58 (m, 2H, CH_2), 1.62-1.98 (m, 2H + 2H, CH_2CH_2), 3.53 (t, J = 6.6 Hz, 2H, CH_2Br), 3.98 (t, J = 7.2 Hz, 2H, $CONCH_2$), 7.45-7.60 (m, 1H, aromatic), 7.67 (d, J = 7.4 Hz, 1H, aromatic), 7.72-7.90 (m, 1H, aromatic), 8.16 (dd, J = 8.0 and 1.0 Hz, 1H, aromatic), 8.41 (s, 1H, NCH). Anal. ($C_{13}H_{15}BrN_2O$) C, H, N.

3-(5-Bromopentyl)-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (34). The title compound was prepared from thienopyrimidinone **31** (1.32 mmol) in acetonitrile (3 mL), 1,5-dibromopentane (2.20 mmol), potassium carbonate (1.98 mmol), and a catalytic amount of potassium iodide following the same procedure for the preparation of **32**. The crude product was purified by flash chromatography using a mixture of cyclohexane/ethyl acetate (7:3, v/v) as eluent, obtaining compound **34** as a pure solid (12%), mp 105.5-108.5 C°. IR (KBr, selected lines) cm^{-1} 2916, 2863, 1654, 1559, 1394, 784, 647. ^1H NMR (DMSO-d_6) δ 1.28-1.55 (m, 2H, CH_2), 1.55-1.93 (m, 2H + 2H, CH_2CH_2), 2.35 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 3.53 (t, J = 6.6 Hz, 2H, CH_2Br), 3.94 (t, J = 7.2 Hz, 2H, $CONCH_2$), 8.34 (s, 1H, NCH). Anal. ($C_{13}H_{17}BrN_2OS$) C, H, N, S.

3-[4-[4-(3-Chlorophenyl)-1-piperazinyl]butyl]-4(3*H*)-quinazolinone (35). A mixture of bromo derivative **32** (0.20 g, 0.85 mmol) and 1-(3-chlorophenyl)piperazine (0.84 g, 4.26 mmol) was heated at 110 °C for 2 hours. The sticky product was dissolved with ethyl acetate and the organic phase was washed with water (20 × 2), dried over anhydrous sodium sulfate, and concentrated to dryness. The crude product was purified by flash chromatography using ethyl acetate/methanol (7:3, v/v) as eluents, obtaining compound **35** as a pure solid (0.042 g, 15%), mp 87.3-89.4 C°. IR (KBr, selected lines) cm^{-1} 1661, 1613, 1598, 1472, 1366, 1243, 771. ^1H NMR ($CDCl_3$) δ 1.52-1.72 (m, 2H, CH_2), 1.73-1.96 (m, 2H, CH_2), 2.44 (t, J = 7.2 Hz, 2H, CH_2N), 2.52-2.64 (m, 4H, piperazine), 3.12-3.25 (m, 4H, piperazine), 4.05 (t, J = 7.0 Hz, 2H, $CONCH_2$), 6.72-6.90 (m, 3H, aromatic), 7.08-7.21 (m, 1H, aromatic), 7.42-7.69 (m, 1H, aromatic), 7.65-7.92 (m, 2H, aromatic), 8.05 (s, 1H, NCH), 8.25-8.38 (m, 1H, aromatic). Anal. ($C_{22}H_{25}ClN_4O$) C, H, N.

3-[5-[4-Phenyl-1-piperazinyl]pentyl]-4(3*H*)-quinazolinone (36). To a solution of bromo derivative **33** (0.29 g, 0.98 mmol) in ethanol (10 mL),

were added 1-phenylpiperazine (0.22 mL, 1.44 mmol), potassium carbonate (0.27 g, 1.95 mmol), and a catalytic amount of potassium iodide. The mixture was refluxed under stirring for 7 hours. After being cooled, the solid was removed by filtration and the filtrate concentrated to dryness. The crude product was purified by flash chromatography using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (5:5, v/v) as eluents, obtaining compound **36** as a pure solid (0.037 g, 10%), mp 85.3-87.2 C°. IR (KBr, selected lines) cm^{-1} 1684, 1662, 1609, 1472, 1376, 1236, 778. ^{1}H NMR (DMSO-d_6) δ 1.20-1.60 (m, 2H + 2H, CH_2CH_2), 1.60-1.85 (m, 2H, CH_2), 2.30 (t, J = 7.0 Hz, 2H, CH_2N), 2.35-2.55 (m, 4H, piperazine), 3.98-3.12 (m, 4H, piperazine), 3.98 (t, J = 7.2 Hz, 2H, $CONCH_2$), 6.70-6.80 (m, 1H, aromatic), 6.82-6.94 (m, 2H, aromatic), 7.14-7.25 (m, 2H, aromatic), 7.48-7.60 (m, 1H, aromatic), 7.66 (d, J = 7.6 Hz, 1H, aromatic), 7.75-7.88 (m, 1H, aromatic), 8.16 (dd, J = 8.2 and 1.2 Hz, 1H, aromatic), 8.41 (s, 1H, NCH). Anal. ($C_{23}H_{28}N_4O$) C, H, N.

3-[5-[4-(Phenylmethyl)-1-piperazinyl]pentyl]-4(3*H*)-quinazolinone (37). The title compound was obtained from bromo derivative **33** (1.78 mmol) and 1-(phenylmethyl)piperazine (1.78 mmol) following the same procedure for the preparation of **36**. The crude product was purified by flash chromatography using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (7:3, v/v) as eluents, obtaining compound **37** as a pure solid (22%), mp 67.2-70.0 °C. IR (KBr, selected lines) cm^{-1} 2808, 1663, 1613, 1471, 1366, 778, 699. ^{1}H NMR (DMSO-d_6) δ 1.18-1.50 (m, 2H + 2H, CH_2CH_2), 1.60-1.80 (m, 2H, CH_2), 2.20 (t, J = 7.4 Hz, 2H, CH_2N), 2.25-2.45 (m, 8H, piperazine), 3.39 (s, 2H, $CH_2C_6H_5$), 3.96 (t, J = 6.8 Hz, 2H, $CONCH_2$), 7.20-7.39 (m, 5H, aromatic), 7.50-7.60 (m, 1H, aromatic), 7.67 (d, J = 7.4 Hz, 1H, aromatic), 7.78-7.90 (m, 1H, aromatic), 8.15 (dd, J = 8.0 and 1.2 Hz, 1H, aromatic), 8.39 (s, 1H, NCH). Anal. ($C_{24}H_{30}N_4O$) C, H, N.

3-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentyl]-4(3*H*)-quinazolinone (38). The title compound was obtained from bromo derivative **33** (0.47 mmol) and 1-(3-chlorophenyl)piperazine (0.56 mmol) following the same procedure for the preparation of **36**. The crude product was purified by flash chromatography using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (7:3, v/v) as eluents, obtaining compound **38** as a pure solid (26%), mp 177.0 °C (dec). IR (KBr, selected lines) cm^{-1} 2924, 1670, 1608, 1474, 776. ^{1}H NMR ($CDCl_3$) δ 1.38-1.79 (m, 2H + 2H, CH_2CH_2), 1.79-2.00 (m, 2H, CH_2), 2.41 (t, J = 7.0 Hz, 2H, CH_2N), 2.58-2.79 (m, 4H, piperazine), 3.10-3.28 (m, 4H, piperazine), 4.02 (t, J = 7.2 Hz, 2H, $CONCH_2$), 6.70-6.98 (m, 3H, aromatic), 7.08-7.25 (m, 1H, aromatic), 7.45-7.65 (m, 1H, aromatic), 7.65-7.90 (m, 2H, aromatic), 8.04 (s, 1H, NCH), 8.25-8.45 (m, 1H, aromatic). Anal. ($C_{23}H_{27}ClN_4O$) C, H, N.

3-[5-[4-(4-Chlorophenyl)-1-piperazinyl]pentyl]-4(3*H*)-quinazolinone (39). The title compound was prepared from bromo derivative **33** (1.35 mmol) and 1-(4-chlorophenyl)piperazine (1.62 mmol) following the same procedure for the preparation of **36**. The crude product was purified by flash chromatography using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent, obtaining compound **39** as a pure solid (10%), mp 153.4-154.9 °C. IR (KBr, selected lines) cm^{-1} 2929, 1661, 1613, 1498, 1471, 1239, 813, 773, 698. ^{1}H NMR ($CDCl_3$) δ 1.30-1.70 (m, 2H + 2H, CH_2CH_2), 1.70-1.95 (m, 2H, CH_2), 2.39 (t, *J* = 7.2 Hz, 2H, CH_2N), 2.50-2.64 (m, 4H, piperazine), 3.10-3.22 (m, 4H, piperazine), 4.02 (t, *J* = 7.4 Hz, 2H, $CONCH_2$), 6.82 (d, *J* = 8.8 Hz, 2H, aromatic), 7.19 (d, *J* = 7.6 Hz, 2H, aromatic), 7.45-7.58 (m, 1H, aromatic), 7.68-7.82 (m, 2H, aromatic), 8.03 (s, 1H, NCH), 8.12-8.18 (m, 1H, aromatic). Anal. ($C_{23}H_{27}ClN_4O$) C, H, N.

3-[5-[4-(2-Ethoxyphenyl)-1-piperazinyl]pentyl]-4(3*H*)-quinazolinone (40). The title compound was prepared from bromo derivative **33** (0.71 mmol) and 1-(2-ethoxyphenyl)piperazine (0.85 mmol) following the same procedure for the preparation of **36**. The crude product was purified by flash chromatography using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent, obtaining compound **40** as a pure solid (10%), mp 91.3-93.2 °C. IR (KBr, selected lines) cm^{-1} 2939, 1664, 1612, 1502, 1475, 1239, 1123, 765, 754. ^{1}H NMR ($CDCl_3$) δ 1.35-1.53 (m, 2H +3H, CH_2 + CH_2CH_3), 1.53-1.71 (m, 2H, CH_2), 1.76-1.94 (m, 2H, CH_2), 2.41 (t, *J* = 7.2 Hz, 2H, CH_2N), 2.56-2.72 (m, 4H, piperazine), 3.04-3.20 (m, 4H, piperazine), 3.96-4.14 (m, 2H + 2H, $CONCH_2$ + CH_2CH_3), 6.80-6.72 (m, 4H, aromatic), 7.45-7.58 (m, 1H, aromatic), 7.65-7.84 (m, 2H, aromatic), 8.04 (s, 1H, NCH), 8.28-8.40 (m, 1H, aromatic). Anal. ($C_{25}H_{32}N_4O_2$) C, H, N.

5,6-Dimethyl-3-[5-[4-phenyl-1-piperazinyl]pentyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (41). The title compound was prepared from bromo derivative **34** and 1-phenylpiperazine following the same procedure for the preparation of **36**. The crude product was purified by filtration on a plug of silica gel using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (5:5, v/v) as eluents, obtaining compound **41** as a pure solid (36%), mp 163.8 °C (dec). IR (KBr, selected lines) cm^{-1} 2945, 1661, 1601, 1568, 1393, 1242, 761, 692. ^{1}H NMR (DMSO-d_6) δ 1.18-1.40 (m, 2H, CH_2), 1.40-1.80 (m, 2H + 2H, CH_2CH_2), 2.34 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 2.40-2.71 (m, 4H + 2H, piperazine + CH_2N), 3.10-3.18 (m, 4H, piperazine), 3.95 (t, *J* = 7.0 Hz, 2H, $CONCH_2$), 6.67-6.85 (m, 1H, aromatic), 6.85-6.98 (m, 2H, aromatic), 7.10-7.30 (m, 2H, aromatic), 8.34 (s, 1H, NCH). Anal. ($C_{23}H_{30}N_4OS$) C, H, N, S.

5,6-Dimethyl-3-[5-[4-(phenylmethyl)-1-piperazinyl]pentyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (42). The title compound was prepared from bromo derivative **34** and 1-(phenylmethyl)piperazine, following the same procedure for the preparation of **36**. The crude product was purified by filtration on a plug of silica gel using a mixture of ethyl acetate/methanol (5:5, v/v) as eluent, obtaining compound **42** as a pure solid (31%), mp 91.7-93.8 °C. IR (KBr, selected lines) cm^{-1} 2937, 2807, 1653, 1558, 1393, 1279, 1160, 738, 698. ^{1}H NMR (DMSO-d_6) δ 1.16-1.50 (m, 2H + 2H, CH_2CH_2), 1.56-1.76 (m, 2H, CH_2), 2.20 (t, J = 7.4 Hz, 2H, CH_2N), 2.24-2.28 (m, 8H, piperazine), 2.35 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 3.39 (s, 2H, $CH_2C_6H_5$), 3.92 (t, J = 7.0 Hz, 2H, $CONCH_2$), 7.18-7.38 (m, 5H, aromatic), 8.32 (s, 1H, NCH). Anal. ($C_{24}H_{32}N_4OS$) C, H, N, S.

3-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentyl]-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (43). The title compound was prepared from bromo derivative **34** and 1-(3-chlorophenyl)piperazine following the same procedure for the preparation of **36**. The crude product was purified by filtration on a plug of silica gel using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (7:3, v/v) as eluents, obtaining compound **43** as a pure solid (33%), mp 117.6-119.8 °C. IR (KBr, selected lines) cm^{-1} 2943, 2818, 1660, 1591, 1562, 1480, 1445, 1238, 759, 692. ^{1}H NMR (DMSO-d_6) δ 1.18-1.58 (m, 2H + 2H, CH_2CH_2), 1.58-1.80 (m, 2H, CH_2), 2.27 (t, J = 6.6 Hz, 2H, CH_2N), 2.33 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 2.38-2.44 (m, 4H, piperazine), 3.00-3.18 (m, 4H, piperazine), 3.94 (t, J = 7.0 Hz, 2H, $CONCH_2$), 6.70-6.90 (m, 3H, aromatic), 7.10-7.23 (m, 1H, aromatic), 8.34 (s, 1H, NCH). Anal. ($C_{23}H_{29}ClN_4OS$) C, H, N, S.

3-[5-[4-(4-Chlorophenyl)-1-piperazinyl]pentyl]-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (44). The title compound was prepared from bromo derivative **34** and 1-phenylpiperazine following the same procedure for the preparation of **36**. The crude product was purified by filtration on a plug of silica gel using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent, obtaining compound **44** (19%), mp 135.5-136.4 °C. IR (KBr, selected lines) cm^{-1} 2939, 2819, 1659, 1564, 1495, 1237, 1093, 818. ^{1}H NMR ($CDCl_3$) δ 1.30-1.70 (m, 2H + 2H, CH_2CH_2), 1.70-1.90 (m, 2H, CH_2), 2.32-2.44 (m, 3H + 2H, CH_3 + CH_2N), 2.49 (s, 3H, CH_3), 2.52-2.62 (m, 4H, piperazine), 3.08-3.20 (m, 4H, piperazine), 3.97 (t, J = 7.2 Hz, 2H, $CONCH_2$), 6.82 (d, J = 8.8 Hz, 2H, aromatic), 6.82 (d, J = 9.0 Hz, 2H, aromatic), 7.89 (s, 1H, NCH). Anal. ($C_{23}H_{29}ClN_4OS$) C, H, N, S.

5,6-Dimethyl-3-[5-[4-(2-ethoxyphenyl)-1-piperazinyl]pentyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (45). The title compound was prepared from bromo derivative **34** and 1-(2-ethoxyphenyl)piperazine following the same procedure for the preparation of **36**. The crude product was purified on flash chromatography using first ethyl acetate 100% and then a mixture of ethyl acetate/methanol (7:3, v/v) as eluents, obtaining compound **45** as a pure solid (31%), mp 97.2-99.4 °C. IR (KBr, selected lines) cm^{-1} 2939, 2816, 1660, 1572, 1501, 1245, 1123, 742. ^{1}H NMR ($CDCl_3$) δ 1.36-1.52 (m, 2H +3H, CH_2 + CH_2CH_3), 1.52-1.72 (m, 2H, CH_2), 1.72-1.92 (m, 2H, CH_2), 2.30-2.47 (m, 3H + 2H, CH_3 + CH_2N), 2.49 (s, 3H, CH_3), 2.60-2.75 (m, 4H, piperazine), 3.06-3.22 (m, 4H, piperazine), 3.98 (t, J = 7.2 Hz, 2H, $CONCH_2$), 4.06 (q, J = 7.0 Hz, 2H, CH_2CH_3), 6.80-6.72 (m, 4H, aromatic), 7.90 (s, 1H, NCH). Anal. ($C_{25}H_{34}N_4O_2S$) C, H, N, S.

3.2.2. In vitro binding assays

Binding assays were done using human cloned 5-$HT_{7(a)}$ and 5-HT_{1A} serotonin receptors (PerkinElmer) expressed on CHO-K1 cells. Radioligand binding assay on 5-HT_7 receptors[3] was carried out in a final incubation volume of 0.51 mL consisting of 250 µL of membrane suspension (15 µg protein/sample in Tris HCl, 50 mM, pH 7.4 containing 10 µM pargiline, 4 mM $MgCl_2$ and 0.05% ascorbic acid,), 250 µL of [^{3}H]-5-HT (final concentration 5 nM, s.a. 106 Ci/mmol, PerkinElmer) in the same buffer used for membrane suspension and 10 µL of tested compounds. Nonspecific binding was obtained in the presence of 10 µM serotonin. Binding assay on 5-HT_{1A} receptors[4] was carried out in a final incubation volume of 0.51 mL consisting of 250 µL of membrane suspension (10 µg protein/sample in Tris HCl, 50 mM, pH 7.4 containing 10 µM pargiline and 4 mM $MgCl_2$), 250 µL of [^{3}H]-8-OH-DPAT (final concentration 1 nM, s.a. 137 Ci/mmol, PerkinElmer) in the same buffer used for membrane suspension and 10 µL of tested compounds. Nonspecific binding was obtained in the presence of 1 µM serotonin. Incubations (30 min at 25 °C) were stopped by rapid filtration under vacuum, through GF/C filters (pre-soaked with 0.3% PEI) for 5-HT_7 receptors or GF/B filters for 5-HT_{1A} receptors, which were then washed with 12 mL (4 × 3 times) of ice-cold buffer (Tris HCl, 50 mM, pH 7.4) using a Brandel M-48R cell harvester. The radioactivity trapped on the filters was counted in 4 mL of Ultima Gold MV (Packard) in a Tri-carb 2800 TR (PerkinElmer) liquid scintillation spectrometer with a counting efficiency of 60%. All compounds were tested in a concentration range from 10^{-5} to 10^{-10} M in triplicate and dose-inhibition curves were analyzed by the "Allfit" program to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding.[5] The K_i values were derived from

IC_{50} values according to the method of Cheng and Prusoff.[6]

3.2.3. cAMP assay protocol

The level of adenylyl cyclase activity was measured using recombinant HEK293 cells stably expressing the human 5-$HT_{7(b)}$ receptor. Cells (prepared with the use of Lipofectamine 2000) were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were grown in Dulbeco's Modifier Eagle Medium containing 10% dialysed foetal bovine serum and 500 mg/mL G418 sulphate. For functional experiments, cells were subcultured in 25 cm diameter dishes, grown to 90% confluence, washed twice with prewarmed to 37 °C phosphate buffered saline (PBS) and were centrifuged for 5 min (160 × g). The supernatant was aspirated, the cell pellet was resuspended in stimulation buffer (1 × HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA). The cAMP level was measured using the LANCE cAMP detection kit (PerkinElmer), according to the manufacture's directions. For the investigation of antagonist effect on 5-HT_7R, the agonist, 5-carboxyamidotryptamine (5-CT; EC_{50} = 1 nM) was used in submaximal concentration (6.2 nM) to stimulate cAMP production and cells (450 per well) were incubated with compound (1 μM) for 30 min at room temperature in 384-well white opaque microtiter plate. After incubation, the reaction was stopped and cells were lysed by the addition of 10 μL working solution (5 μL Eu-cAMP and 5 μL ULight-anti-cAMP). The assay plate was incubated for 1 hour at room temperature. Time-resolved fluorescence resonance energy transfer (TR-FRET) was detected by an Infinite M1000 Pro (Tecan) using instrument settings from LANCE cAMP detection kit manual.

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4. Manuscript I

New *N*- and *O*-long-chain arylpiperazine derivatives as 5-HT_{1A} and 5-HT_7 receptor ligands: studies on quinazolin-4(3*H*)-one system

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Abstract

Based on our earlier works of structure-activity relationships and molecular modeling studies on long-chain arylpiperazine ligands, a series of new derivatives were synthesized. This paper reports a further investigation on the quinazolinone system with the purpose of thoroughly exploring if some structural modifications of this scaffold can influence the affinity for the 5-HT_7 and 5-HT_{1A} receptors. In the new derivatives, the quinazolinone was modified in a 6-phenylpyrimidine, which represents a novelty among the LCAP derivatives, and a 2-methylquinazoline systems. A 4-arylpiperazine moiety through a pentyl chain was anchored at the nitrogen or oxygen atom of the heterocyclic scaffolds. The substituents at the piperazine nucleus are phenyl, phenylmethyl, 3- or 4-chlorophenyl, and 2-ethoxyphenyl. Binding tests performed on human cloned 5-HT_7 and 5-HT_{1A} receptors showed that, among these derivatives, the 4-[5-[4-(2-ethoxyphenyl)-1-piperazinyl]pentoxy]-6-phenyl-pyrimidine (**13**) and the 3-[5-[4-(2-ethoxyphenyl)-1-piperazinyl]pentyl]-2-methyl-4(3*H*)-

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quinazolinone (**20**) displayed the best affinity values, with K_i = 23.5 and 8.42 nM for 5-HT_7 and 6.96 and 2.99 nM for 5-HT_{1A} receptors, respectively. Molecular modeling study has been done on 5-HT_7 and 5-HT_{1A} receptors to fully investigate the binding mode of the new and previous reported ligands. Docking studies revealed coherent binding mode for all compounds in both receptors. This observation is well matched with our previous model and confirmed that L-shape is more suitable than extended conformation for 5-HT_7R. In addition, it was outlined that a planar bicyclic system is preferable over a single heterocyclic ring with a bulky substituent that interacted with both receptors.

Keywords: 5-HT_7 receptor ligands, 5-HT_{1A} receptor ligands; long-chain arylpiperazines; *N*-alkylated derivatives, *O*-alkylated derivatives, molecular modeling.

4.1. Introduction

Two of the most frequently encountered heterocyclic scaffolds in medicinal chemistry are the quinazoline and quinazolinone, which have wide pharmacological applications such as antibacterial, antidiabetic, anti-inflammatory, and many others.[1] The quinazoline system has been found in many natural products, such as alkaloids,[2, 3] which showed medicinal applications.[4-6] Accordingly, a lot of comprehensive studies regarding the quinazolines/quinazolinones functionalization to synthesize new effective drugs have been done.[7-9] Furthermore, quinazoline derivatives were reported as CNS depressants[10] and anticonvulsants,[11] and this system is also present in an extensively studied class of serotoninergic ligands, called LCAPs.[12-16] The serotonin neurotransmitter interacts with a large number of receptors, classified into seven families (5-HT_{1-7}), following the IUPHAR classification.[17] With the exception of 5-HT_3, they are all GPCRs of family A. The 5-HT_7R was the last to be discovered in 1993, and it is positively coupled with adenylyl cyclase via a G_s protein.[18] This receptor is located in the brain and peripheral tissues. In particular, it is largely distributed in hippocampus and thalamus suggesting its involvement in CNS disorders such as schizophrenia, anxiety, and depression.[12, 18, 19]

Recently, we have reported new LCAPs bearing a quinazolinone system as a terminal fragment, which displayed high-to-low affinity for 5-HT_7R (K_i 6.88–1135 nM, Table 2) and high-to-moderate affinity for 5-HT_{1A}R (K_i 1.04–268 nM, Table 2).[20, 21] Here we present the synthesis of a novel series of LCAPs, to thoroughly research the quinazolinone as a terminal fragment, to explore how its modifications influence both the affinity for 5-HT_7R and 5-HT_{1A}R and binding modes at receptor homology models. Following the results from the previous investigation only

derivatives with pentyl linker were prepared and the same arylpiperazine fragments (*i.e.* phenyl, phenylmethyl, 3-chloro-, 4-chloro-, and 2-ethoxyphenyl) (Fig. 1) were used. The structural changes concerning the terminal part (benzo cracking,[22] methylation) and in shifting the anchoring point of the arylpiperazinylalkyl moiety to the nitrogen or oxygen atom. Due to alkylation reaction regioselectivity, *O*-alkylated pyrimidine and 2-methylquinazoline derivatives were more difficult to be obtained but they were isolated from the reaction mixture and used in preparation of the final compounds.

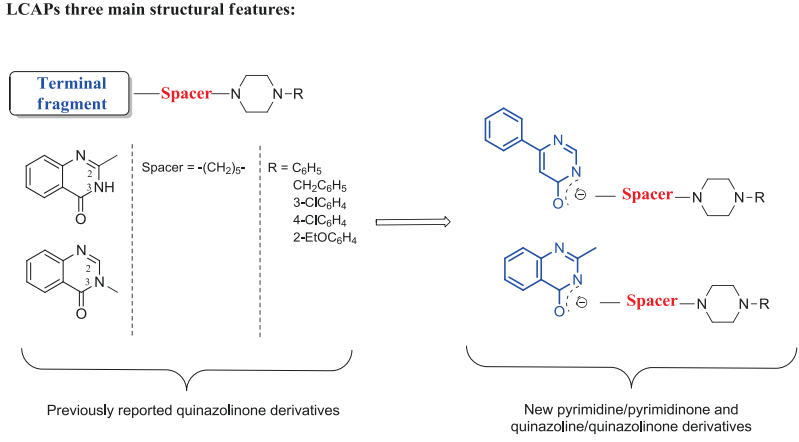


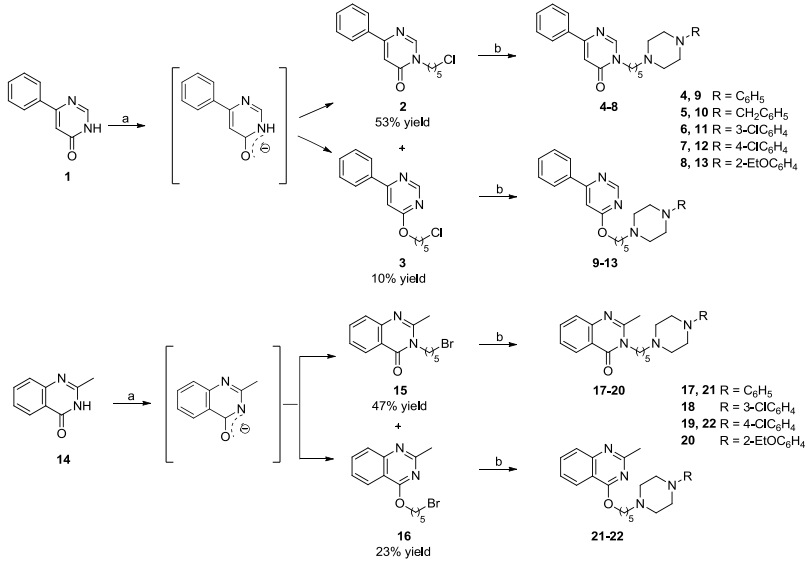
Figure 1. Structural features of previously and new synthesized LCAPs, (the new modifications on the terminal fragment are outlined in blue).

To the best of our knowledge, this is the first time that a 6-phenylpyrimidine (the result of splitting bicyclic quinazolinone system) is used as a scaffold for the preparation of 5-HT_{1A}R and 5-HT_7R ligands. No hits within sets of 4166 5-HT_{1A}R and 720 5-HT_7R ligands with K_i < 100 nM stored in ChEMBL v11 database[23] for 4-oxy-pyrimidine/pyrimidinone substractural query (using Instant JChem[24]) was found. On the other hand, both fragments represent versatile synthetic intermediates and several articles describing their synthesis and various biological activities continue to appear in literature.[25]

4.2. Results and discussion

4.2.1. Chemistry

The synthetic procedure adopted for the preparation of the new pyrimidine **4-13** and quinazoline **17-22** derivatives is outlined in Scheme 1. The 6-phenyl-4(3*H*)-pyrimidinone (**1**) and the 2-methyl-4(3*H*)-quinazolinone (**14**) reacted with an excess of 1,5-dibromopentane or 1-chloro-5-bromopentane in the presence of potassium carbonate and a catalytic amount of potassium iodide to obtain derivatives **2**, **3**, **15**, and **16**. Halo derivatives **2** and **3** were prepared by using traditional heating method, **15** and **16** by using microwave irradiation.

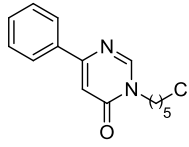
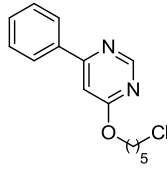
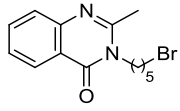
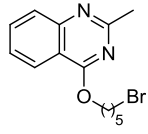


Scheme 1. Reagents and conditions: (a) $X(CH_2)_5Br$, K_2CO_3, KI catalytic amount, CH_3CN or $CO(CH_3)_2$, mw or reflux; (b) substituted piperazines, K_2CO_3, KI catalytic amount, CH_3CN, mw or reflux.

From the reaction mixture, derivatives **2** and **15** (alkylated at the nitrogen atom) were isolated, and it was also possible to isolate derivatives **3** and **16** in low yields, alkylated at the oxygen atom as confirmed by ^{1}H NMR and IR spectral data (Table 1). ^{1}H NMR spectra of compounds **2** and **15** show a signal of two protons of a methylene unit at δ 3.92 and 4.03, respectively, attributable to a *N*-alkylation ($CONCH_2$). The shift of these signals for compounds **3** and **16** at δ 4.53 and 4.39, respectively, is due to an *O*-alkylation (OCH_2). IR spectra further confirmed such findings, compounds **2** and **15** display peaks at 1671 and 1673 cm^{-1},

respectively, due to the C=O stretching, peaks that are absent in compounds **3** and **16**.

Table 1. Spectral data of halo derivatives **2**, **3**, **15**, and **16**.

Compound		^{1}H NMR, δ (ppm)		IR, (cm^{-1})
				C=O
		$CONCH_2$	OCH_2	
2		3.92	-	1673
3		-	4.53	-
15		4.03	-	1671
16		-	4.39	-

It has been well-established that regioselectivity varies from one scaffold to another and within the same scaffold. Also, different synthetic outcomes can be obtained depending on solvent, nature of electrophiles, and other conditions such as temperature, base used for deprotonation, and nature of the substituent at the 2-position of the quinazoline.[26-28]

For these reasons, it is very difficult to establish predictable and robust protocols for *N*- versus *O*-alkylation reactions, which could proceed (depending on the chain length) via an intramolecular mechanism involving cyclic 1,3-azaoxonium intermediates.[29, 30] The different percentage in the alkylated product mixtures are a result of competing pathways. For these reasons, it is very difficult to establish predictable and robust to different products. The application of thermodynamic or kinetic control determines the final composition of the product.[31]

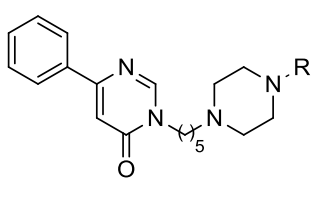
Compounds **4**-**13** and **17**-**22** were prepared from halo derivatives **2**, **3**, **15**, and **16** by reaction with an excess of the properly substituted piperazine, in the presence of potassium carbonate and a catalytic amount

of potassium iodide using traditional heating or by microwave irradiation. The difficulty of isolating some *O*-alkylated final compounds was limiting and therefore only two quinazoline derivatives were prepared(Scheme 1).

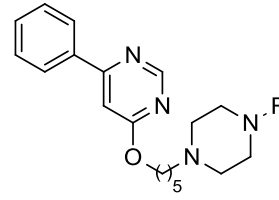
4.2.2. Binding tests

New derivatives **4-13** and **17-22** were tested on human cloned 5-HT_{1A} and 5-$HT_{7(a)}$ serotonin receptors expressed in CHO-K1 cells following a previously reported procedure.[20] Binding assays on 5-HT_7 and 5-HT_{1A} receptors were carried out by using [^{3}H]-5-HT and [^{3}H]-8-OH-DPAT as radioligands, respectively. Results, expressed as K_i (nM), are summarized in Table 2.

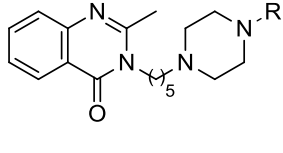
Table 2. Binding properties of derivatives **4-13**, **7-22**, **23-30**, and reference compounds SB 269970 and 8-OH- DPAT.



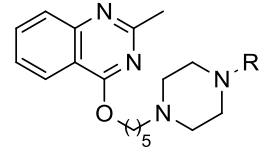
4-8



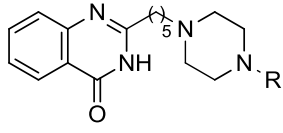
9-13



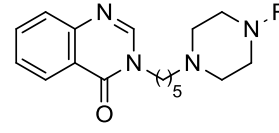
17-20



21, 22



23-25



26-30

Comp.	R	K_i[a] (nM)	
		5-HT_7	5-HT_{1A}
4	C_6H_5	613 ± 68	111 ± 21
5	$CH_2C_6H_5$	1328 ±192	772 ± 156
6	3-ClC_6H_4	94.7 ± 10	17.8 ± 2.8
7	4-ClC_6H_4	1310 ± 179	167 ± 20
8	2-$EtOC_6H_4$	45.1 ± 4.6	25.9 ± 3.7
9	C_6H_5	143 ± 17	33.5 ± 4.1
10	$CH_2C_6H_5$	990 ± 177	1032 ± 114
11	3-ClC_6H_4	44.8 ± 7.8	17.4 ± 2.0
12	4-ClC_6H_4	1377 ± 252	90.3 ± 6.5
13	2-$EtOC_6H_4$	23.5 ± 2.9	6.96 ± 0.76
17	C_6H_5	53.7 ± 12	18.6 ± 2.1
18	3-ClC_6H_4	35.1 ± 6.9	8.21 ± 1.1
19	4-ClC_6H_4	327 ± 47	100 ± 14
20	2-$EtOC_6H_4$	8.42 ± 0.78	2.99 ± 0.26
21	C_6H_5	276 ± 27	24.7 ± 2.0
22	4-ClC_6H_4	181 ± 20	52.1 ± 4.4
23[b]	C_6H_5	228 ± 12	43.5 ± 5.4
24[b]	3-ClC_6H_4	11.9 ± 3.2	7.33 ± 0.77
25[b]	4-ClC_6H_4	101 ± 26	116 ± 20
26[b]	C_6H_5	307 ± 57	28.9 ± 4.6
27[b]	$CH_2C_6H_5$	1082 ± 100	268 ± 25
28[b]	3-ClC_6H_4	35.8 ± 7.0	6.28 ± 0.86
29[b]	4-ClC_6H_4	12.9 ± 0.85	51.5 ± 11
30[b]	2-$EtOC_6H_4$	6.88 ± 0.66	1.04 ± 0.13
SB-269970		0.71 ± 0.06	9024 ± 181
8-OH-DPAT		388 ± 58	2.65 ± 0.10

[a]Each value is the mean ± SD of the data from three separate experiments.
[b]Data from Ref. [20].

4.2.3. Structure–affinity relationship studies

The influence of terminal moiety can be directly traced in *N*-alkylated derivatives, comparing appropriately substituted arylpiperazines from quinazolinone series **26**–**30** with 2-methylquinazolinones **17**–**20** and 6-phenylpyrimidinones **4**–**8** (Table 2). The introduction of the methyl substituent at the 2-position of the quinazolinone system did not significantly change affinities for both receptors, with the exceptions of the phenyl derivative **17**, which showed a 6-fold increase of affinity for the 5-HT_7R compared to **26**[20] (K_i = 53.7 *vs* 307 nM) and the 4-chlorophenyl derivatives **19** *vs* **29**,[20] that conversely demonstrated a 25-fold decrease of affinity for this receptor (K_i = 327 and 12.9 nM, respectively). On the other hand, the benzo-cracking strategy caused a decrease in affinity for 5-HT_7R [ranging from 1.2-fold (**5** *vs* **27**) to 100-fold (**7** *vs* **29**)] and 5-HT_{1A}R [ranging from 2.8-fold (**6** *vs* **28**) to 25-fold (**8** *vs* **30**)].

Regarding pirimidinone derivatives, the 3-chloro and 2-ethoxyphenyl derivatives **6** and **8** show affinity values in the nanomolar range for both 5-HT_7 and 5-HT_{1A} receptors. The introduction of an unsubstituted phenyl ring (compound **4**) is detrimental for affinity on both receptors. The substitution of phenyl with a 4-chlorophenyl and phenylmethyl moiety (**5** and **7**) induces a further decrease of affinity on both receptors.

The anchoring at the oxygen atom of the alkyl spacer in the pyrimidine derivatives **9**-**13** leads to a slight increase of affinity for 5-HT_7R for phenyl, 3-chlorophenyl, and 2-ethoxyphenyl substituted derivatives **9**, **11**, and **13**, and also for 5-HT_{1A}R, except for the 3-chlorophenyl derivative **11**. Interestingly, among 6-phenylpirimidines, compound **13** displays a higher affinity value with respect to the corresponding *N*-substitued derivative **8**. The phenylmethyl and 4-chlorophenyl derivatives **10** and **12** display low affinity for both receptors comparable to that of corresponding *N*-substituted derivatives (**5** and **7**), conversely compound **12** exhibits an improved affinity for 5-HT_{1A}R.

Regarding quinazolinone derivatives, phenyl, 3-chlorophenyl, and 2-ethoxyphenyl substituted compounds **17**, **18**, and **20** show the higher affinity for 5-HT_7 and 5-HT_{1A} receptors. Among them, the 2-ethoxyphenyl **20** displays the higher affinity values in this and the other series. The substitution with a 4-chlorophenyl in compound **19** leads to a decrease of affinity for 5-HT_7 and 5-HT_{1A} receptors.

The anchoring at the 4-position of the alkyl spacer on phenyl derivative **21** is detrimental for the affinity for the 5-HT_7R, but not for 5-HT_{1A}R, while the 4-chlorophenyl derivative **22** demonstrates a slightly increased affinity for both receptors with respect to **19**.

Finally, comparison of results of binding tests of *N*- and *O*-substituted derivatives shows a similar general trend regarding the substituents introduced on the piperazine ring. The benzo-cracking performed in compounds **4**-**13** produces a decrease in affinity for 5-HT_7 and 5-HT_{1A}

receptors. Therefore, these findings demonstrate that 5-HT_7 and 5-HT_{1A} binding sites can accommodate a 6-phenylpyrimidine or pyrimidinone to a lesser degree than a quinazoline or 2-methylquinazoline and quinazolinone or 2-methylquinazolinone terminal fragment.

Comparing these results with those of the previous work (23-30),[20] the introduction of the methyl substituent at the 2-position of the quinazolinone system does not determine significant changes of affinity for the 5-HT_{1A}R and 5-HT_7R, with the exceptions of the phenyl derivative **17**, which when compared to **26**[20] shows a 6-fold increase of affinity for the 5-HT_7R (K_i = 53.7 nM) and the 4-chlorophenyl derivative **19**, that conversely compared to **29**[20] demonstrates a 25-fold decrease of affinity for the 5-HT_7R (K_i = 327 nM) (Table 2).[20]

4.2.4. Molecular modeling studies

A molecular modeling study has been done on the new **4**-**13**, **17**-**22**, and previous reported **23**-**30**[20] ligands (Table 2), to investigate their binding mode on 5-HT_7 and 5-HT_{1A} receptors. Homology models of 5-HT_{1A} and 5-HT_7 receptors were built on crystal structures of the D_3 (PDB ID: 3PBL) receptor,[33] and used for the study of the binding mode of the new *N*-/*O*-alkyl derivatives. LigPrep was used to prepare the structures of the molecules[34] and Epik to assign the appropriate ionization states at pH = 7.4.[35] Docking was performed by using Glide at SP level.[36] A spatial constrain was imposed for the creation of an ionic interaction between the protonated nitrogen of the ligand and Asp3.32 side chain. For each compound, five top-scored complexes were considered, of which the best one was selected.

Binding modes and residue interactions for title compounds were similar, despite the fact that the adopted ligand conformations were different for 5-HT_7R and 5-HT_{1A}R. Generally, the synthesized compounds preferred a L-shape conformation when bound to 5-HT_7R and an extended conformation when bound to 5-HT_{1A}R. In agreement with our previous study,[33] this result could be attributable to the different size of the cavity within transmembrane helices (TMHs) 2, 3, 7 and the first extracellular *loop 1 (*EL1), which was smaller in 5-HT_7R than in 5-HT_{1A}R. Comparison on both receptors of the docking pose of ligand **20** (green), which possess the highest affinity at the 5-HT_7R and 5-HT_{1A}R, shows different orientations for the terminal 2-methyl-4(3*H*)-quinazolinone fragment. This system in the 5-HT_7R points towards the extracellular loops and interacts with Thr2.64, Arg7.36, and Cys146 of the EL2 (Fig. 4A left), while in the 5-HT_{1A}R points towards the cavity and interacts with Tyr2.64, Phe3.28, Asn7.39, and Trp7.40 (Fig. 4A right). The 2-ethoxyphenylpiperazine portion of **20** is hosted in a small cavity between TMHs 5 and 6, in which the aromatic ring establishes a CH-π or π-π interaction with the Phe6.51 in

both receptors (Fig. 4B). The orientation of the 2-ethoxygroup is different in the two receptors, in the 5-HT_7R the orto substituent seems to interact with Cys3.36 (Fig. 4B left), in the 5-HT_{1A}R with Ser5.42 and Thr5.43 (Fig. 4B right).

We studied from a molecular modeling point of view the 3-chloro and 4-chlorophenylpiperazine derivatives (**6**, **7**, **11**, **12**, **18**, and **19**). It was found that the introduction of a chloro atom at the meta position of the phenylpiperazine ring increases the affinity for both receptors with respect to the para-chloro analogous. This increase of affinity could be justified by the binding pose of the 3-chlorophenylpiperazine derivative (see **6** *vs* **7**), which shows a favourable orientation towards the Cys3.36, establishing an additional halogen bond interaction (Fig. 4C). As general trend, a similar behaviour was found for the chlorophenylpiperazine derivatives (**11** *vs* **12** and **18** *vs* **19**) along the series (Table 2).

Moreover, we report in particular the binding mode of unsubstituted phenylpiperazine analogues (**4**, **9**, **17**, **21**, **23**,[20] and **26**;[20] Table 2). Generally, the subset analogues are docked with similar conformations (L-shape for the 5-HT_7R and extended for 5-HT_{1A}, Fig. 4D). The 2-methyl-4(3*H*)-quinazolinone planar fragment (compound **17**) is better accommodated into the 5-HT_7R cavity within TMHs 2, 3, 7, and EL1, than the 6-phenyl-4(3*H*)-pyrimidinone (**4**), 6-phenylpyrimidine (**9**), and 2-methylquinazoline (**21**) scaffolds. In fact, compounds **4**, **9**, and **21** show lower affinity values with respect to **17** for 5-HT_7R, whose binding site cavity presents more voluminous amino acids (*i.e.* Val2.61, Arg7.36, and Leu7.39) than 5-HT_{1A}R. Therefore, for the 5-HT_7R a planar bicyclic system is preferable over a single heterocyclic ring with a bulky substituent like a phenyl.

The terminal planar bicyclic fragment is also preferred for the 5-HT_{1A}R (Fig. 4D right). Comparing conformations and binding poses of the phenylpiperazinepyrimidine derivatives (**4** and **9**) and the analogous quinazoline derivatives (**17**, **21**, **23**,[20] and **26**[20]) it is possible to observe the same behaviour. The planar conformation allows to the quinazoline derivatives a closer interaction with hydrophobic residues such as Ala2.61, Ala7.36, and Trp7.40. Moreover, the spatial orientation of the carbonyl group of the quinazolinone and pyrimidinone, and also of the aryloxy moiety of the quinazoline and pyrimidine system seems to play a key role in their accommodation into the binding pocket. In all selected compounds in this subset, with the exception of derivative **4**, the plane of terminal fragment adopts a vertical orientation (seeing the binding site from the extracellular side) (Fig. 4D right). The pyrimidinone ring of derivative **4**, which has the highest K_i value among them, is placed horizontally with the carbonyl group perpendicular to the TMHs (Fig. 4D right, cyan). This finding could influence the polar interaction with Asn7.39, which is located deeper in the TMH7.

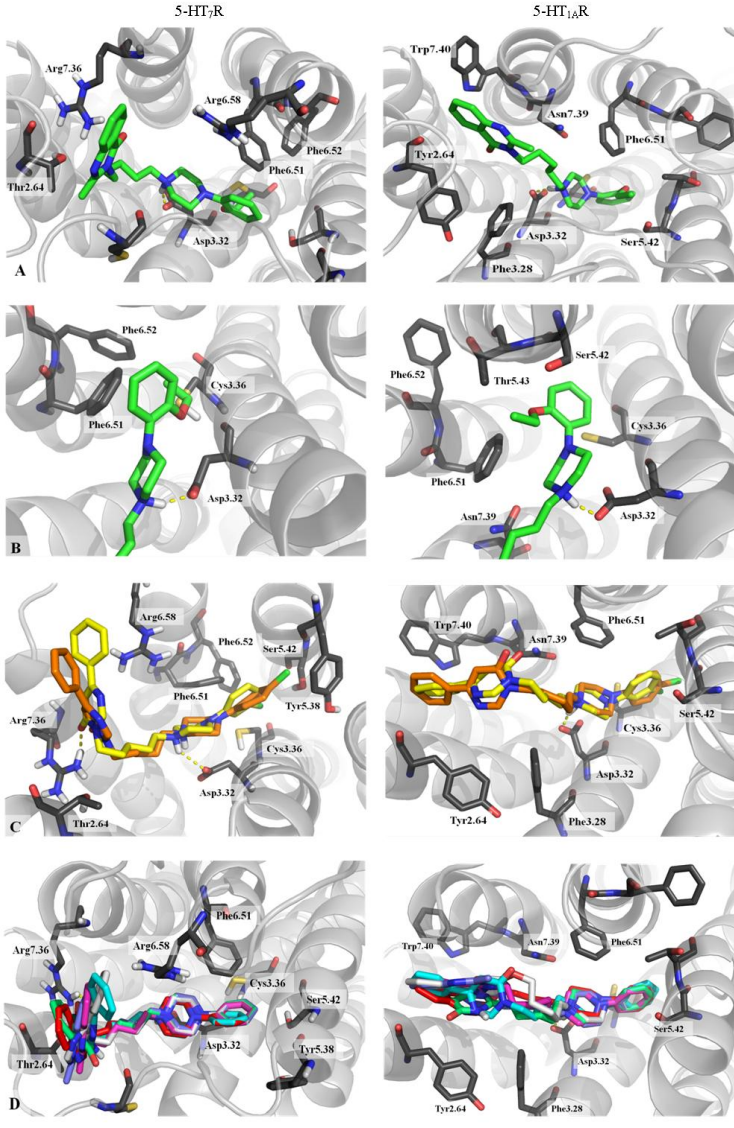


Figure 4. Panel illustrating molecular modeling results of the new *N*-/*O*-alkyl derivatives, and of the previously reported for 5-HT_7 (left) and 5-HT_{1A} receptors (right). (A) Docking pose of the high-affinity ligand **20** (green) on 5-HT_7R and 5-HT_{1A}R. (B) Detail of the orientation into the binding pocket of the 2-ethoxyphenyl moiety. (C) Orientation of the 3-chloro **6** (yellow) and 4-chlorophenylpiperazine derivatives **7** (orange). (D) Comparison of the binding modes of phenylpiperazine derivatives **4** (cyan), **9** (grey), **17** (magenta), **21** (purple blue), **23**[20](limegreen), and **26**[20](red).

4.3. Conclusion

In conclusion, we described the synthesis of new LCAPs with structural modifications in the terminal fragment and in the anchoring position of the arylpiperazinylalkyl moiety. We report the simultaneous preparation and isolation of *N*- and *O*-alkylated pyrimidine and 2-methylquinazoline derivatives, which might be used as intermediate products for the synthesis of novel potential biological agents. New derivatives have been evaluated for binding affinities at the human cloned 5-HT_{1A}R and 5-HT_7R and the main structure-affinity relationships were outlined. Despite the fact that the new **8**, **11**, **13**, **17**, **18**, and **20** show affinity values for the 5-HT_7R in the nanomolar range, they also have comparable affinity for the 5-HT_{1A}R. Therefore, they act as dual ligands. The discovery, among 6-phenylpyrimidines, of high-affinity ligands for 5-HT_7 and 5-HT_{1A} receptors allows us to conclude that this new scaffold was a useful tool in the development of new LCAPs and may be an interesting pharmacophoric terminal fragment for novel serotonin receptor ligands.

Acknowledgments

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4.4. Experimental section

4.4.1. Chemistry

Melting points were determined in an Electrothermal IA9200 apparatus using glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin Elmer series FTIR 1600 spectrometer in KBr disks. Elemental analyses for C, H, and N were within ± 0.4% of theoretical values and were obtained with a Carlo Erba Elemental Analyzer Mod. 1108 apparatus. ^{1}H NMR spectra were performed on a Varian Inova Unity 200 spectrometer (200 MHz) in DMSO-d_6 or $CDCl_3$ solution. Tetramethylsilane was used as the internal standard; chemical shifts and coupling constants (*J*) are given in δ values (ppm) and in Hertz (Hz), respectively. Signal multiplicities are abbreviated as follow: s (singlet), d (doublet), t (triplet), m (multiplet). Microwave irradiation experiments were carried out with a CEM Discover instrument using closed Pyrex glass tubes (ca. 10 mL) with Teflon-coated septa. Thin-layer chromatography was utilized to monitor the progress of reactions and to test the purity of all the synthesized compounds, using Merck aluminium sheet coated with silica gel 60 F_{254} and detection with ultraviolet light at

254 and 366 nm of wavelength. Purification of synthesized compounds was performed by flash column chromatography using Merck silica gel (0.040-0.063 mm). All chemicals and solvents were reagent grade and were purchased from commercial source.

4.4.1.1. General procedure for the preparation 3-(5-chloropentyl)-6-phenyl-4(3*H*)-pyrimidinone (2) and 4-(5-chloropenthoxy)-6-phenyl-pyrimidine (3). To a mixture of pirimidinone **1** (2.90 mmol), 1-bromo-5-chloro-pentane (5.80 mmol), potassium carbonate (4.34 mmol), and of a catalytic amount of potassium iodide, acetone was added (30 mL) and the mixture was refluxed under stirring for 9 hours. After being cooled, the solid was removed by filtration and the solution was concentrated to dryness. Recrystallization with cyclohexane gave pure compound **2**. From the solution compound **3** was isolated by flash chromatography using a mixture of cyclohexane/ethyl acetate (9:1, v/v).

Compound **2**: yield 53%, mp 103.9-104.5 °C. IR (KBr, selected lines) cm^{-1} 2944, 2361, 1673, 1593, 1450, 691. ^1H NMR (DMSO-d_6) δ 1.30-1.50 (m, 2H, CH_2), 1.60-1.85 (m, 2H + 2H, CH_2CH_2), 3.65 (t, J = 6.6 Hz, 2H, CH_2Cl), 3.92 (t, J = 7.2 Hz, 2H, $CONCH_2$), 6.97 (s, 1H, NCH), 7.40-7.58 (m, 3H, aromatic), 7.98-8.15 (m, 2H, aromatic), 8.59 (s, 1H, CCH). Anal. ($C_{15}H_{17}ClN_2O$) C, H, N.

Compound **3**: yield 10%, mp 41.0-44.0 °C. IR (KBr, selected lines) cm^{-1} 2956, 1592, 1541, 1466, 1219, 868, 696. ^1H NMR (DMSO-d_6) δ 1.44-1.65 (m, 2H, CH_2), 1.70-1.90 (m, 2H + 2H, CH_2CH_2), 3.67 (t, J = 6.6 Hz, 2H, CH_2Cl), 4.39 (t, J = 6.4 Hz, 2H, OCH_2), 7.45-7.60 (m, 3H + 1H, aromatic + NCH), 8.15-8.25 (m, 2H, aromatic), 8.84 (d, J = 1.2 Hz, 1H, CCH). Anal. ($C_{15}H_{17}ClN_2O$) C, H, N.

4.4.1.2. General procedure for the synthesis of 3-[5-(4-substituted-1-piperazinyl)pentyl]-4(3*H*)-pyrimidinones (4-8) and 4-[5-(4-substituted-1-piperazinyl)pentoxy]-pyrimidines (9-13). Acetonitrile (4 mL) was added to a mixture of chloroderivative **2** or **3** (0.90 mmol), properly substituted piperazine (1.08 mmol), sodium carbonate (1.08 mmol), and a catalytic amount of potassium iodide. The mixture was refluxed under stirring for 9 hours. After being cooled, the solid was removed by filtration and the solution was concentrated to dryness. The following new compounds were obtained using this procedure.

4.4.1.3. 3-[5-(4-Phenyl-1-piperazinyl)pentyl]-6-phenyl-4(3*H*)-pyrimidinone (4). The title compound was obtained by recrystallization from acetonitrile (51%), mp 139.6-140.4 °C. IR (KBr, selected lines) cm^{-1} 2929, 2823, 1665, 1592, 1451, 1239, 750, 695. ^1H NMR ($CDCl_3$) δ 1.30-1.58 (m, 2H, CH_2), 1.60-1.95 (m, 2H + 2H, CH_2CH_2), 2.60 (t, J = 7.2 Hz, 2H, CH_2N), 2.70-2.95 (m, 4H, piperazine), 3.20-3.45 (m, 4H, piperazine),

3.98 (t, J = 7.4 Hz, 2H, $CONCH_2$), 6.82-6.98 (m, 3H + 1H, aromatic + NCH), 7.20-7.34 (m, 2H, aromatic), 7.42-7.52 (m, 3H, aromatic), 7.88-8.10 (m, 2H, aromatic), 8.19 (s, 1H, CCH). Anal. ($C_{25}H_{30}N_4O$) C, H, N.

4.4.1.4. 3-[5-[4-(Phenylmethyl)-1-piperazinyl]pentyl]-6-phenyl-4(3*H*)-pyrimidinone (5). The title compound was purified by flash chromatography using a mixture of ethyl acetate/methanol (8:2, v/v) as eluent (26%), mp 106.2-106.5 °C. IR (KBr, selected lines) cm^{-1} 2947, 2802, 1673, 1596, 1451, 688. ^1H NMR (DMSO-d_6) δ 1.19-1.55 (m, 2H + 2H, CH_2CH_2), 1.60-1.79 (m, 2H, CH_2), 2.22 (t, J = 7.4 Hz, 2H, CH_2N), 2.30-2.42 (m, 8H, piperazine), 3.39 (s, 2H, $CH_2C_6H_5$), 3.90 (t, J = 7.0 Hz, 2H, $CONCH_2$), 6.96 (s, 1H, NCH), 7.18-7.38 (m, 5H, aromatic), 7.42-7.55 (m, 3H, aromatic), 8.00-8.15 (m, 2H, aromatic), 8.58 (s, 1H, CCH). Anal. ($C_{26}H_{32}N_4O$) C, H, N.

4.4.1.5. 3-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentyl]-6-phenyl-4(3*H*)-pyrimidinone (6). The title compound was obtained by recrystallization from acetonitrile (14%), mp 112.2-112.7 °C. IR (KBr, selected lines) cm^{-1} 2938, 1666, 1596, 1489, 1449, 1245, 692. ^1H NMR (DMSO-d_6) δ 1.22-1.42 (m, 2H, CH_2), 1.42-1.62 (m, 2H, CH_2), 1.62-1.82 (m, 2H, CH_2), 2.31 (t, J = 6.8 Hz, 2H, CH_2N), 2.38-2.49 (m, 4H, piperazine), 3.18-3.22 (m, 4H, piperazine), 3.93 (t, J = 7.0 Hz, 2H, $CONCH_2$), 6.75-6.88 (m, 1H, aromatic), 6.88-6.97 (m, 1H, aromatic), 6.98 (s, 1H, NCH), 7.18-7.25 (m, 1H, aromatic), 7.42-7.78 (m, 1H, aromatic), 8.00-8.15 (m, 1H, aromatic), 8.61 (s, 1H, CCH). Anal. ($C_{25}H_{29}ClN_4O$) C, H, N.

4.4.1.6. 5-[4-(4-Chlorophenyl)-1-piperazinyl]pentyl]-6-phenyl-4(3*H*)-pyrimidinone (7). The title compound was obtained by recrystallization from acetonitrile (10%), mp 153.6-154.0 °C. IR (KBr, selected lines) cm^{-1} 2936, 1664, 1594, 1495, 1450, 1237, 812, 696. ^1H NMR (DMSO-d_6) δ 1.20-1.40 (m, 2H, CH_2), 1.40-1.60 (m, 2H, CH_2), 1.60-1.80 (m, 2H, CH_2), 2.29 (t, J = 6.6 Hz, 2H, CH_2N), 2.40-2.49 (m, 4H, piperazine), 3.02-3.15 (m, 4H, piperazine), 3.91 (t, J = 7.4 Hz, 2H, $CONCH_2$), 6.84-6.95 (m, 2H, aromatic), 6.96 (s, 1H, NCH), 7.15-7.25 (m, 2H, aromatic), 7.43-7.53 (m, 3H, aromatic), 7.99-8.11 (m, 2H, aromatic), 8.59 (s, 1H, CCH). Anal. ($C_{25}H_{29}ClN_4O$) C, H, N.

4.4.1.7. 3-[5-[4-(2-Ethoxyphenyl)-1-piperazinyl]pentyl]-6-phenyl-4(3*H*)-pyrimidinone (8). The title compound was obtained using 16 hours of reflux and was purified by flash chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluent (32%), mp 103.5-104.1 °C. IR (KBr, selected lines) cm^{-1} 2927, 2804, 1664, 1499, 1455, 1239, 699. ^1H NMR (DMSO-d_6) δ 1.20-1.40 (m, 2H + 3H, CH_2 + CH_2CH_3), 1.40-1.60 (m, 2H, CH_2), 1.60-1.81 (m, 2H, CH_2), 2.30 (t, J =

6.8 Hz, 2H, CH_2N), 2.38-2.58 (m, 4H, piperazine), 2.85-3.04 (m, 4H, piperazine), 3.88-4.10 (m, 4H, CH_2CH_3 + $CONCH_2$), 6.78-6.95 (m, 4H, aromatic), 6.97 (s, 1H, NCH), 7.40-7.55 (m, 3H, aromatic), 8.00-8.15 (m, 2H, aromatic), 8.60 (s, 1H, CCH). Anal. ($C_{27}H_{34}N_4O_2$) C, H, N.

4.4.1.8. 4-[5-(4-Phenyl-1-piperazinyl)pentoxy]-6-phenyl-pyrimidine (9). The title compound was purified by flash chromatography ethyl acetate as eluent (53%), mp 70.0-70.4 °C. IR (KBr, selected lines) cm^{-1} 2946, 2817, 1592, 1360, 1227, 1006, 756, 689. ^1H NMR (DMSO-d_6) δ 1.35-1.65 (m, 2H + 2H, CH_2CH_2), 1.70-1.90 (m, 2H, CH_2), 2.28-240 (m, 2H, CH_2N), 2.40-2.60 (m, 4H, piperazine), 3.02-3.18 (m, 4H, piperazine), 4.41 (t, J = 6.4 Hz, 2H, OCH_2), 6.70-6.81 (m, 1H, aromatic), 7.18-7.28 (m, 2H, aromatic), 7.48-7.60 (m, 1H + 3H, NCH + aromatic), 8.15-8.25 (m, 2H, aromatic), 8.85 (d, J = 1.8 Hz, 1H, CCH). Anal. ($C_{25}H_{30}N_4O$) C, H, N.

4.4.1.9. 4-[5-[4-(Phenylmethyl)-1-piperazinyl]pentoxy]-6-phenyl-pyrimidine (10). The title compound was purified by flash chromatography using ethyl acetate and then a mixture of ethyl acetate/methanol (9.5:0.5, v/v)) as eluents (46%), mp 63.8-64.3 °C. IR (KBr, selected lines) cm^{-1} 2941, 2810, 1578, 1541, 1345, 1214, 1005, 696. ^1H NMR (DMSO-d_6) δ 1.30-1.58 (m, 2H + 2H, CH_2CH_2), 1.68-1.82 (m, 2H, CH_2), 2.25 (t, J = 6.6 Hz, 2H, CH_2N), 2.30-2.42 (m, 8H, piperazine), 3.41 (s, 2H, $CH_2C_6H_5$), 4.38 (t, J = 6.6 Hz, 2H, OCH_2), 7.18-7.38 (m, 5H, aromatic), 7.49 (s, 1H, NCH), 7.50-7.60 (m, 3H, aromatic), 8.18-8.22 (m, 2H, aromatic), 8.83 (d, J = 0.8 Hz, 1H, CCH). Anal. ($C_{26}H_{32}N_4O$) C, H, N.

4.4.1.10. 4-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentoxy]-6-phenyl-pyrimidine (11). The title compound was purified by flash chromatography using ethyl acetate as eluent to obtain an oil (64%). IR (neat, selected lines) cm^{-1} 2940, 2818, 1591, 1461, 1352, 1237, 987, 758, 694. ^1H NMR ($CDCl_3$) δ 1.42-1.61 (m, 2H, CH_2), 1.63-1.98 (m, 2H + 2H, CH_2CH_2), 2.58 (t, J = 7.6 Hz, 2H, CH_2N), 2.70-2.90 (m, 4H, piperazine), 3.25-3.43 (m, 4H, piperazine), 4.42 (t, J = 6.6 Hz, 2H, $CONCH_2$), 6.72-6.90 (m, 3H, aromatic), 7.08-7.24 (m, 1H + 2H, NCH + aromatic), 7.42-7.53 (m, 3H, aromatic), 7.95-8.08 (m, 2H, aromatic), 8.82 (d, J = 1.0 Hz, 1H, CCH). Anal. ($C_{25}H_{29}ClN_4O$) C, H, N.

4.4.1.11. 4-[5-[4-(4-Chlorophenyl)-1-piperazinyl]pentoxy]-6-phenyl-pyrimidine (12). The title compound was obtained by recrystallization from acetonitrile (19%), mp 104.2-104.5 °C. IR (KBr, selected lines) cm^{-1} 2943, 1590, 1496, 1354, 1236, 1003, 811. ^1H NMR (DMSO-d_6) δ 1.38-1.61 (m, 2H + 2H, CH_2CH_2), 1.70-1.85 (m, 2H, CH_2), 2.32 (t, J = 6.8 Hz, 2H, CH_2N), 2.41-2.49 (m, 4H, piperazine), 3.02-3.15 (m, 4H, piperazine), 4.40 (t, J = 6.6 Hz, 2H, OCH_2), 6.85-6.95 (m, 2H, aromatic), 7.18-7.25 (m, 2H, aromatic), 7.44-7.58 (m, 1H + 3H, NCH +

aromatic), 8.16-8.23 (m, 2H, aromatic), 8.84 (d, *J* = 1.0 Hz, 1H, CCH). Anal. ($C_{25}H_{29}ClN_4O$) C, H, N.

4.4.1.12. 4-[5-[4-(2-Ethoxyphenyl)-1-piperazinyl]pentoxy]-6-phenyl-pyrimidine (13). The title compound was purified by flash chromatography using ethyl acetate as eluent to obtain an oil (27%). IR (neat, selected lines) cm^{-1} 2939, 2814, 1590, 1499, 1455, 1239, 737. ^1H NMR ($CDCl_3$) δ 1.40-1.63 (m, 2H +3H, CH_2 + CH_2CH_3), 1.63-1.98 (m, 2H + 2H, CH_2CH_2), 2.65 (t, *J* = 6.2 Hz, 2H, CH_2N), 2.78-3.08 (m, 4H, piperazine), 3.15-3.42 (m, 4H, piperazine), 4.06 (q, *J* = 6.8 Hz, 2H, CH_2CH_3), 4.42 (t, *J* = 6.6 Hz, 2H, OCH_2), 6.80-7.08 (m, 4H, aromatic), 7.11 (s, 1H, NCH), 7.42-7.60 (m, 3H, aromatic), 7.98-8.15 (m, 2H, aromatic), 8.82 (d, *J* = 1.0 Hz, 1H, 1H, CCH). Anal. ($C_{27}H_{34}N_4O_2$) C, H, N.

4.4.1.13. General procedure for the preparation of 3-(5-bromopentyl)-2-methyl-4(3*H*)-quinazolinone (15) and 4-(5-bromopenthoxy)-2-methylquinazoline (16). Acetonitrile (4 mL) was added to mixture of compound **14** (1.55 mmol), 1,5-dibromopentane (4.67 mmol), potassium carbonate (2.32 mmol), and a catalytic amount of potassium iodide. The mixture and a magnetic bar was sealed in a Pyrex tube and was heated at 90 °C by microwave irradiation for 90 min (run time 3 min, microwave max power 150 W and max pressure 150 Psi). After being cooled, the solid was removed by filtration and the solution was concentrated to dryness. From the obtained residue, compound **15** as a solid and compound **16** as an oil were isolated by flash chromatography using ethyl acetate/cyclohexane (5:5, v/v) as eluent.

Compound **15**: yield 47%, mp 52.0-54.6 °C. IR (KBr, selected lines) cm^{-1} 3052, 1671, 1626, 1467, 1424, 1265, 737. ^1H NMR (DMSO-d_6) δ 1.40-1.58 (m, 2H, CH_2), 1.58-1.78 (m, 2H, CH_2), 1.78-1.95 (m, 2H, CH_2), 2.61 (s, 3H, CH_3), 3.56 (t, *J* = 6.6 Hz, 2H, CH_2Br), 4.03 (t, *J* = 7.2 Hz, 2H, $CONCH_2$), 7.42-7.52 (m, 1H, aromatic), 7.52-7.60 (m, 1H, aromatic), 7.72-7.83 (m, 1H, aromatic), 8.05-8.12 (m, 1H, aromatic).

Compound **16**: yield 23%, IR (neat, selected lines) cm^{-1} 1619, 1577, 1503, 1434, 1369, 1318, 1168, 1112, 782. ^1H NMR (DMSO-d_6) δ 1.54-1.68 (m, 2H, CH_2), 1.78-1.99 (m, 2H + 2H, CH_2CH_2), 2.61 (s, 3H, CH_3), 3.58 (t, *J* = 6.8 Hz, 2H, CH_2Br), 4.53 (t, *J* = 6.4 Hz, 2H, OCH_2), 7.53-7.65 (m, 1H, aromatic), 7.76-7.95 (m, 2H, aromatic), 8.05-8.13 (m, 1H, aromatic). Anal. ($C_{14}H_{17}BrN_2O$) C, H, N.

4.4.1.14. General procedure for the synthesis of 2-methyl-3-[5-(4-substituted-1-piperazinyl)pentyl]-4(3*H*)-quinazolinones (17-20) and 2-methyl-4-[5-(4-substituted-1-piperazinyl)pentoxy]-quinazolines (21, 22). Acetonitrile (4 mL) was added to a mixture of bromoderivative **15** or **16** (0.73 mmol), properly substituted piperazine (0.88 mmol), sodium

carbonate (0.88 mmol), and a catalytic amount of potassium iodide. The mixture and a magnetic bar was sealed in a Pyrex tube and was heated at 90 °C by microwave irradiation for 1 hour (run time 3 min, microwave max power 150 W and max pressure 150 Psi). After being cooled, the solid was removed by filtration and the solution was concentrated to dryness. The following new compounds were obtained using this procedure:

4.4.1.15. 2-Methyl-3-[5-(4-phenyl-1-piperazinyl)pentyl]-4(3*H*)-quinazolinone (17). The crude product was purified by flash chromatography using ethyl acetate 100%, a mixture of ethyl acetate/methanol (9:1, v/v), and then ethyl acetate/methanol (8:2, v/v) as eluents (37%), mp 78.0-79.0 °C. IR (KBr, selected lines) cm^{-1} 2934, 2820, 1678, 1600, 1476, 1402, 1240, 780, 762. ^1H NMR (DMSO-d_6) δ 1.35-1.78 (m, 2H + 2H + 2H, $CH_2CH_2CH_2$), 2.32 (t, J = 6.8 Hz, 2H, NCH_2), 2.41-2.58 (m, 4H, piperazine), 2.62 (s, 3H, CH_3), 3.03-3.17 (m, 4H, piperazine), 4.04 (t, J = 7.4 Hz, 2H, $CONCH_2$), 6.71-6.82 (m, 1H, aromatic), 6.86-6.98 (m, 2H, aromatic), 7.14-7.26 (m, 2H, aromatic), 7.41-7.53 (m, 1H, aromatic), 7.53-7.62 (m, 1H, aromatic), 7.72-7.84 (m, 1H, aromatic), 8.05-8.14 (m, 1H, aromatic). Anal. ($C_{24}H_{30}N_4O$) C, H, N.

4.4.1.16. 3-[5-[4-(3-Chlorophenyl)-1-piperazinyl]pentyl]-2-methyl-4(3*H*)-quinazolinone (18). The crude product was purified by flash chromatography using ethyl acetate 100%, a mixture of ethyl acetate/methanol (9:1, v/v), and then ethyl acetate/methanol (8:2, v/v) as eluents (13%), mp 113.0-114.8 °C. IR (KBr, selected lines) cm^{-1} 2928, 2815, 1678, 1595, 1471, 1462, 1393, 1243, 770. ^1H NMR (DMSO-d_6) δ 1.25-1.78 (m, 2H + 2H + 2H, $CH_2CH_2CH_2$), 2.31 (t, J = 6.8 Hz, 2H, NCH_2), 2.40-2.55 (m, 4H, piperazine), 2.61 (s, 3H, CH_3), 3.05-3.20 (m, 4H, piperazine), 4.03 (t, J = 7.0 Hz, 2H, $CONCH_2$), 6.72-6.81 (m, 1H, aromatic), 6.81-6.94 (m, 2H, aromatic), 7.12-7.25 (m, 1H, aromatic), 7.41-7.60 (m, 2H, aromatic), 7.70-7.82 (m, 1H, aromatic), 8.04-8.15 (m, 1H, aromatic). Anal. ($C_{24}H_{29}ClN_4O$) C, H, N.

4.4.1.17. 3-[5-[4-(4-Chlorophenyl)-1-piperazinyl]pentyl]-2-methyl-4(3*H*)-quinazolinone (19). The crude product was recrystallized from water (49%), mp 101.2-102.2 °C. IR (KBr, selected lines) cm^{-1} 2937, 1678, 1597, 1499, 1467, 1394, 1357, 1240, 768. ^1H NMR ($CDCl_3$) δ 1.25-1.78 (m, 2H + 2H, $CH_2CH_2CH_2$), 2.31 (t, J = 7.2 Hz, 2H, NCH_2), 2.40-2.55 (m, 4H, piperazine), 2.61 (s, 3H, CH_3), 3.02-3.15 (m, 4H, piperazine), 4.03 (t, J = 7.8 Hz, 2H, $CONCH_2$), 6.91 (d, J = 9.2 Hz, 2H, aromatic), 7.21 (d, J = 8.8 Hz, 2H, aromatic), 7.40-7.52 (m, 1H, aromatic), 7.52-7.62 (m, 1H, aromatic), 7.70-7.82 (m, 1H, aromatic), 8.04-8.15 (m, 1H, aromatic). Anal. ($C_{24}H_{29}ClN_4O$) C, H, N.

4.4.1.18. 3-[5-[4-(2-Ethoxyphenyl)-1-piperazinyl]pentyl]-2-methyl-4(3*H*)-quinazolinone (20). The crude product was purified by flash chromatography using ethyl acetate 100%, a mixture of ethyl acetate/methanol (9:1, v/v), and then ethyl acetate/methanol (8:2, v/v) as eluents, obtaining compound **10** as a pure oil (12%). IR (neat, selected lines) cm^{-1} 2940, 1671, 1594, 1500, 1474, 1394, 1266, 1241, 736. ^1H NMR ($CDCl_3$) δ 1.39-1.60 (m, 2H + 3H, CH_2 + CH_2CH_3), 1.71-1.95 (m, 2H + 2H, CH_2CH_2), 2.65 (s, 3H, CH_3), 2.70 (t, *J* = 7.6 Hz, 2H, NCH_2), 2.80-3.10 (m, 4H, piperazine), 3.25-3.45 (m, 4H, piperazine), 3.98-4.20 (m, 2H + 2H, $CONCH_2$ + CH_2CH_3), 6.80-7.10 (m, 4H, aromatic), 7.38-7.50 (m, 1H, aromatic), 7.55-7.62 (m, 1H, aromatic), 7.62-7.80 (m, 1H, aromatic), 8.18-8.27 (m, 1H, aromatic). Anal. ($C_{26}H_{34}N_4O_2$) C, H, N.

4.4.1.19. 2-Methyl-4-[5-(4-phenyl-1-piperazinyl)pentoxy]-quinazoline (21). The crude product was recrystallized from water (26%), mp 64.9-66.5 °C. IR (KBr, selected lines) cm^{-1} 2960, 2833, 1575, 1500, 1422, 1356, 1239, 1163, 1112, 777. ^1H NMR (DMSO-d_6) δ 1.42-1.70 (m, 2H + 2H, CH_2CH_2), 1.75-1.95 (m, 2H, CH_2), 2.33 (t, *J* = 6.4 Hz, 2H, NCH_2), 2.40-2.55 (m, 4H, piperazine), 2.61 (s, 3H, CH_3), 3.00-3.15 (m, 4H, piperazine), 4.53 (t, *J* = 6.4 Hz, 2H, OCH_2), 6.70-6.81 (m, 1H, aromatic), 6.83-6.96 (m, 2H, aromatic), 7.12-7.25 (m, 2H, aromatic), 7.53-7.62 (m, 1H, aromatic), 7.75-7.96 (m, 2H, aromatic), 8.02-8.18 (m, 1H, aromatic). Anal. ($C_{24}H_{30}N_4O$) C, H, N.

4.4.1.20. 4-[5-(4-(4-Chlorophenyl)-1-piperazinyl)pentoxy]-2-methyl-quinazoline (22). The crude product was recrystallized from acetonitrile (12%), mp 109.0-111.9 °C. IR (KBr, selected lines) cm^{-1} 2941, 1578, 1497, 1424, 1351, 1163, 1109, 768. ^1H NMR ($CDCl_3$) δ 1.48-1.70 (m, 2H, CH_2), 1.70-2.00 (m, 2H + 2H, CH_2CH_2), 2.63 (t, *J* = 7.4 Hz, 2H, NCH_2), 2.71 (m, 3H, CH_3), 2.75-2.90 (m, 4H, piperazine), 3.25-3.39 (m, 4H, piperazine), 4.56 (t, *J* = 6.4 Hz, 2H, OCH_2), 6.78-6.90 (m, 2H, aromatic), 7.15-7.30 (m, 2H, aromatic), 7.42-7.58 (m, 1H, aromatic), 7.73-7.87 (m, 2H, aromatic), 8.05-8.18 (m, 1H, aromatic). Anal. ($C_{24}H_{29}ClN_4O$) C, H, N.

4.5. In vitro binding assays

Binding assays were performed using human cloned 5-$HT_{7(a)}$ and 5-HT_{1A} serotonin receptors (PerkinElmer) expressed on CHO-K1 cells. Radioligand binding assays were carried out using the condition reported on technical data sheet with some modifications. Briefly, 5-$HT_{7(a)}$ receptors[37] were resuspended in Tris HCl 50 mM pH 7.4 containing 4 mM $MgCl_2$ and incubated for 40 min at 27 °C in a final volume of 0.51 ml, consisting of 250 µL of membrane suspension (15 µg protein/sample), 250 µL of [^3H]-5-HT (final concentration 5 nM, s.a. 106 Ci/mmol,

PerkinElmer) prepared in the same buffer used for membrane suspension and 10 μL of tested compounds. Nonspecific binding was obtained in the presence of 10 μM serotonin.

For 5-HT_{1A} binding assay,[38] receptors were resuspended in Tris HCl 50 mM pH 7.4 containing 4 mM $CaCl_2$ and incubated (10 μg protein/sample) for 60 min. at 27 °C in the same volume using for 5-$HT_{7(a)}$ receptors but in presence of [^{3}H]-8-OH-DPAT (final concentration 1 nM, s.a. 137 Ci/mmol, PerkinElmer). Nonspecific binding was obtained in presence of 10 μM serotonin and, for both binding assays, a reference drug was tested. Incubations were stopped by rapid filtration under vacuum, through GF/C filters (pre-soaked with 0.3% PEI) and washed with 12 mL (4 × 3 times) of ice-cold washing buffer (Tris HCl, 50 mM, pH 7.4) using a Brandel M-48R cell harvester. The radioactivity trapped on the filters was counted in 4 mL of Ultima Gold MV (Packard) in a Tri-carb 2800 TR (PerkinElmer) liquid scintillation spectrometer with a counting efficiency of 60%. All compounds were tested in a concentration range from 10^{-5} to 10^{-10} M in triplicate and dose-inhibition curves were analyzed by the "Allfit" program to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding.[39] The K_i values were derived from IC_{50} values according to the Cheng and Prusoff equation.[40]

4.6. Molecular modeling

The building of homology models of 5-HT_{1A} and 5-HT_7 receptors, validation of these models, and ligand-directed optimization of the binding sites were performed according to the details presented in our previous work.[33] The 5-HT_{1A} and 5-HT_7 receptor models selected in IFD procedure were used to study the binding mode of the synthesized ligands. These compounds were docked using Glide at SP level. The spatial constrain was imposed on the creation of an ionic interaction between the protonated amine group of the ligand and the Asp3.32 side chain. Next, ligand-receptor complexes were analysed, and only those models were kept for which coherent, for the whole set of compounds, and closest compliance with common binding mode for monoaminergic receptor ligands was observed.[41] Final figures of the docking pose in both receptors were generated using PyMOL.[42]

4.7. References

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4.8. Supporting material

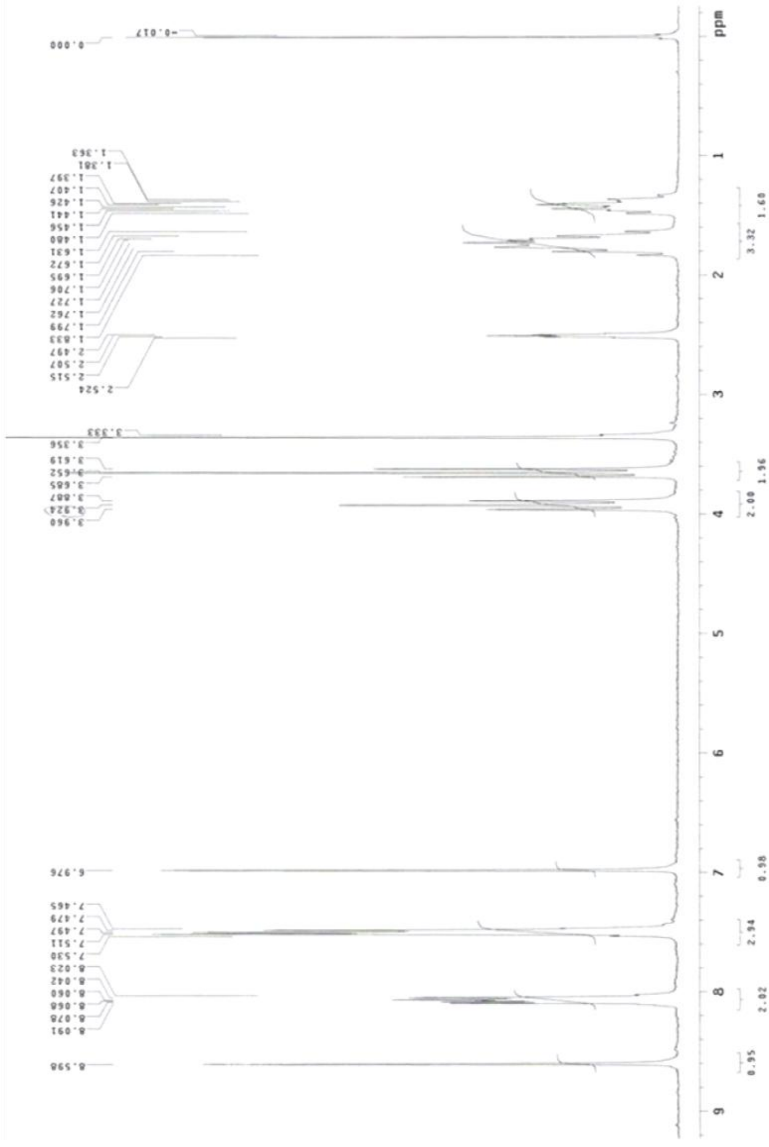

Figure 1. ^{1}H NMR of 3-(5-chloropentyl)-6-phenyl-4(3*H*)-pyrimidinone (**2**).

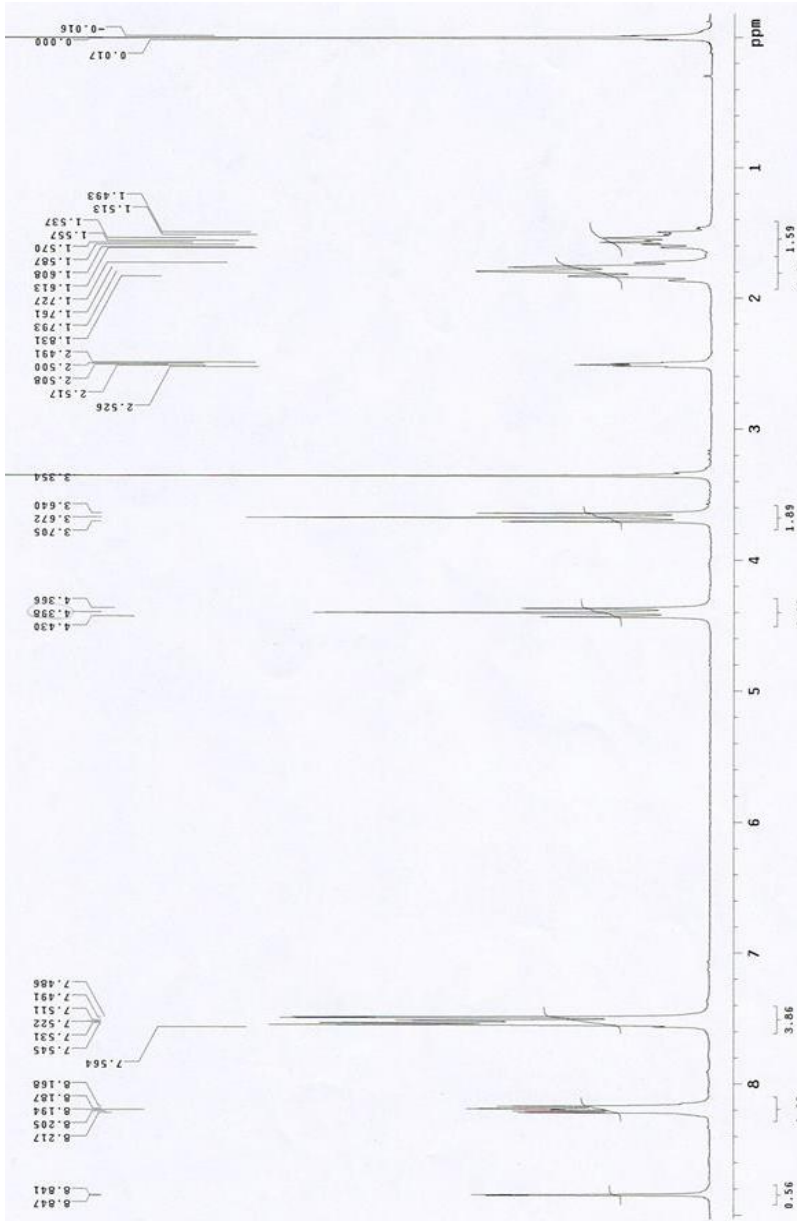

Figure 2. ^{1}H NMR of 4-(5-chloropenthoxy)-6-phenyl-pyrimidine (**3**).

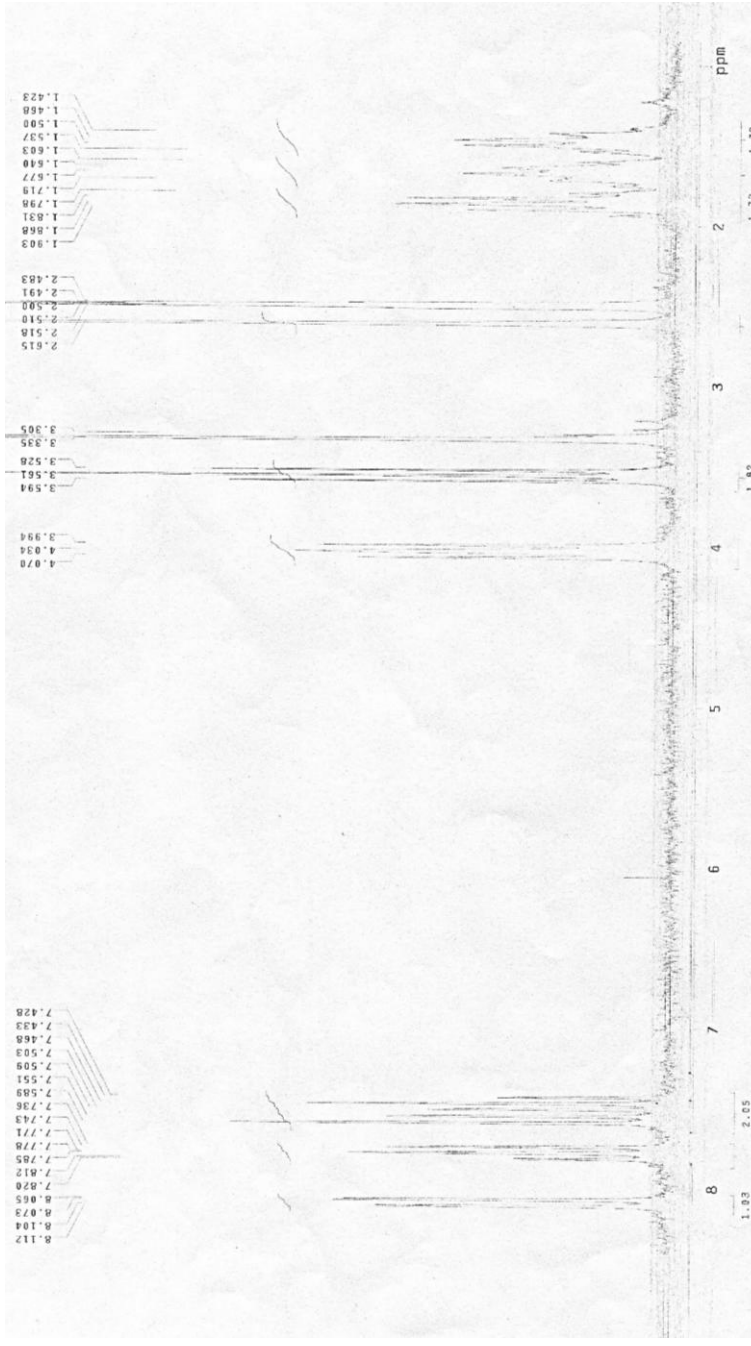

Figure 3. ^{1}H NMR of 3-(5-bromopentyl)-2-methyl-4(3*H*)-quinazolinone (**15**).

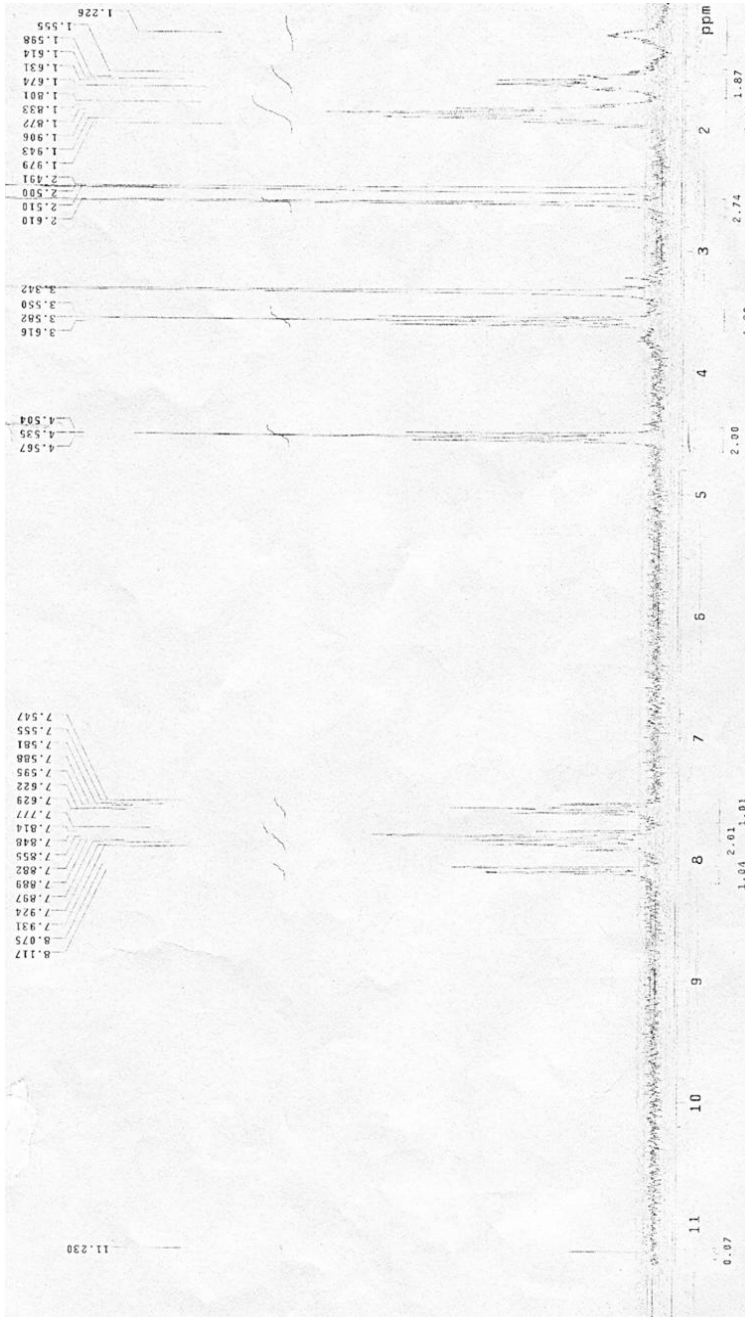

Figure 4. ^{1}H NMR of 4-(5-bromopenthoxy)-2-methylquinazoline (**16**).

5. Manuscript II

Bivalent ligand approach to the design of new 1-(4-aryl-1-piperazinyl)-3-[4-(phenylmethy)-1-piperazinyl]-1-propanone derivatives as selective ligands for 5-HT$_7$ over the 5-HT$_{1A}$ receptor

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Abstract

In this work we report the discovery of new selective ligands for 5-HT$_7$ over the 5-HT$_{1A}$ receptor using the bivalent ligand approach. These new synthesized compounds possess a 4-arylpiperazine linked through an acyl spacer to another substituted piperazine system and were tested for their binding properties on human cloned 5-HT$_{1A}$ and 5-HT$_7$ serotonin receptors. Among these, phenyl, 4- or 2-chlorophenyl, 2-methoxyphenyl, 2-pyridyl, and 2-pyrimidyl derivatives **15**, **24**, **25**, and **27**-**29** displayed nanomolar affinity values for the 5-HT$_7$ receptor (K_i 24.2-52.0 nM) and no affinity for the 5-HT$_{1A}$ receptor.

Keywords: Bivalent ligand approach; dual ligands; selective 5-HT$_7$R ligands; bis-piperazines; binding properties.

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[#] These authors contributed equally to the work.

The 1-arylpiperazine scaffold is one of the most studied in medicinal chemistry. It is present in many agents acting on CNS, which possess a broad range of receptors targets including serotoninergic, adrenergic, and dopaminergic receptors. Several studies of various classes of ligands with an arylpiperazine moiety in their structure have been reported, and these effort have led to successful drugs such as buspirone, perospirone, ziprasidone, and aripiprazole.[1-4] Generally, CNS agents with arylpiperazine-based template are characterized by three structural moieties (*i.e.* substituted phenyl ring linked to the piperazine; alkyl chain with different length; heterocyclic terminal fragment), which can be modified to modulate specific properties such as pharmacokinetic, affinity, and selectivity for different targets (Fig.1).

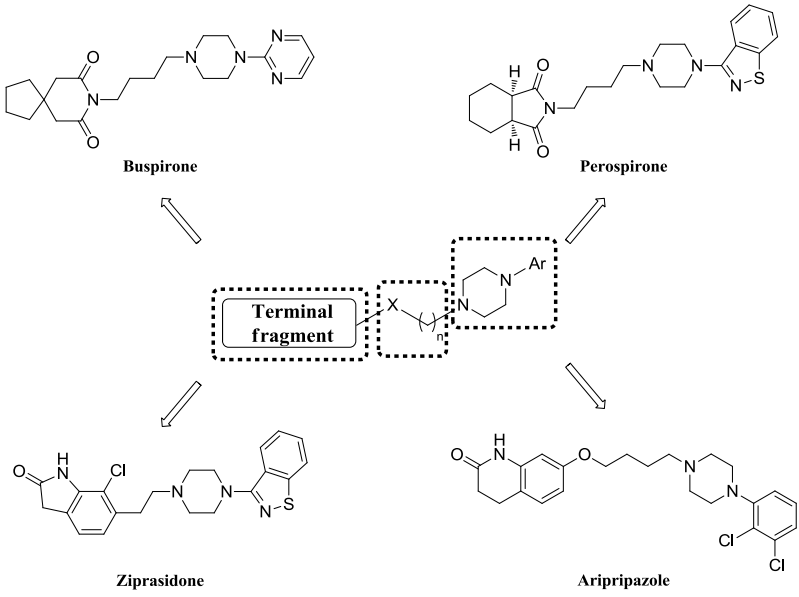


Figure 1. Structure of some commercial drugs containing the arylpiperazine template. The three structural moieties are marked by the dotted boxes.

Regarding the large family of serotonin receptors, several 1-arylpiperazine derivatives belonging to the LCAPs class have been developed, in particular they have been widely used as 5-HT_{1A}R and 5-HT_7R ligands.[5, 6] These serotonin receptor subtypes are co-expressed in major brain structures (limbic areas, hippocampus, and raphe nuclei), however they differ for the type of G protein-coupled, thus G_i for 5-HT_{1A}R and G_s for 5-HT_7R. For that reason, they modulate the same second messenger systems even though in the opposite way and could be involved in an interesting functional cross-talk between them as it has been

suggested.[7] The 5-HT_7R was the last discovered member of serotonin receptors and the interest in this receptor is due to its involvement in disorders like anxiety, schizophrenia, and depression.[8, 9] In addition, an increasing number of studies demonstrates a role for the 5-HT_7R on cognitive processes (particularly on hippocampal-dependent learning and memory) and in the regulation of structural plasticity in adolescent and mature brain circuits.[10-13]

In the frame of our study on serotonin receptor ligands we developed different series of 5-HT_{1A}R and 5-HT_7R ligands containing the arylpiperazine scaffold.[14, 15] More recently, our effort was focused on the discovery of 5-HT_7R selective ligands over the 5-HT_{1A}R, since the level of homology of their transmembrane domains is fairly high.[16]

In this work, the new compounds were designed according to the "bivalent ligand" approach, an extensive method used to achieve the selectivity among different receptor subtypes. There are several papers describing the "bivalent ligand" approach, which have produced good results in terms of affinity and selectivity,[17-20] and to the best of our knowledge, in the field of the 5-HT_7/5-HT_{1A} receptor ligands it was used only once before.[21]

Initially, we synthesized the 1-(4-phenyl-1-piperazinyl)-3-(4-phenyl-1-piperazinyl)-1-propanone and the 1-(4-phenyl-1-piperazinyl)-5-(4-phenyl-1-piperazinyl)-1-pentanone (**13** and **14**) for the purposes of evaluating the effect of the linker length between the two phenylpiperazines moiety. Both, propanone and pentanone analogous, possess the essential triplet (PI, ARn, and X) proposed for the pharmacophore model of the 5-HT_7R antagonism by Kołaczkowski *et al*. in 2006 (Fig. 2).[22]

Subsequently, setting the propionyl as the better linker chain, we introduced different substituents at the HYD/Ar_2 domain. Then, we chose the benzyl system in Ar_2 and we decided to use different substituents at the Ar_1 domain of piperazine such as: 4-, 3- and 2-ClC_6H_4, 4- and 2-$MeOC_6H_4$, 2-pyridyl, and 2-pyrimidyl (Fig. 3, compounds **13-29**). Moreover, we synthesized two derivatives containing only one piperazine moiety (Fig. 3, compounds **30** and **32**) and finally one derivative in which we substituted both piperazine moieties with two benzylamines (Fig. 3, compound **33**).

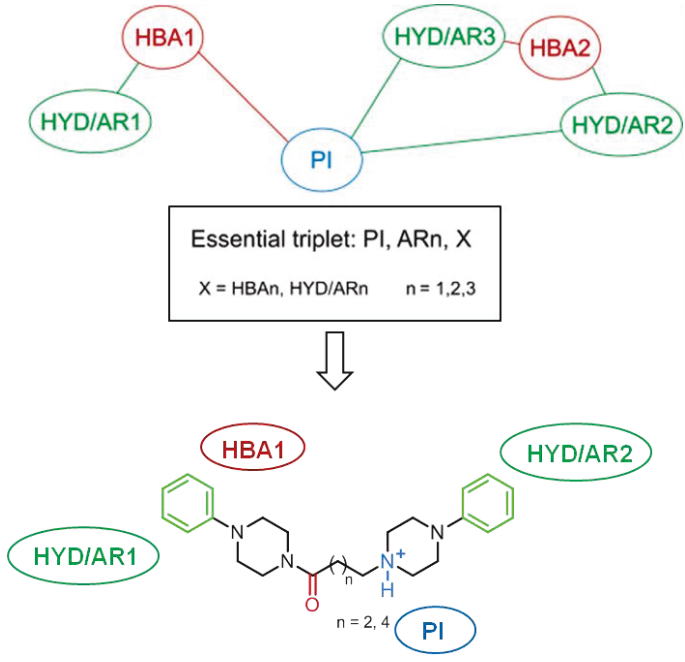
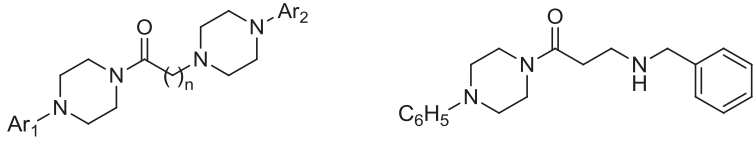
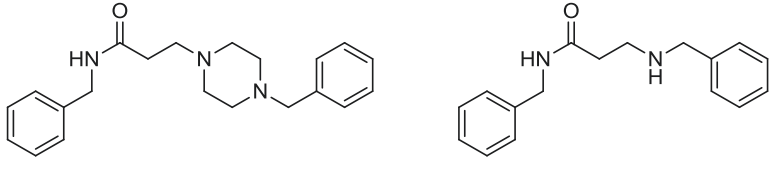


Figure 2. Pharmacophore model proposed for 5-HT_7R antagonism (Kołaczkowski *et al*., 2006) and general structure of the new bivalent ligands. The essential triplet was included in the title compounds: protonated nitrogen (positive ion, PI), hydrophobic/aromatic region (HYD/AR), and H-bond acceptor (HBA).



13-29 **30**



32 **33**

Figure 3. Structure of new synthesized compounds.

The straightforward synthesis of compounds **13**-**29**, **30**, **32**, and **33** is summarized in Scheme 1. Commercially available phenylpiperazines free bases **1**, **3**, and hydrochloride salt **2** reacted with 3-chloropropionyl chloride or 5-chloropentanoyl chloride in dichloromethane and in presence of potassium carbonate to give chloro derivative **5**, **8**, and **12**. Chloro derivatives **4**, **6**, **7**, **9**-**11** are prepared following known procedures.[23-25] Final compounds **13**-**29** and **32** were prepared from chloro derivatives **4**-**12** by reacting with an excess of the properly substituted piperazine using microwave irradiation at 90 °C for 30-60 min in the presence of potassium carbonate, a catalytic amount of potassium iodide, and acetonitrile as solvent. Compounds **30** and **33** were obtained from chloro derivative **4**[23] and **31**,[26] respectively, by reacting with an excess of benzylamine at reflux without solvent. Compound **33**[27] was claimed in a patent but its preparation and the experimental properties were not reported.

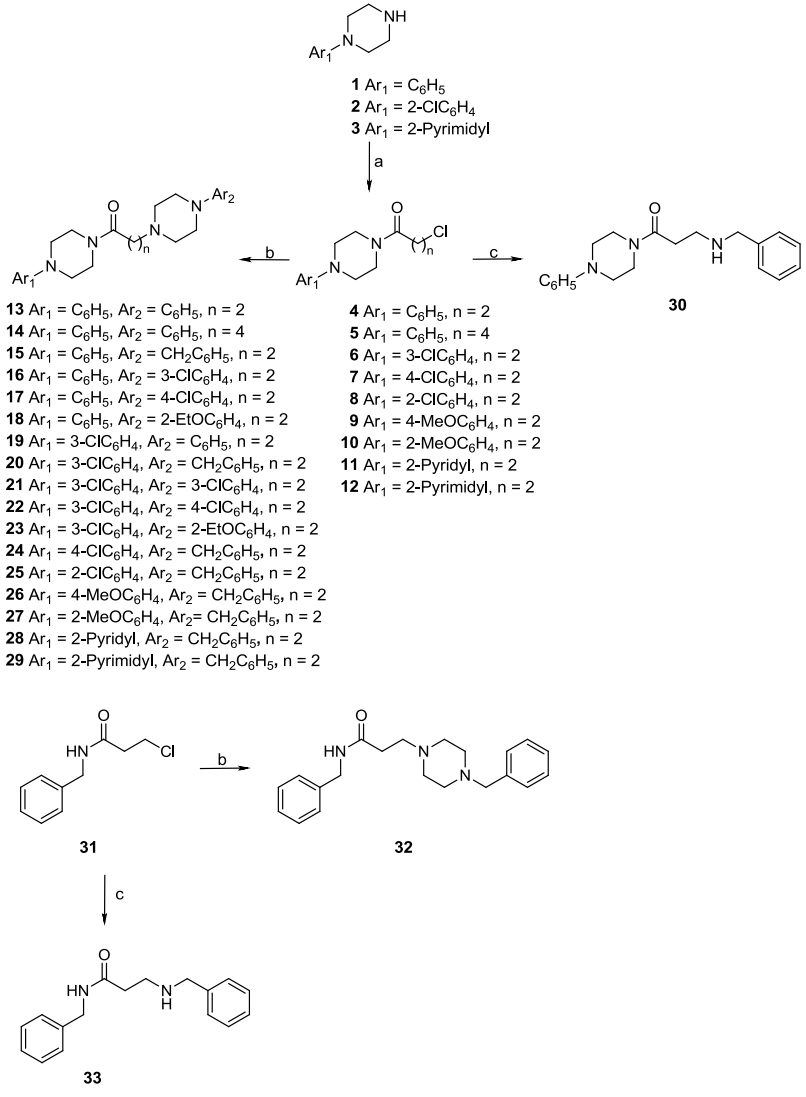
Radioligand binding assays were performed on new derivatives **13**-**30**, **32**, and **33** in order to determine the affinity and selectivity profile of the synthesized compounds for cloned human 5-HT_{1A} and 5-HT_7 receptors expressed in CHO-K1 cells. According to the previously reported procedure,[16] the experiments were carried out using [^{3}H]-5-HT and [^{3}H]-8-OH-DPAT as radioligands for 5-HT_7R and 5-HT_{1A}R, respectively (Table 1).

Looking at the binding data, it emerges that some derivatives act as high-affinity selective ligands for the 5-HT_7R over 5-HT_{1A}R (compounds **15**, **24**, **25**, **27**-**29**, K_i range values from 23.5 to 52.0 nM), in particular, among them compound **29** showed the best affinity value. So, these results led us to outline some structure-affinity relationships.

The unsubstituted phenylpiperazine **13** displayed good selectivety for the 5-HT_7R over 5-HT_{1A}R (K_i = 26.2 and 272 nM, respectively), while the 3-chlorophenylpiperazine analogous **19** did not show affinity for the 5-HT_7R and showed very low affinity for the 5-HT_{1A}R. Elongation of the acyl spacer from two to four methylene units (compounds **13** and **14**) decreased the affinity for both receptors (Table 1).

The benzyl derivative **15** displayed a good affinity for the 5-HT_7R (K_i = 52.0 nM) and no affinity for the 5-HT_{1A}R being one of the selective 5-HT_7R ligand in the series. The 3-chlorophenyl analogous **20** retained the affinity for the 5-HT_7R, but not the selectivity over the 5-HT_{1A}R (Table 1). A similar trend was observed for all 3-chlorophenyl derivatives **19**-**23** (Table 1). Therefore, the introduction of a 3-chloro atom on the phenyl moiety of the terminal fragment was detrimental for affinity and selectivity to 5-HT_7R.

Replacement of the phenyl (HYD/Ar_2) with a benzyl led to high increase of selectivity for 5-HT_7R over 5-HT_{1A}R (**13** *vs* **15**, Table 1).



Scheme 1. Reagents and conditions: (a) 3-chloropropionyl chloride or 5-chloropentanoyl chloride, CH_2Cl_2, 2 or 4 hours, reflux or room temperature; (b) substituted piperazine, K_2CO_3, KI, CH_3CN, mw, 90 °C, 30-60 min; (c) excess of benzylamine, reflux.

Table 1. Binding properties of derivatives **13-30**, **32**, **33**, and reference compounds SB 269970, 8-OH-DPAT, and 5-HT.

Comp.	Ar_1	Ar_2	n	K_i[a] (nM)	
				5-HT_7	5-HT_{1A}
13	C_6H_5	C_6H_5	2	26.2 ± 5.7	272 ± 19
14	C_6H_5	C_6H_5	4	197 ± 18	350 ± 46
15	C_6H_5	$CH_2C_6H_5$	2	52.0 ± 15	NA[b]
16	C_6H_5	3-ClC_6H_4	2	32.7 ± 5.8	33.5 ± 4.1
17	C_6H_5	4-ClC_6H_4	2	462 ± 114	1684 ± 313
18	C_6H_5	2-$EtOC_6H_4$	2	80.3 ± 16	81.8 ± 15
19	3-ClC_6H_4	C_6H_5	2	NA[b]	1074 ± 75
20	3-ClC_6H_4	$CH_2C_6H_5$	2	54.7 ± 9.2	101± 18
21	3-ClC_6H_4	3-ClC_6H_4	2	2761 ± 828	142 ± 11
22	3-ClC_6H_4	4-ClC_6H_4	2	2366 ± 545	302 ± 38
23	3-ClC_6H_4	2-$EtOC_6H_4$	2	119 ± 23	15.2 ± 2.2
24	4-ClC_6H_4	$CH_2C_6H_5$	2	36.6 ± 3.92	NA[b]
25	2-ClC_6H_4	$CH_2C_6H_5$	2	50.2 ± 12.3	NA[b]
26	4-$MeOC_6H_4$	$CH_2C_6H_5$	2	NA[b]	NA[b]
27	2-$MeOC_6H_4$	$CH_2C_6H_5$	2	24.2 ± 4.34	NA[b]
28	2-Pyridyl	$CH_2C_6H_5$	2	29.5 ± 8.21	NA[b]
29	2-Pyrimidyl	$CH_2C_6H_5$	2	23.5 ± 2.32	NA[b]
30	-	-	-	NA[b]	938 ± 126
32	-	-	-	4.34 ± 0.21	0.34 ± 0.04
33	-	-	-	176 ± 36.1	3750 ± 521
SB-269970				0.71 ± 0.06	9024 ± 181
8-OH-DPAT				388 ± 58	2.65 ± 0.10
5-HT				2.12 ± 0.41	0.91 ± 0.10

[a]Each value is the mean ± SD of the data from three separate experiments.
[b]NA = < 50% inhibition at 10^{-5} M.

Taking into account the appropriate ionization states of the piperazine ring, we studied the influence of the PI on the piperazine in HYD/Ar_2 (Table 1). We selected compounds **13** and **15** in order to calculate the pK_a value and the percentage of the protonated species, setting the pH = 7.4 and using MarvinSketch 6.2.1.[28] Our outcomes showed that compound **13** was mainly protonated at nitrogen N_4 (84%, pK_a = 8.11). On the other hand, compound **15** was protonated primarily to nitrogen N_1 (47%, pK_a = 8.55). Accordingly, this could influence the binding mode of the new compounds inside the receptor cavity within transmembrane helices (TMHs) 2, 3, 7 and the first extracellular loop 1 (EL1) of the 5-HT_7R. In fact, an ionic interaction between the protonated nitrogen (N_1) of the

ligand and the Asp3.32 side chain should be formed, so as a result, the terminal fragment could be closer to the extracellular loop and interacts with amino acids, which are only on the 5-HT_7R (Trp63 from EL1 and Lys144, Cys146, and Leu148 from EL2), thus achieving the selectivity.[15] Consequently, the next step of the work was focused on the modification of the substituents at the piperazine, labeled as HYD/Ar_1 domain. The introduction of a 4-ClC_6H_4, 2-ClC_6H_4, 2-$MeOC_6H_4$, 2-pyridyl, and 2-pyrimidyl substituent on the piperazine (**24**, **25**, and **27**-**29**) led to high-affinity values for 5-HT_7R and, as expected, no affinity for 5-HT_{1A}R. On the contrary, the introduction of the 4-$MeOC_6H_4$ on the piperazine (compound **26**) led to a lack of affinity for both receptors. In particular, the affinity values were ranked: 2-pyrimidyl > 2-$MeOC_6H_4$ > 2-pyridyl > 4-ClC_6H_4 > 2-ClC_6H_4.

The introduction of a benzylamine in HYD/Ar_2 had significantly affected the binding to 5-HT_{1A}R, especially for 5-HT_7R (**13** *vs* **30**, Table 1).

Noteworthy, derivative **32** does not have selectivity towards either receptors subtypes, however, it possesses very low K_i value for the 5-HT_{1A}R (K_i = 0.34 nM). Actually, the lack of rigidity of the terminal fragment (generally a heterocyclic nucleus) in addition to the increase of spatial freedom of the phenyl ring led to a total loss of selectivity. Therefore, it acts as dual 5-HT_7R/5-HT_{1A}R ligand.

In conclusion, the bivalent ligand approach has been successfully applied to design and synthesize the bis-piperazine derivative **13**, which was subsequently optimized to achieve the selectivity for 5-HT_7R over the 5-HT_{1A}R. The introduction of different substituents in the HYD/Ar_2 domain has led to the identification of the highly-selective ligand **15**. The best result was obtained while keeping the benzyl in the HYD/Ar_2 domain and introducing further arylsubstituents (*i.e.* 4-ClC_6H_4, 2-ClC_6H_4, 2-$MeOC_6H_4$, 2-pyridyl, and 2-pyrimidyl) at HYD/Ar_1. In summary, we managed to reach our goal of developing more selective compounds. We obtained the benzylpiperazine derivatives **15**, **24**, **25**, and **27**-**29**, which possess good affinity and are selectivity for 5-HT_7R (K_i values from 23.5 to 52.0 nM) over 5-HT_{1A}R.

Acknowledgments

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5.1. References and notes

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5.2. Supplementary material

5.2.1. Experimental protocols

Melting points were determined in an Electrothermal IA9200 apparatus in glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin Elmer series FT-IR 1600 spectrometer in KBr disks. Elemental analyses for C, H, N, and S were within ± 0.4% of theoretical values and were performed on a Carlo Erba Elemental Analyzer Mod. 1108 apparatus. ^{1}H NMR spectra were recorded on a Varian Inova Unity 200 spectrometer (200 MHz) in DMSO-d_6 or $CDCl_3$ solution. Chemical shifts are given in δ values (ppm), using tetramethylsilane as the internal standard; coupling constants (*J*) are given in Hz. Signal multiplicities are characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). Microwave irradiation experiments were carried out with a CEM Discover instrument using closed Pyrex glass tubes (ca. 10 mL) with Teflon-coated septa. All the synthesized compounds were tested for purity on TLC (aluminium sheet coated with silica gel 60 F_{254}, Merck) and visualized by UV light (λ = 254 and 366 nm). Purification of synthesized compounds by flash column chromatography was performed using Merck silica gel (0.040-0.063 mm). All chemicals and solvents were reagent grade and were purchased from commercial vendors.

General procedure for the synthesis of chloro-1-(4-substituted-1-piperazinyl)-1-oxoalkanes (**5**, **8**, **12**). To a mixture of properly 1-substituted piperazine free bases **1** and **3** or hydrochloride salt **2** (9.23 mmol) and potassium carbonate (10.13 mmol) in dichloromethane (30 mL) was added 3-chloropropanoyl chloride or 5-chloropentanoyl chloride (10.19 mmol). The suspension was stirred for 4 hours at refluxed (compounds **5** and **8**) or for 2 hours at room temperature (compound **12**).

After being cooled (**5** and **8)** or stopped the stirring (**12**), the suspension was washed with water (20 mL × 3). The organic phase was dried on anhydrous sodium sulphate, filtered, and evaporated in vacuum to dryness. Using this procedure the following new compounds were obtained:

5-Chloro-1-(4-phenyl-1-piperazinyl)-1-pentanone (5). The title compound was obtained by recrystallization from n-hexane as a pure solid (77%), mp 73.6-73.9 °C. IR (KBr, selected lines) cm^{-1} 1639, 1597, 1498, 1464, 1443, 1233, 1201, 1019. ^{1}H NMR (DMSO-d_6) δ 1.50-1.88 (m, 2H + 2H, CH_2CH_2), 2.39 (t, J = 7.4 Hz, 2H, $COCH_2$), 3.00-3.20 (m, 4H, piperazine), 3.50-3.70 (m, 4H + 2H, piperazine + CH_2Cl), 6.75-6.88 (m, 1H, aromatic), 6.88-7.02 (m, 2H, aromatic), 7.15-7.30 (m, 2H, aromatic). Anal. ($C_{15}H_{21}ClN_2O$) C, H, N.

3-Chloro-1-[4-(2-chlorophenyl)-1-piperazinyl]-1-propanone (8). The title compound was purified by column chromatography using a mixture of cyclohexane/ethyl acetate (6:4, v/v) as eluent and was obtained as a pure oil (50%). IR (neat, selected lines) cm^{-1}1646, 1588, 1480, 1442, 1229, 1205, 1026, 762. ^{1}H NMR (DMSO-d_6) δ 2.80-3.05 (m, 2H + 4H, $COCH_2$ + piperazine), 3.56-3.70 (m, 4H, piperazine), 3.82 (t, J = 6.6 Hz, 2H, CH_2Cl), 7.00-7.20 (m, 2H, aromatic), 7.25-7.39 (m, 1H, aromatic), 7.39-7.47 (m, 1H, aromatic). Anal. ($C_{13}H_{16}Cl_2N_2O$) C, H, N.

3-Chloro-1-[4-(2-pyrimidyl)-1-piperazinyl]-1-propanone (12). The title compound was purified by column chromatography using ethyl acetate as eluent and was obtained as a pure solid (63%), mp 137.5-141 °C. IR (KBr, selected lines) cm^{-1} 1646, 1625, 1584, 1548, 1496, 1441, 1356, 1254, 981. ^{1}H NMR (DMSO-d_6) δ 2.90 (t, J = 6.6 Hz, 2H, $COCH_2$) 3.48-3.60 (m, 4H, piperazine), 3.60-3.80 (m, 4H, piperazine), 3.81 (t, J = 6.6 Hz, 2H, CH_2Cl), 6.6 (t, J = 4.8 Hz, 1H, aromatic), 8.38 (d, J = 4.6 Hz, 2H, aromatic). Anal. ($C_{11}H_{15}ClN_4O$) C, H, N.

General procedure for the synthesis of 1-(4-substituted-1-piperazinyl)-3-(4-substituted-1-piperazinyl)-1-propanones (13, 15-29), 1-(4-phenyl-1-piperazinyl)-3-(4-phenyl-1-piperazinyl)-1-pentanone (14) and *N*-(1-phenylmethyl)-4-(phenylmethyl)-1-piperazinepropanamide (32). Acetonitrile (3 mL) was added to a mixture of chroro derivative **2-4** or **16** (1.18 mmol), properly substituted piperazine (1.42 mmol), potassium carbonate (1.42 mmol), and of a catalytic amount of potassium iodide. The mixture and a magnetic bar was sealed in a Pyrex tube and was heated at 90 °C by microwave irradiation for 30-60 min (run time 3 min, microwave max power 150 W and max pressure 150 Psi). After being cooled, the solid was removed by filtration and the solution was concentrated to dryness. Using this procedure the following new compounds were obtained:

1-(4-Phenyl-1-piperazinyl)-3-(4-phenyl-1-piperazinyl)-1-propanone (13). The title compound was obtained by recrystallization from cyclohexane as a pure solid (65%), mp 107.0-107.9 °C. IR (KBr, selected lines) cm^{-1} 2820, 1635, 1599, 1498, 1465, 1451, 1237, 1006, 758. ^1H NMR (DMSO-d_6) δ 2.47-2.63 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N + piperazine), 3.02-3.19 (m, 8H, piperazine), 3.55-3.67 (m, 4H, piperazine), 6.70-6.85 (m, 2H, aromatic), 6.85-7.00 (m, 4H, aromatic), 7.17-7.28 (m, 4H, aromatic). Anal. ($C_{23}H_{30}N_4O$) C, H, N.

1-(4-Phenyl-1-piperazinyl)-5-(4-phenyl-1-piperazinyl)-1-pentanone (14). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluent and was obtained as a pure solid (28%), mp 75.0-76.9 °C. IR (KBr, selected lines) cm^{-1} 2938, 2819, 1651, 1630, 1560, 1496, 1445, 1235, 752. ^1H NMR ($CDCl_3$) δ 1.68-1.98 (m, 2H + 2H, CH_2 + CH_2), 2.46 (t, J = 6.6 Hz, 2H, $COCH_2$), 2.79 (t, J = 6.6 Hz, 2H, CH_2N), 2.96-3.06 (m, 4H, piperazine), 3.10-3.24 (m, 4H, piperazine), 3.38-3.50 (m, 4H, piperazine), 3.58-3.68 (m, 2H, piperazine), 3.72-3.83 (m, 2H, piperazine), 6.86-6.98 (m, 5H, aromatic), 7.23-7.37 (m, 5H, aromatic). Anal. ($C_{25}H_{34}N_4O$) C, H, N.

1-(4-Phenyl-1-piperazinyl)-3-[4-(phenylmethy)-1-piperazinyl]-1-propanone (15). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (8:2, v/v) as eluents and was obtained as a pure solid (32%), mp 69.3-71.2 °C. IR (KBr, selected lines) cm^{-1} 2713, 1628, 1595, 1448, 1235, 1155, 760, 730. ^1H NMR (DMSO-d_6) δ 2.30-2.70 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.03-3.20 (m, 4H, piperazine), 3.46 (s, 2H, $CH_2C_6H_5$), 3.50-3.65 (m, 4H, piperazine), 6.75-6.85 (m, 1H, aromatic), 6.85-7.00 (m, 2H, aromatic), 7.15-7.39 (m, 7H, aromatic). Anal. ($C_{24}H_{32}N_4O$) C, H, N.

1-(4-Phenyl-1-piperazinyl)-3-[4-(3-chlorophenyl)-1-piperazinyl]-1-propanone (16). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluents and was obtained as a pure solid (15%), mp 59.6-60.5 °C. IR (KBr, selected lines) cm^{-1} 2819, 1633, 1598, 1473, 1449, 1237, 758. ^1H NMR (DMSO-d_6) δ 2.42-2.70 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N + piperazine), 3.02-3.22 (m, 8H, piperazine), 3.55-3.70 (m, 4H, piperazine), 6.73-7.00 (m, 6H, aromatic), 7.15-7.30 (m, 3H, aromatic). Anal. ($C_{23}H_{29}ClN_4O$) C, H, N.

1-(4-Phenyl-1-piperazinyl)-3-[4-(4-chlorophenyl)-1-piperazinyl]-1-propanone (17). The title compound was obtained by recrystallization from n-hexane as a pure solid (41%), mp 115.2-116.6 °C. IR (KBr, selected lines) cm^{-1} 2808, 1625, 1598, 1496, 1436, 1232, 816, 754. ^1H NMR (DMSO-d_6) δ 2.43-2.65 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N +

piperazine), 3.02-3.20 (m, 8H, piperazine), 3.50-3.68 (m, 4H, piperazine), 6.76-6.85 (m, 1H, aromatic), 6.85-7.00 (m, 4H, aromatic), 7.15-7.28 (m, 4H, aromatic). Anal. ($C_{23}H_{29}ClN_4O$) C, H, N.

1-(4-Phenyl-1-piperazinyl)-3-[4-(2-ethoxyphenyl)-1-piperazinyl]-1-propanone (18). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluents and was obtained as a pure solid (66%), mp 85.6-86.7 °C. IR (KBr, selected lines) cm^{-1} 2820, 1645, 1598, 1498, 1446, 1236, 1156, 1122, 756. ^1H NMR (DMSO-d_6) δ 1.33 (t, J = 7.0 Hz, 3H, CH_2CH_3), 2.43-2.67 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N + piperazine), 2.90-3.02 (m, 4H, piperazine), 3.02-3.20 (m, 4H, piperazine), 3.50-3.70 (m, 4H, piperazine), 3.99 (q, J = 7.0 Hz, 2H, CH_2CH_3), 6.74-7.00 (m, 7H, aromatic), 7.18-7.30 (m, 2H, aromatic). Anal. ($C_{25}H_{34}N_4O_2$) C, H, N.

1-[4-(3-Chlorophenyl)-1-piperazinyl]-3-(4-phenyl-1-piperazinyl)-1-propanone (19). The title compound was purified column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluents and was obtained as a pure solid (40%), mp 171.8-172.9 °C. IR (KBr, selected lines) cm^{-1} 2830, 1643, 1594, 1497, 1442, 1283, 1233, 773, 760. ^1H NMR (DMSO-d_6) δ 2.43-2.62 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N + piperazine), 3.02-3.24 (m, 8H, piperazine), 3.55-3.65 (m, 4H, piperazine), 6.70-6.85 (m, 2H, aromatic), 6.85-7.00 (m, 4H, aromatic), 7.16-7.28 (m, 3H, aromatic). Anal. ($C_{23}H_{29}ClN_4O$) C, H, N.

1-[4-(3-Chlorophenyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (20). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluent and was obtained as a pure oil (68%). IR (neat, selected lines) cm^{-1} 2813, 1644, 1594, 1486, 1444, 1232, 1155, 1009, 737. ^1H NMR (DMSO-d_6) δ 2.32-2.65 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.10-3.25 (m, 4H, piperazine), 3.45 (s, 2H, $CH_2C_6H_5$), 3.50-3.62 (m, 4H, piperazine), 6.79-7.00 (m, 3H, aromatic), 7.19-7.38 (m, 6H, aromatic). Anal. ($C_{24}H_{31}ClN_4O$) C, H, N.

1-[4-(3-Chlorophenyl)-1-piperazinyl]-3-[4-(3-chlorophenyl)-1-piperazinyl]-1-propanone (21). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluent and was obtained as a pure solid (50%), mp 201.6-202.6 °C. IR (KBr, selected lines) cm^{-1} 1636, 1593, 1560, 1489, 1438, 1235. ^1H NMR (DMSO-d_6) δ 2.43-2.75 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N + piperazine), 3.02-3.29 (m, 8H, piperazine), 3.55-3.65 (m, 4H, piperazine), 6.75-6.85 (m, 2H, aromatic), 6.85-7.00 (m, 4H, aromatic), 7.16-7.29 (m, 2H, aromatic). Anal. ($C_{23}H_{28}Cl_2N_4O$) C, H, N.

1-[4-(3-Chlorophenyl)-1-piperazinyl]-3-[4-(4-chlorophenyl)-1-piperazinyl]-1-propanone (22). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluents and was obtained as a pure solid (40%), mp 179.0-181.4 °C. IR (KBr, selected lines) cm^{-1} 1648, 1591, 1496, 1442, 1227, 823, 773. ^1H NMR ($CDCl_3$) δ 3.10-3.30 (m, 2H + 4H, $COCH_2$ + piperazine), 3.30-3.42 (m, 4H, piperazine), 3.51 (t, J = 6.4 Hz, 2H, CH_2N), 3.56-3.62 (m, 4H, piperazine), 3.62-3.80 (m, 4H, piperazine), 6.70-6.90 (m, 5H, aromatic), 7.13-7.28 (m, 3H, aromatic). Anal. ($C_{23}H_{28}Cl_2N_4O$) C, H, N.

1-[4-(3-Chlorophenyl)-1-piperazinyl]-3-[4-(2-ethoxyphenyl)-1-piperazinyl]-1-propanone (23). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (9:1, v/v) as eluent and was obtained as an oil (80%). IR (neat, selected lines) cm^{-1} 1643, 1594, 1499, 1447, 1265, 1239, 738, 704. ^1H NMR (DMSO-d_6) δ 1.33 (t, J = 6.8 Hz, 3H, CH_2CH_3), 2.43-2.62 (m, 2H + 2H + 4H, $COCH_2$ + CH_2N + piperazine), 2.90-3.05 (m, 4H, piperazine), 3.10-3.25 (m, 4H, piperazine), 3.50-3.65 (m, 4H, piperazine), 4.00 (q, J = 7.0 Hz, 2H, CH_2CH_3), 6.77-7.00 (m, 7H, aromatic), 7.18-7.30 (m, 1H, aromatic). Anal. ($C_{25}H_{33}ClN_4O_2$) C, H, N.

1-[4-(4-Chlorophenyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (24). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent and was obtained as a pure solid (64%), mp 141.2-143.0 °C. IR (KBr, selected lines) cm^{-1} 2808, 2767, 1649, 1496, 1441, 1229, 1205, 1158, 1011, 738. ^1H NMR (DMSO-d_6) δ 2.22-2.45 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.00-3.20 (m, 4H, piperazine), 3.41 (s, 2H, $CH_2C_6H_5$), 3.50-3.65 (m, 4H, piperazine), 6.90-7.05 (m, 2H, aromatic), 7.18-7.40 (m, 2H + 5H, aromatic). Anal. ($C_{24}H_{31}ClN_4O$) C, H, N.

1-[4-(2-Chlorophenyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (25). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (8:2, v/v) as eluent and was obtained as a pure solid (60%), mp 93.6-95.8 °C. IR (KBr, selected lines) cm^{-1} 2811, 1644, 1479, 1435, 1266, 1222, 1147, 1007, 765. ^1H NMR (DMSO-d_6) δ 2.25-2.65 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 2.82-3.05 (m, 4H, piperazine), 3.44 (s, 2H, $CH_2C_6H_5$), 3.50-3.65 (m, 4H, piperazine), 7.00-7.48 (m, 4H + 5H, aromatic). Anal. ($C_{24}H_{31}ClN_4O$) C, H, N.

1-[4-(4-Methoxyphenyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (26). The title compound was obtained by recrystallization from cyclohexane as a pure solid (50%), mp 127.0-129.7 °C. IR (KBr, selected lines) cm^{-1} 2807, 1637, 1511, 1458, 1247, 1033,

1010, 823. ^{1}H NMR (DMSO-d_6) δ 2.25-2.60 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 2.85-3.05 (m, 4H, piperazine), 3.43 (s, 2H, $CH_2C_6H_5$), 3.50-3.65 (m, 4H, piperazine), 3.68 (s, 3H, OCH_3), 6.78-6.98 (m, 4H, aromatic), 7.20-7.40 (m, 5H, aromatic). Anal. ($C_{25}H_{34}N_4O_2$) C, H, N.

1-[4-(2-Methoxyphenyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (27). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent and was obtained as a pure semisolid product (72%). IR (neat, selected lines) cm^{-1} 2952, 2816, 1639, 1503, 1439, 1237, 1156, 1031, 1008, 755. ^{1}H NMR (DMSO-d_6) δ 2.25-2.60 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 2.80-3.00 (m, 4H, piperazine), 3.43 (s, 2H, $CH_2C_6H_5$), 3.50-3.65 (m, 4H, piperazine), 3.78 (s, 3H, OCH_3), 6.80-7.05 (m, 4H, aromatic), 7.15-7.20 (m, 5H, aromatic). Anal. ($C_{25}H_{34}N_4O_2$) C, H, N.

1-[4-(2-Pyridyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (28). The title compound was obtained by recrystallization from cyclohexane as a pure solid (40%), mp 67.4-69.8 °C. IR (KBr, selected lines) cm^{-1} 2812, 1611, 1483, 1426, 1252, 1007, 743. ^{1}H NMR (DMSO-d_6) δ 2.10-2.60 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.40-3.62 (m, 2H + 8H, $CH_2C_6H_5$ + piperazine), 6.60-6.70 (m, 1H, aromatic), 6.80-6.90 (m, 1H, aromatic), 7.18-7.40 (m, 5H, aromatic), 7.50-7.61 (m, 1H, aromatic), 8.05-8.17 (m, 1H, aromatic). Anal. ($C_{23}H_{31}N_5O$) C, H, N.

1-[4-(2-Pyrimidyl)-1-piperazinyl]-3-[4-(phenylmethyl)-1-piperazinyl]-1-propanone (29). The title compound was obtained by recrystallization from cyclohexane as a pure solid (28%), mp 102.9-104.7 °C. IR (KBr, selected lines) cm^{-1} 2812, 1636, 1585, 1546, 1490, 1438, 1357, 1258, 982. ^{1}H NMR (DMSO-d_6) δ 2.20-2.60 (m, 2H + 2H + 8H, $COCH_2$ + CH_2N + piperazine), 3.43 (s, 2H, $CH_2C_6H_5$), 3.45-3.60 (m, 4H, piperazine), 3.60-3.83 (m, 4H, piperazine), 6.66 (t, J = 4.8 Hz, 1H, aromatic), 7.18-7.20 (m, 5H, aromatic), 8.38 (t, J = 4.6 Hz, 2H, aromatic). Anal. ($C_{22}H_{30}N_6O$) C, H, N.

***N*-(1-Phenylmethyl)-3-(4-benzylpiperazin-1-yl)propanamide (32)**. The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (8:2, v/v) as eluent and was obtained as a pure solid (19%), mp 62.3-62.8 °C. IR (KBr, selected lines) cm^{-1} 3386, 2806, 1656, 1633, 1536, 1452, 1295, 1154, 1011, 739. ^{1}H NMR (DMSO-d_6) δ 2.32 (t, J = 6.8 Hz, 2H, $COCH_2$), 2.33-2.45 (m, 8H, piperazine), 2.54 (t, J = 6.8 Hz, 2H, CH_2N), 3.45 (s, 2H, $CH_2C_6H_5$), 4.26 (d, J = 5.8 Hz, 2H, $CH_2NHC_6H_5$), 7.18-7.39 (m, 5H + 5H, aromatic), 8.22 (t, J = 5.8 Hz, 1H,

NH). Anal. ($C_{21}H_{27}N_3O$) C, H, N.

General procedure for the synthesis of *N*-(1-phenylmethyl)-3-substitutedpropanamides (30) and (33). A mixture of the appropriate chroro derivative **4** or **31** (3.96 mmol) and benzylamine (18.31 mmol) was refluxed under stirring for 1-2 hours until a sticky residue was obtained. After being cooled, water was added to the residue and the mixture was dissolved with ethyl acetate and washed with water (20 mL × 3). The organic phase was dried on anhydrous sodium sulphate, filtered, and concentrated in vacuum to dryness. Using this procedure the following new compounds were obtained:

3-(Phenylmethyl)-1-(4-phenylpiperazin-1-yl)propan-1-one (30). The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent and was obtained as a pure oil (44%). IR (neat, selected lines) cm^{-1} 2823, 1640, 1598, 1495, 1441, 1231, 1027, 759. ^1H NMR (DMSO-d_6) δ 2.45-2.60 (m, 2H, $COCH_2$), 2.70 (t, J = 7.0 Hz, 2H, CH_2NH), 3.00-3.18 (m, 4H, piperazine), 3.52-3.65 (m, 4H, piperazine), 3.69 (s, 2H, $CH_2C_6H_5$), 6.75-6.85 (m, 1H, aromatic), 6.85-7.00 (m, 2H, aromatic), 7.15-7.39 (m, 7H, aromatic). Anal. ($C_{20}H_{25}N_3O$) C, H, N.

***N*-(Phenylmethyl)-3-[(phenylmethyl)amino]propanamide (33)**. The title compound was purified by column chromatography using a mixture of ethyl acetate/methanol (7:3, v/v) as eluent and was obtained as a pure oil (65%). IR (neat, selected lines) cm^{-1} 3298, 3029, 2833, 1645, 1547, 1454, 1452, 1029, 740. ^1H NMR (DMSO-d_6) δ 2.31 (t, J = 6.8 Hz, 2H, $COCH_2$), 2.72 (t, J = 7.0 Hz, 2H, CH_2NH), 3.68 (s, 2H, $CH_2NHCH_2C_6H_5$), 4.26 (d, J = 6.0 Hz, 2H, $CONHCH_2C_6H_5$), 7.15-7.38 (m, 5H + 5H, aromatic), 8.45 (t, J = 5.6 Hz, 1H, NH). Anal. ($C_{17}H_{20}N_2O$) C, H, N.

5.2.2. In vitro binding assays

The binding assays were performed using human cloned 5-$HT_{7(a)}$ and 5-HT_{1A} serotonin receptors (PerkinElmer) expressed on CHO-K1 cells. Radioligand binding assay on 5-HT_7 receptors[1] was carried out in a final incubation volume of 0.51 mL consisting of 250 µL of membrane suspension (15 µg protein/sample in Tris HCl, 50 mM, pH 7.4 containing 10 µM pargiline, 4 mM $MgCl_2$ and 0.05% ascorbic acid,), 250 µL of [^3H]-5-HT (final concentration 5 nM, s.a. 106 Ci/mmol, PerkinElmer) in the same buffer used for membrane suspension and 10 µL of tested compounds. Nonspecific binding was obtained in the presence of 10 µM serotonin. Binding assay on 5-HT_{1A} receptors[2] was carried out in a final incubation volume of 0.51 mL consisting of 250 µL of membrane suspension (10 µg protein/sample in Tris HCl, 50 mM, pH 7.4 containing

10 μM pargiline and 4 mM $MgCl_2$), 250 μL of [^{3}H]-8-OH-DPAT (final concentration 1 nM, s.a. 137 Ci/mmol, PerkinElmer) in the same buffer used for membrane suspension and 10 μL of tested compounds. Nonspecific binding was obtained in the presence of 1 μM serotonin. Incubations (30 min at 25 °C) were stopped by rapid filtration under vacuum, through GF/C filters (pre-soaked with 0.3% PEI) for 5-HT_7 receptors or GF/B filters for 5-HT_{1A} receptors, which were then washed with 12 mL (4 × 3 times) of ice-cold buffer (Tris HCl, 50 mM, pH 7.4) using a Brandel M-48R cell harvester. The radioactivity trapped on the filters was counted in 4 mL of Ultima Gold MV (Packard) in a Tri-carb 2800 TR (PerkinElmer) liquid scintillation spectrometer with a counting efficiency of 60%. A concentration range from 10^{-5} to 10^{-10} M were used for testing all compounds in triplicate and dose-inhibition curves were analyzed by the "Allfit" program to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding.[3] According to the method of Cheng and Prusoff,[4] the K_i values were derived from IC_{50} values.

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6. Conclusions

In this thesis I have addressed the serotoninergic system and in particular my efforts were focused on the selectivity issue for two receptor subtypes: the 5-HT_7R and the 5-HT_{1A}R.

The primary objective of my research work has been to identify novel thienopyrimidinone and quinazolinone derivatives with the purpose of exploring how some structural changes in the terminal fragment, in the alkyl chain length, and in the substituents on the piperazine fragment could influence affinity and selectivity for 5-HT_{1A}R and 5-HT_7R. The 2-ethoxy derivatives **40** and **45** (Table 1, pag. 55) were the best ligands in the series, showing high affinity for both receptors, but they did not display any 5-HT_7R selectivity over the 5-HT_{1A}R. In addition, preliminary data on functional activity indicate that compounds **40** and **45** act as antagonists at 5-HT_7R.

Secondly, further investigation on the quinazolinone system revealed that the new compounds **8**, **11**, **13**, **17**, **18**, and **20** (Table 2, pag. 81) showed affinity values for the 5-HT_7R in the nanomolar range. They also have comparable affinity for the 5-HT_{1A}R, thus acting as dual 5-HT_7R/5-HT_{1A}R ligands. The docking studies on the receptor homology models outlined a similar ligands conformation. This study confirmed that L-shape was more suitable than extended conformation for 5-HT_7R and in addition it was established that a planar bicyclic system (quinazoline) was preferable over a single heterocyclic ring with a bulky substituent (phenyl-pyrimidine) in the interaction with both receptors. The simultaneous preparation and isolation of *N*- and *O*-alkylated pyrimidine and 2-methylquinazoline derivatives was described, which might be used as intermediate products for the synthesis of novel potential biological agents.

Finally, new 1-(4-aryl-1-piperazinyl)-3-[4-(phenylmethy)-1-piperazinyl]-1-propanone derivatives were designed, according to the "bivalent ligand" approach. Among the new compounds, phenyl, 4- or 2-chlorophenyl, 2-methoxyphenyl, 2-pyridyl, and 2-pyrimidyl derivatives **15**, **24**, **25**, and **27-29** (Table 1, pag. 107) displayed nanomolar affinity values for the 5-HT_7R (K_i 24.2-52.0 nM) and no affinity for the 5-HT_{1A}R. The most important SARs, concerning the new bis-piperazine derivatives, were outlined. In particular, the outcomes showed that the introduction of the benzyl group in the HYD/Ar_2 domain was essential for the selectivity for 5-HT_7 over 5-HT_{1A} receptors.

In conclusion, I managed to reach my goal of developing selective 5-HT_7R ligands.

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