CHAPTER II

INTERACTION OF BIVALENT LIGANDS WITH 5-HT$_3$ RECEPTOR

2.1 New antagonists and the multivalency in 5-HT$_3$

Application of selective 5-HT$_3$ agonists like 2-methyl-5-hydroxytryptamine, phenylbiguanide and *meta*-chlorophenylbiguanide (mCPBG) (Figure 8)causes unfavourable effects like nausea and anxiety. As previously mentioned, no therapeutic use of the agonists is targeted at present. In contrast, several 5-HT$_3$ antagonists are currently available on the market: tropisetron, ondansetron, granisetron, dolasetron, palonosetron. All of these are highly potent compounds which can induce complete competitive block of peripheral and central 5-HT$_3$ receptors, showing noticeable efficacy in preventing acute CINV and PONV. The research activity performed on 5-HT$_3$ inhibitors is still being developed, producing drug molecules that show efficacy at the 5-HT$_3$R level.

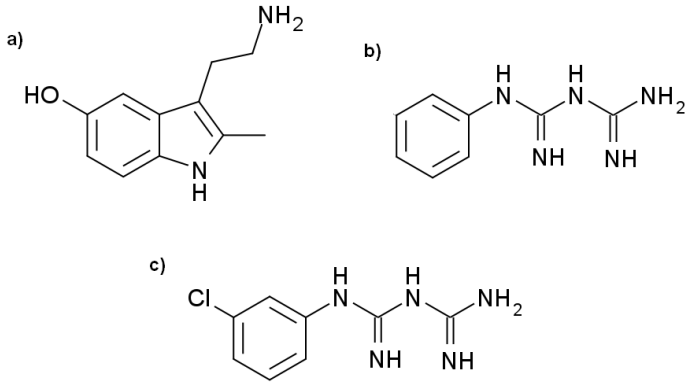

Figure 8- Structure of the 5-HT3 agonists: a) 2-methyl-5-hydroxytryptamine; b) phenylbiguanide; c) *meta*-chlorophenylbiguanide (mCPBG).

Simple 3-point pharmacophoric models were proposed for a variety of 5-HT$_3$ antagonists. Particularly, potent 5-HT$_3$ antagonists comprise three necessary components [Clark *et al.*, 1993]: an aromatic ring, a carbonyl group or H-bond acceptor attached to the aromatic ring (HA), and a basic nitrogen, as shown in Figure 9. Subsequently, it has been suggested the presence of two important hydrophobic sites where the aromatic ring acts as a "spacer" for 5-HT$_3$ antagonists to reach the hydrophobic sites [Heidempergher *et al.*, 1997]. The scope of hydrophobic sites was further explored by

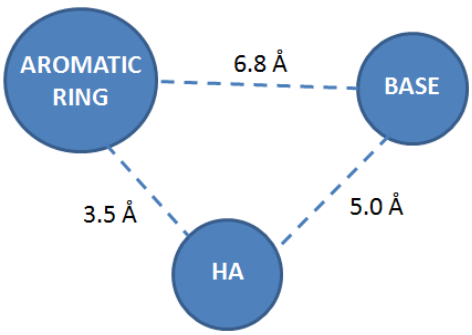

Figure 9- 3-ponit pharmacophoric model for the 5-HT$_3$ receptor.

Cappelli and co-workers [Cappelli *et al.*, 1998] through extensive studies to map the binding interactions of arylpiperazine derivatives.

The interest of the research group of Professor Cappelli in the development of 5-HT$_3$R ligands stems from the study of arylpiperazine derivatives related to quipazine and has produced a considerable amount of information on the interaction of this class of ligands with their receptor, including SAFIR (structure affinity relationship) and SAR (structure–activity relationship) data [Cappelli *et al.,* 2002, 2005].

Interesting, Cappelli and co-workers [Cappelli *et al.*, 2010] described the design, synthesis and preliminary pharmacological characterization of the tacrine-related heterobivalent ligand (see below, compound **2b**; Figure 7)**,** a compound that showed a nanomolar potency for both 5-HT$_3$ receptor and human acetylcholinesterase (AChE) and represented the first example of a rationally designed high affinity 5-HT$_3$ receptor ligand showing nanomolar AChE inhibitory activity. During the structure-activity study of granisetron derivates carried out by Vernekar and co-workers [Vernekar *et al.*, 2010], a similar compound, showing nanomolar potency for 5-HT$_3$R, was synthesised (Figure 10). In particular, the N1-position of granisetron was supposed to be the most appropriate for substitution of large biophysical tags, and this hypothesis was confirmed by attaching a fluorophore at that position to create a fluorescent analogue. This represents a further confirmation of the possibility to functionalise ligands according to the various targets.

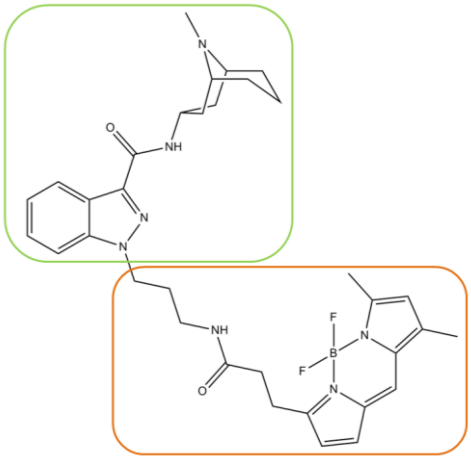

Figure 10- Structure of the fluorescent analogue of granisetron, composed by granisetron (in the green square) and the fluorophore (in the orange square).

Furthermore, recently a non-covalent approach to dynamic multivalent complexes has been reported [Galeazzi *et al.*, 2010]. The complexes are composed of a hydrophobic urea adamantyl-functionalised poly(propylene imine) dendrimer, a solubilising

oligo(ethylene glycol)-based guest molecule, and an arylpiperazine heterobivalent 5-HT$_3$ receptor ligand (see below, compound **3b,d**) [Galeazzi *et al*., 2010]. This work opened new perspectives in fabricating dynamic and adjustable non-covalent complexes showing a pharmacological potential. However, the stability of the supramolecular complexes in the biological environment should be increased further to allow for the in vivo evaluation of the construct. From that arose the interest in preparing new guest molecules showing an increased interaction strength with the host and producing supramolecular constructs able to bind multiple biological receptors simultaneously (multivalent binding) in order to explore multivalency in 5-HT$_3$ receptor.

2.2 Bivalent ligands

Appropriately designed bivalent ligands can be used as tools for a large variety of studies involving the 5-HT$_3$ receptor, however the rational design of tailored bivalent 5-HT$_3$ receptor ligands requires a deep understanding of their interaction with the receptor.

In this thesis we focused on the characterization of the interaction of the 5-HT$_3$ receptors with bivalent ligands assembled by (a) an optimised arylpiperazine 5-HT$_3$ receptor ligand (playing the role of an anchor in interacting with the main binding site of the 5-HT$_3$ receptor), (b) a spacer showing different length and (c) a probe showing different stereoelectronic and functional features (Figure 11). On the basis of their functional features, the probes can be classified into chemofunctional (e. g. the ureido acetic acid (UAA) moiety of heterobivalent guests) or biofunctional (a group capable of interacting with receptors or enzymes, e. g. tacrine in the heterobivalent ligand shown in figure 7, or the same arylpiperazine anchor (MPQC) in the corresponding homobivalent ligands).

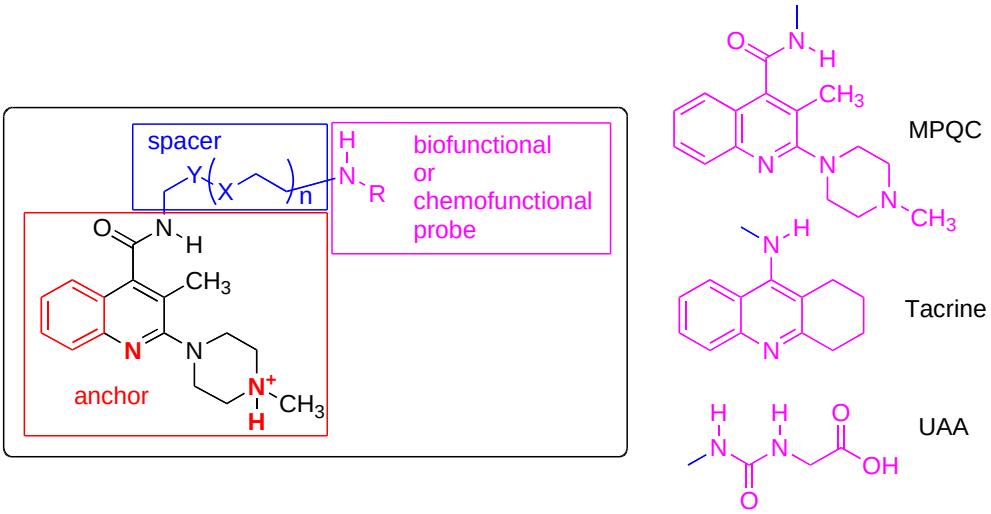

Figure 11- Design of arylpiperazine bivalent ligands **1-3**. The elements in red are the pharmacophoric groups, which allow the arylpiperazine moiety (anchor) to interact with the 5-HT$_3$R binding site; in magenta the probe responsible for the interaction with a biological target (biofunctional probe: MPQC and Tacrine) or for a particular chemical property (chemofunctional probe: UAA); in bleu the spacer.

Bivalent ligands **1-3** were synthesised in the laboratory of Professor Cappelli and their affinity for the 5-HT$_3$ receptor located in the central nervous system was measured by means of displacement studies performed with radiolabeled granisetron specifically bound to the 5-HT$_3$ receptor in rat cortical membranes [Nelson & Thomas, 1989]. Results are summarised in Table 2.

Table 2- Effects of the Variation of Probe and Spacer on the 5-HT$_3$ Receptor Binding Affinities of Bivalent Ligands **1-3**. [a] See Figure 10; [b] X-CH2: -$(CH_2)_X$ -; X-PEG: -CH_2CH_2-$(OCH_2CH_2)_{X-2}$-CH_2CH_2-; [c] Each value is the mean ± SEM of 3 determinations and represents the concentration giving half the maximum inhibition of [^{3}H]granisetron (final concentration 1 nM) specific binding to rat cortical membranes; [d] Relative potency with respect to the compound bearing the heptamethylene spacer (expressed as the ratio between the K_i value of the compound and that of the analogue showing the same probe and the heptamethylene spacer).

Compound	Probe[a]	Spacer (methylene equival.)[b]	K_i (nM)[c]	RP[d]
1a	MPQC	6-CH2 (6)	28 ± 7.1	10
1b	MPQC	7-CH2 (7)	2.7 ± 0.7	1
1c	MPQC	8-CH2 (8)	67 ± 15	25
1d	MPQC	3-PEG (8)	7.7 ± 1.5	3
1e	MPQC	8-PEG (23)	27 ± 8.2	10
1f	MPQC	10-PEG (29)	15 ± 2.4	5
2a	Tacrine	6-CH2 (6)	71 ± 22	13
2b	Tacrine	7-CH2 (7)	5.6	1
2c	Tacrine	8-CH2 (8)	69 ± 17	12
3b	UAA	7-CH2 (7)	26	1
3d	UAA	3-PEG (8)	141	5
3e	UAA	8-PEG (23)	106 ± 18	4
3f	UAA	10-PEG (29)	154 ± 21	6

The analysis of the structure-affinity relationships in this class of arylpiperazine ligands demonstrate that the 5-HT$_3$ receptor is capable of accommodating bivalent ligands showing different spacers and probes, which modulate the affinity in a significant manner. Most notably, homobivalent ligands **1** are among the most potent ligands in each subgroup characterised by the same spacer. This was considered an outstanding result, which deserved a detailed rationalization by means of computational studies. However, other probes are compatible with the interaction with 5-HT$_3$R in the low nanomolar range.

Moreover, the spacer features appear to affect the interaction of the different probes. Thus, the heptamethylene tether seems to behave as the optimal spacer, and either its shortening to the hexamethylene, or the elongation to the octamethylene counterparts decrease the 5-HT$_3$R affinity of about one order of magnitude in compounds **1** and **2**. The replacement of two methylene units in the octamethylene spacers of homobivalent ligand **1c** with two oxygen atoms as in **1d** produces a potency increase of about one order

of magnitude. When long spacers are considered, a possible fluctuation in the extra-receptorial aqueous environment can be assumed, but the differences observed in the structure-affinity relationships between the compounds bearing the longest spacers (23 or 29 methylene equivalents) appear to suggest that the probes interact with different environments. It is noteworthy that the bivalent ligands bearing the longest spacer (29 methylene equivalents) show relative potency (with respect to the compounds bearing the heptamethylene spacer) values around 4 and are more potent than the bivalent ligands tethered by the shortest chain (relative potency values ranging from 10 to 19).

2.3 Aim of the work

The homology model of the homomeric 5-HT$_{3A}$R, previously built in our laboratory [Moura Barbosa *et al*., 2010], were used as an instrument to check the possibility of the receptor to accommodate bivalent ligands and to identify eventual putative additional binding pockets. The binding modalities of the arylpiperazine bivalent ligands were analysed on the basis of three-dimensional models of the ligand-receptor complexes.

Among all the compounds, only the most representative and interesting compounds (**1a-d** and **1f**) were docked into the homomeric 5-HT$_{3A}$R model.

2.4 Computational Methods

2.4.1 Homology modelling

Homology protein modelling is a theoretical approach to predict a protein three-dimensional structure when is not known and/or the experimental methods (i.e. NMR, X-rays) fail due to the crystallization problems or difficulty to obtain enough quantity of protein. This approach relies on the sequence identity that exists among proteins of the same family, taking advantage of the fact that during evolution, the three-dimensional structure of homologous proteins has been much more preserved than their primary

sequences. Therefore, comparative or homology protein modelling uses experimentally determined structures as templates to predict the 3D structure of another protein that has a similar amino acid sequence. More generally, the program derives spatial restraints from the template structure(s) and transfers them to the target sequence, on the bases of the sequence alignment [Sali & Blundell, 1993; Sali, 1995; Sanchez & Sali, 1997]. A 3D model is obtained by optimization of a molecular probability density function (pdf). The molecular pdf for comparative modelling is optimised with the variable target function procedure in Cartesian space that employs methods of conjugate gradients and molecular dynamics with simulated annealing.

The homology models of the extracellular portion of the human 5-HT$_3$A and B subunits were previously built in our laboratory (see Moura Barbosa *et al*., 2010 for a detailed description) by the program Modeller [Sali & Blundell, 1993]. Briefly, models were built using the nAChR structure as a template, differently from previous modelling studies, and using a sequence alignment recently modified by Price *et al.* 2008 (Figure 12) to be in agreement with the experimental data available.

Figure 12- Multiple sequence alignment of n-acetylcholine receptor subunit A (nAChRA) and 5-HT$_3$ receptor subunits A (mouse: m5HT3RA, human: h5HT3RA) and B (human: h5HT3RB). The position of Loops A to F in the sequences is also indicated. In bold, residues mutated experimentally in the mouse 5-HT$_3$R sequence; in bold and grey highlighting, the residues which were clearly demonstrated to be determinant for the mouse 5-HT$_3$R binding affinity of agonists and/or antagonists, and the corresponding residues in the human 5-HT$_3$R A and B sequences.

Each monomer was superimposed to each subunit of the nAChR template pentamer in order to create a 5-HT$_3$A homopentamer and a BABBA heteropentamer, according to the Atomic Force Microscopy studies by Barrera *et al.* [2005]. However, in this work docking studies were performed only at the AA interface.

2.4.2 Molecular docking

Molecular docking tries to predict the structure of the intermolecular complex formed between two or more molecules. In terms of protein-ligand docking methods, the docking

problem can be rationalised as the search for the precise ligand conformations and orientations (commonly referred as posing) within a given targeted protein when the structure of the protein is known or can be estimated.

The protonated structures of the bivalent ligands (**1a-d** and **1f**) were built and fully optimised using Dreiding minimization in the Discovery Studio program of Accelrys [*Accelrys Software Inc.*]. Then they were docked into the interface of the minimised average structure of the human 5-HT$_{3A\text{-}A}$ dimer built as previously described [Moura Barbosa *et al.*, 2010].

Flexible docking of the bivalents ligands into the interface of the 5-HT$_3$ dimer was performed with Autodock 4.2 [Morris *et al.*, 1998]. The protein matrix was instead treated as rigid. A grid of 59×43×127 points with a grid spacing of 0.375 Å, manually centred to an arbitrarily chosen point in the serotonin active site was built to perform docking of ligands into the whole subunit interface; a grid of the same spacing but of larger size (127×59×181 points) was instead built and positioned on the subunit surface, in order to perform explore eventual active docking sites on the receptor surface. Docking of the ligands was performed using the Lamarckian Genetic Algorithm with a population of 150 individuals, during 200 runs for 27000 generations with 25 million energy evaluations.

The docking results were afterward clustered setting an rms threshold value of 5.0 (for the docking into the dimer interface) and of 2.0 (for the docking onto the receptor surface), to identify the principal ligand poses inside the protein subunit interfaces and the principal probe poses on the surface. The most representative structure (i.e. that with the lowest docking energy) of each of the principal clusters for every ligand/docking system was selected for further analysis.

2.4.3 Molecular Dynamics Simulations

Molecular Dynamics (MD) simulations allow the study of the dynamic of a system. In particular, MD simulations enable the complex and dynamic processes that take place in biological systems to be analyzed and provide the ultimate detail concerning the individual atom motion as a function of time. Classical MD generates successive configurations of the system (trajectory) by integrating Newton's laws of motion (Equation 1).

$$\frac{d^2 x_i}{dt^2} = -\frac{F_{x_i}}{m_i} \qquad \text{Eq1}$$

This equation determines the trajectory of a given particle of mass mi along one coordinate (xi), with F_{xi} representing the force acting on the particle in that direction.

The most populated low energy docking pose of compound **1f** was used as the starting conformation for the dynamic simulation; in this structure, the ligand anchor lies into the serotonin binding site, while the probe lies outside the receptor surface.

The 5-HT$_{3A\text{-}A}$/**1f** complex was immersed in solvation box whose boundaries extend 2 Å beyond the furthest edges of the protein and which contains 61838 water molecules (with density 1 g/l box; SPCE model). 4 Na^+ ions were added for electrostatic neutrality. All simulations were performed with the GROMACS suite of programs version 4.0 [Berendsen *et al*., 1995; Hess *et al*., 2008] using the Gromos 96 FF united atom force field.

Non-covalent interactions (van der Waals and short-range electrostatics) were truncated at 10 Å; longer-range electrostatic interactions were computed using the particle-mesh Ewald (PME) method [Essmann *et al*., 1995] with an interpolation order of 6. At the end, all restraints were removed from the system, allowing completely free motion of the solute in the successive equilibration phase.

First, energy minimization of the water was performed with 100000 steps of steepest descent, with the aim of relaxing the solvent structure around the solute. Then, the whole

system was equilibrated for 1 ns, with a timestep of 1 fs, in the NPT ensemble (P=1 atm; T=300 K) using the Berendsen algorithm.

Finally, a free simulation run of 20 ns was performed, collecting system snapshots every 1 ps. For results analysis and for images it was used VMD program [Humphrey *et al*., 1996].

2.5 Results and Discussions

2.5.1 Docking of homobivalent ligand at the AA interface

The protonated structures of homobivalent ligands with different length spacers (**1a** with $-[CH_2]_6-$, **1b** with $-[CH_2]_7-$, **1c** with $-[CH_2]_8-$, **1d** with $-[CH_2CH_2O]_2-CH_2CH_2-$ see Table 1) were docked into the interface of the 5-HT$_{3A-A}$ dimer, where the serotonin binding site is located. Briefly, the binding site of the natural agonist is characterised mainly by polar and aromatic residues such as Trp^{183}, Tyr^{234}, Trp^{90}, Tyr^{153} and Trp^{95} involved in cation-π and π-π interactions with the ligand aromatic portion [Beene *et al.,* 2002; Price & Lummis, 2004]; also a negative-charged residue is present, Glu^{129}, which may establish hydrogen bond and charge-assisted hydrogen bond interactions [Brejc *et al.*, 2001]. In all the cases, the main poses of the ligand anchor moiety lie within the serotonin binding site and share similar features: the charged head of the piperazine moiety establishes a charged-reinforced H-bond interaction with the Glu^{129} side chain, the nearby amide NH is hydrogen-bonded to the hydroxyl side chain of Tyr^{153} and the ligand aromatic portion and the quinoline nitrogen make π-interactions with the aromatic side chains of Trp^{183} and Trp^{90}, respectively (Figure 13).

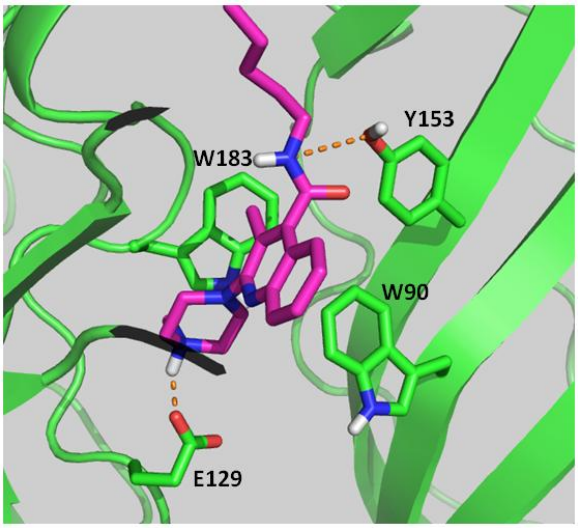

Figure 13- Docking pose of the ligand anchor moiety (in magenta) lying within the serotonin binding site. The main residues that interact with ligand are represented in sticks.

On the contrary, the docking poses of the arylpiperazine probe can be clustered into two main groups depending on the probe position in the dimer interface. The first putative binding site for the probe is located below the known active site, close to the membrane (Figure 14d) and is characterised by both aromatic and polar residues: Tyr^{73}, Tyr^{88}, and Phe^{130}, which has been proved to be a key residue for receptor function [Price *et al.*, 2008], and Ser^{177}, which establishes H-bond interaction with the piperazine ring. In previous docking studies, Tyr^{73}, Tyr^{88}, and Phe^{130} are found to interact with antagonists, and in particular, two of them (Tyr^{73} and Phe^{130}) have been hypothesised to take part in important interactions within a putative allosteric binding site, found for palonosetron and not for the other antagonists [Moura Barbosa *et al.*, 2010].

The second putative binding site was instead found in the upper extracellular region of the dimer interface (in the opposite direction with respect to the membrane) (Figure 14c) and it is characterised by: Asp^{189}, which forms a charge-reinforced hydrogen bond with the charged head of the piperazine moiety; polar and aromatic residues, such as Tyr^{143} and Gln^{151}, whose role in antagonist binding, particularly granisetron, was previously proven by single point mutagenesis experiments [Price & Lummis, 2004; Venkataraman *et al.*, 2002]. The involvement of Tyr^{143} and Gln^{151} in interactions with agonists or antagonists has also been shown by other docking studies [Maksay *et al.*, 2003; Moura Barbosa *et al.*, 2010; Reeves *et al.*, 2003]. The planar rings of antagonists seem to be frequently intercalated between aromatic side-chains including that of Tyr^{143} [Maksay et al., 2003; Moura Barbosa *et al.,* 2010], while the hydroxyl of 5-HT appears to be in a hydrophilic pocket which comprises the Gln^{151} side chain [Reeves *et al.*, 2003].

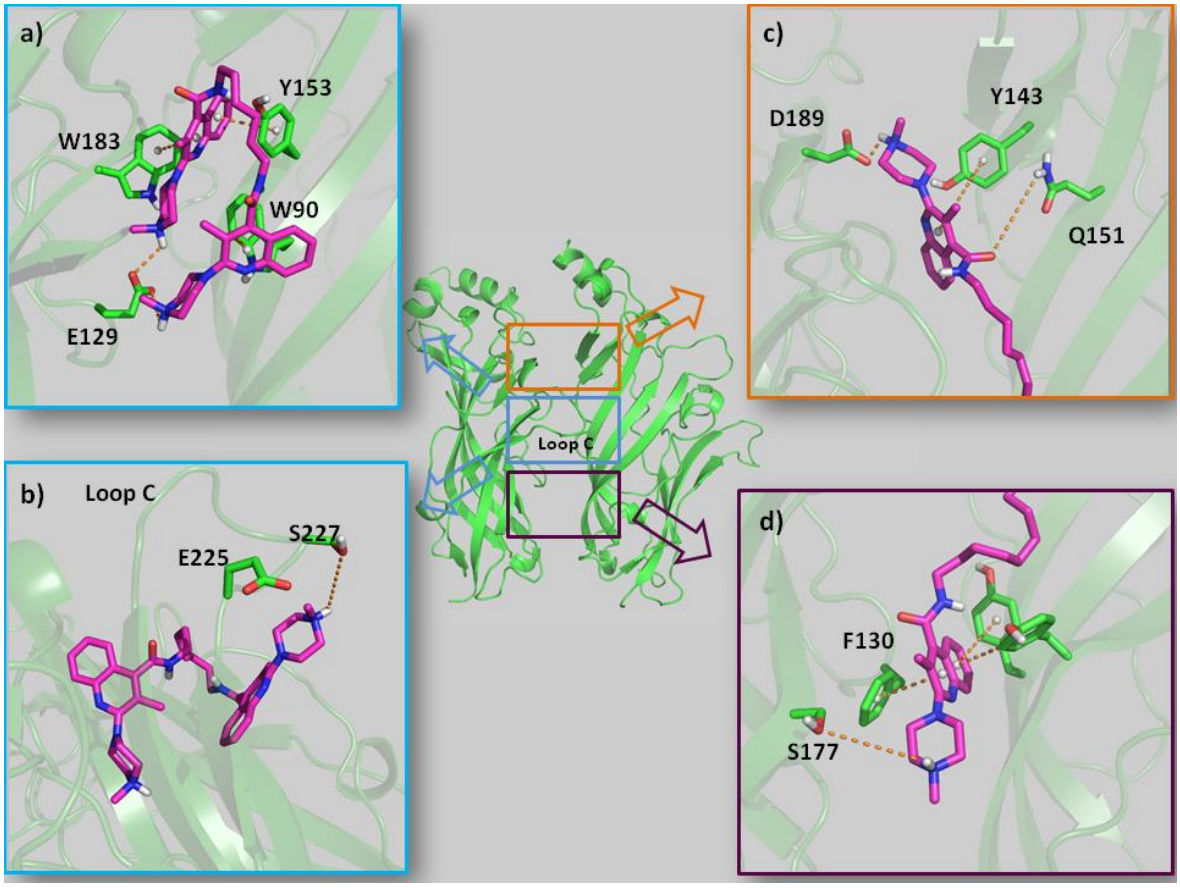

Figure 14- Docking of compounds **1a**-**c** into the 5-HT$_{3A-A}$ receptor dimer: binding mode of the probe in a pincer-like manner (a), on the receptor surface (b), in the upper extracellular region (c) and close to the membrane (d).

Although all the studied homobivalent ligands can dock into these two putative binding pockets, they show binding peculiarities worthy of note: in fact, in its most frequent low energy poses, the heptamethylene-spacer ligand (**1b**) shows its spacer stretched out and its probe lying into one of the two putative probe binding pockets (Figure 14c,d). These poses may indicate the presence of regions with a secondary or allosteric binding site, as previously hypothesised by Moura Barbosa and co-workers [2010], which could explain the greater affinity of this homobivalent ligand for the 5-HT$_3$R. On the contrary, the homobivalent ligand with the shortest hexamethylene spacer (**1a**) prefers the docking pose where both the cationic amines of the piperazines interact with Glu^{129} in a pincer-like manner, (Figure 14a) while the ligand with the longer octamethylene spacer (**1c**) prefers a pose where the probe extends outside the receptor surface (Figure 14b),

reaching a polar surface area near loop C. The molecular dynamic simulation performed on this latter docking pose of **1c** shows that the probe progressively moves from the receptor interface towards the receptor surface, partially modifying the loop C conformation. In particular, this is mainly due to the formation of persistent hydrogen bonds between the probe and the side chains of Ser^{227} and Glu^{225}, on the receptor surface. The partial unfolding of Loop C might be responsible for the different binding mode of the anchor moiety observed during dynamics: the anchor moves away from Glu^{129} (a key residue for the agonist/antagonist binding), preventing the formation of the important charged-reinforced H-bond interaction. This behaviour might explain the lower affinity of compound **1c** for the 5-HT$_3$R that is 25 times less active compared with compound **1b**.

Finally, it is worth noting that the docking poses of the homobivalent ligand **1d**, (which has a spacer -CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-), are different from those of **1c**, which is characterised by the same length of the atom spacer. In the most populated low energy clusters, the orientation of compound **1d** is very similar to that of the heptamethylene-spacer ligand (**1b**), so the probe does not extends outside the receptor surface, but lies into one of the two putative probe binding pockets (Figures 14c,d). In fact, the replacement of two methylene units in the octamethylene spacers of homodimer **1c** with two oxygen atoms in the homodimer **1d** produces an increase of interactions between the oxygens in the spacer and the interface residues, which stabilise the complex, decreasing the binding energy. These results could justify the increase in potency of the homobivalent ligand **1d** with respect to compound **1c**, which is about one order of magnitude less active.

The results of the molecular dynamics simulations suggest the possibility that the probe of bivalent ligands with longer spacers (e. g. 23 or 29 methylene equivalents) reaches other binding sites in the same receptor. To check this hypothesis, the compound with the longest spacer (**1f**, see Table 2) was manually docked in the 5-HT$_3$ dimer: despite the spacer was forced to assume an unrealistic all-extended conformation, the probe couldn't reach the adjacent binding interface. Even though these long bivalent ligands cannot bind

into two consecutive active sites meanwhile and in the same receptor, they may either intercept active sites of other receptors, thus behaving as biofunctional ligands, or dock into other probable binding pockets on the same receptor surface.

2.5.2 Docking of the arylpiperazine probe on the receptor surface

In the attempt to discover new possible binding sites, rigid docking of the probe was performed on the whole receptor surface. The results showed the presence of three main surface areas capable of accommodating the arylpiperazine probe (Figure 15).

The main pose of the probe is found in the extracellular receptor portion near the channel entrance, opposite to the membrane (Figure 15a); here, the probe occupies a pocket that shows very similar features to those of the serotonin site, being the main residues involved in ligand binding aromatic or negatively charged: Asn^{49} (whose carboxylic group is hydrogen-bonded to the cationic head of the piperazine ring), Glu^{98} (whose side chain forms a H-bond interaction with the ligand amino group), Tyr^{50}, Phe^{99} and Trp^{102} (which stabilise the complex interacting with the aromatic group).

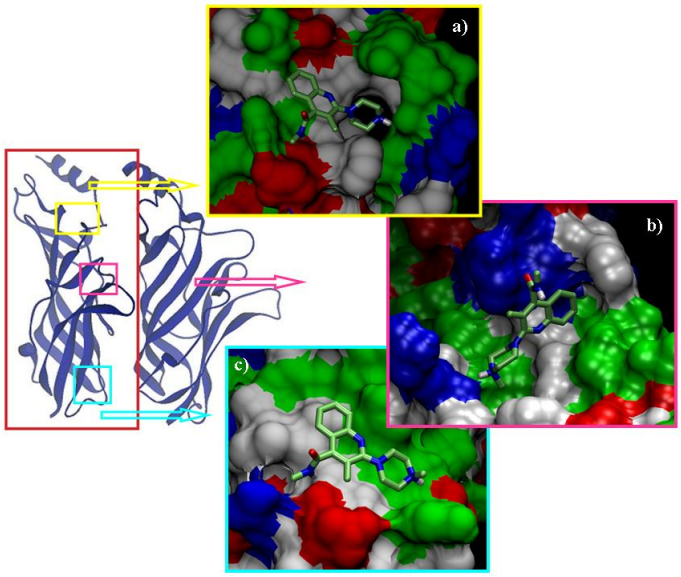

Figure 15- Docking of the arylpiperazine probe on the 5-HT$_{3A}$ receptor surface: charged surface of the putative sites in the extracellular receptor portion (a), above the loop C (b) and near the membrane (c).

The second putative surface site shows different features from the others; in fact, it is a polar pocket (Pro^{63}, Ile^{187}, Asn^{191}, Gln^{188}) surrounded by a few positively charged residues: Arg^{61}, Arg^{196} (whose guanine group is hydrogen-bonded to the cationic head of the

piperazine ring), Lys[62] (which may form a H-bond with the ligand carbonyl group) (Figure 15b). This secondary site is localised in a surface region just above the loop C, very near the active site, therefore it may be exploited by bivalent ligands with relatively short spacer, as in the case discussed above of compound **1c**. In addition, as this site is positively charged, it may easily accommodate negative chemofunctional probes, such as that in compound **3e**,**f** where a deprotonated ureido acetic moiety is present. Finally, the least important pose shows the ligand probe lying in a polar and hydrophobic surface pocket near the membrane: the main residues involved in ligand binding are Ile[242], Asn[175], Asp[172], Val[173], Tyr[167] and Gln[174] (Figure 15c). Therefore, depending on the spacer-length, a bivalent ligand probe may interact with one of the putative sites on the surface or reach other receptor and act as biofunctional or chemofunctional ligand on the basis of its stereoelectronic and functional features.

2.6 Conclusions

In conclusions, the study of the interactions of the 5-HT$_3$R with arylpiperazine ligands led to conceive a design approach to bivalent ligands in which an arylpiperazine moiety is linked by means of a spacer to a probe showing different functional (chemofunctional or biofunctional) features. The high 5-HT$_3$R affinity shown by homobivalent and heterobivalent ligands provides evidence on the viability of this approach, which can be considered of broad applicability in the design of bivalent ligands tailored for specific applications. In fact, the conjugation of a biofunctional probe, as in the case of tacrine derivatives **2a**-**c**, can be used to provide interaction opportunities with an additional biological target such as acetylcholinesterase. On the other hand, the conjugation with an ureido acetic acid moiety (the chemofunctional probe of heterobivalent ligands **3b**,**d**-**f**) provides binding capabilities toward a synthetic receptor localised on the surface of urea adamantyl-functionalised poly(propylene imine) dendrimer. In this regard, the affinity of bivalent ligands **3e**,**f** with the longest spacers (23 or 29 methylene equivalents, respectively) suggests that their arylpiperazine moieties find an high affinity binding site within 5-HT$_3$R leaving the ureido acetic moiety available for the interaction with either

the positively charged site on the receptor surface or the synthetic receptor localised on the dendrimer surface. Moreover, the finding of secondary binding sites could explain the greater affinity of this bivalent ligand for the 5-HT$_3$R and may indicate the presence of an allosteric binding site, as previously hypothesised by Moura Barbosa and co-workers [2010]. Finally, the existence on the receptor surface of three potential accessory binding sites for the arylpiperazine moiety shown by the molecular modelling studies implies that multivalency in 5-HT$_3$R could involve receptor domains different from the main binding site. The high affinity shown by homobivalent ligands **1a**-**f** suggests that bivalency is a promising approach in 5-HT$_3$R modulation. Since bivalency can be considered the first step of multivalency, the results obtained provide the rational basis for applying the concepts of multivalency to the study of 5-HT$_3$R function.