Homology modeling and structural analysis of the antipsychotic drugs receptorome.

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(CEXS Deparment)

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A Pedro y Emilia

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Abstract

The work of this thesis was focused on the study of the molecular mechanisms implicated in the multireceptor binding affinity profile of antipsychotic drugs. As the first step, we obtained homology models for all the receptors putatively involved in the antipsychotic drugs receptorome, suitable for building consistent drug-receptor complexes. These complexes were structurally analyzed and compared using multivariate statistical methods, which in turn allowed the identification of the relationship between the pharmacological properties of the antipsychotic drugs and the structural differences in the receptor targets. The results can be exploited for the design of safer and more effective antipsychotic drugs with an optimum binding profile.

Resumen

El trabajo de la presente tesis se ha centrado en el estudio de los mecanismos moleculares que determinan el perfil de afinidad de unión por múltiples receptores de los fármacos antipsicóticos. Como primer paso se construyeron modelos de homología para todos los receptores potencialmente implicados en la actividad farmacológica de dichos fármacos, usando una metodología adecuada para construir complejos fármaco-receptor consistentes. La estructura de estos complejos fue analizada y se llevó a cabo una comparación mediante métodos estadísticos multivariantes, que permitió la identificación de asociaciones entre la actividad farmacológica de los fármacos antipsicóticos y diferencias estructurales de los receptores diana. Los resultados obtenidos tienen interés para ser explotados en el diseño de fármacos antipsicóticos con un perfil farmacológico óptimo, más seguros y eficaces.

Preface

The classic model which compared a drug with a key and a pharmacological target with a keyhole assumes that the compounds with therapeutic effects exert their action interacting with a single biomolecule. Much on the contrary, the fast-growing volume of data in genomics, proteomics and systems biology depicts a far more complex scenario in which the biochemical mechanisms of diseases and therapies involve many different biological receptors. Schizophrenia is an example of complex disease, and clinical useful antipsychotic drugs are characterized by interacting with a wide range of receptors with different affinity, mainly G protein-coupled-receptors (GPCRs). Here we will present a detailed study based on close examination of known antipsychotic drugs and multiple receptors potentially involved in their therapeutic effects, in order to understand the mechanisms of the antipsychotic pharmacologic profile.

First, we will present our study on structural characterization of the receptor binding pockets, which required building comparable homology models for a large set of GPCRs putatively involved in psychosis treatment. Then we will show results of a comparative analysis of the receptorome binding site using novel methodologies, which link the molecular diversity of the structures under study with the available clinical data. Furthermore, we introduce our findings of the identification of regions and ligand-receptor interactions associated to the optimum multireceptor binding profile of antipsychotic drugs, which could be exploited for the design of improved antipsychotic compounds. In addition, we will report other particular applications of the obtained structures and models with relevance for the rationalization of diverse experimental results.

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Abbreviations

5-HT_{1A}	Subtype 1A serotonin receptor
5-HT_{2A}	Subtype 2A serotonin receptor
5-HT_{2B}	Subtype 2B serotonin receptor
5-HT_{2C}	Subtype 2C serotonin receptor
5-HT_{6}	Subtype 6 serotonin receptor
5-HT_{7}	Subtype 7 serotonin receptor
ADA_1	Subtype 1 alfa adrenergic recepor
ADA_2	Subtype 2 alfa adrenergic recepor
D_1	Subtype D1 dopamine receptor
D_2	Subtype D2 dopamine receptor
D_3	Subtype D3 dopamine receptor
D_4	Subtype D4 dopamine receptor
H_1	Subtype 1 histamine receptor
M_1	Subtype 1 cholinergic muscarinic receptor
M_2	Subtype 2 cholinergic muscarinic receptor
M_3	Subtype 3 cholinergic muscarinic receptor
M_4	Subtype 4 cholinergic muscarinic receptor
APD	Antipsychotic drug
β-AR	Beta-adrenergic receptor
CPCA	Consensus principal component analysis
EL	Extracellular loop
GPCR	G protein-coupled receptor
MIF	Molecular interaction field
PCA	Principal component analysis
PC	Principal component
PLS	Partial least squares
RMSD	Root-mean-square deviation
3D-QSAR	3D quantitative structure-activity relationship
TM	Transmebrane helix

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Articles:

- Comparative Structural Analysis of the Risperidone Receptorome Using GRID/CPCA Methodology.

  López L, Bauer-Mehren A, Selent J and Manuel Pastor.
  (Manuscript in preparation)

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  López L, Selent J, Ortega R, Masaguer CF, Domínguez E, Areias F, Brea J, Loza MI, Sanz F, Pastor M.
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- A Novel Multilevel Statistical Method for the Study of the Relationships between Multireceptorial Binding Affinity Profiles and *in vivo* Endpoints.

  Selent J, Bauer-Mehren A, **López L**, Loza MI, Sanz F, Pastor M.
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  Ortega R, Raviña E, Masaguer CF, Areias F, Brea J, Loza MI, **López L**, Selent J, Pastor M, Sanz F.
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  April 29 – 30, 2008, Erlangen, Germany

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  Bauer-Mehren A, Selent J, **López L**, Sanz F, Pastor M.
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  April 29 – 30, 2008, Erlangen, Germany

- In silico Multireceptor Profiling of Antipsychotic Drugs.

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  III Congreso de la Sociedad española de Farmacogenética y Farmacogenómica,
  November 15 – 17, 2007, Santiago de Compostela, Spain

- Computational Modeling Supporting the Multireceptor Profiling of Anitpsychtotic Drugs.

  Sanz F, Selent J, **López L**, Pastor M
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  February 23-25th, 2008, Granada, Spain

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- Computational Docking Studies of Benzolactam Derivatives as Selective D_3 Receptor Ligands.

  López L, Selent J, Ortega R, Masaguer CF, Sanz F, Pastor M.
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"La ciencia no nos ha enseñado aún si la locura es o no lo más sublime de la inteligencia."

(Edgar Allan Poe)

1. INTRODUCTION

1.1. Paradigm Shift in Drug Discovery

The strategies used for discovery of new drugs have evolved in the history of mankind. The centenary Ehrlich's concept of producing highly selective compounds or 'magic bullets' has been cornerstone of most drug discovery. Indeed, this concept yielded notable successes, such as the selective cyclooxygenase 2 (COX-2) inhibitors that exhibit favorable efficacy.[1,2] However, as the pathogenesis of many diseases involves multiple factors, often unknown, selective single-target compounds tend to fail in the fight against multigenic and complex diseases.[3,4] Thus, during the last decade we assisted a paradigm shift in drug discovery, which accounts for a wide awareness of the complexity of the phenomena involved in the therapeutic effects of drugs, and in which the focus on a single privileged target has moved to take into account that: (i) diseases pathogenesis are more complex than one single gen,[5] hence several targets must be considered for the therapeutic effect of the drug,[6] and (ii) the contribution of the drugs in other targets must be also considered (side effects).[7]

Unfortunately, the simple awareness of the complexity involved in multi-target prototype drugs does not help to obtain better compounds and there is a pushing need for methods able to identify the receptors that must be targeted by the drugs and the optimum activation profile. Probably the best approach to the problem is represented by system biology methods[8-10] that provide a detailed description of all the relationships between the implicated biomolecules. However, because of the extreme complexity of the involved mechanisms, we will not achieve such detailed understanding for many important diseases, in the immediate future. Hence, an alternative approach is the analysis of the multireceptor binding affinity profile of known drugs for elucidating hints about the binding profiles associated to efficacy or side effects. With the application of structure-based methods, such binding profiles can be linked to ligand structural features that could be exploited for obtaining new drugs with higher efficacy and less undesirable effects.

Schizophrenia is a good example of complex and polygenic disease[11] for which the pathogenesis is unknown, even if available antipsychotic drugs (APD) have a proven clinical efficacy.

1.2. Schizophrenia

Schizophrenia (from the Greek "schizo", to tear or split, and from "phren", the intellect or mind) is a chronic, debilitating mental disorder affecting 1-2%

of the global population.[12] It has an average lifetime prevalence of 0.7-0.8%[13] and is associated with mortality rates two-three times higher than those in the general population.[14]

Simptomatology

The symptomatology of schizophrenia can be clustered into positive, negative and cognitive symptoms (Figure 1). Positive psychotic symptoms are the most prominent ones, involving an excess or distortion of normal function. These include auditory and visual hallucinations, paranoia, and other delusional states together with disorganized thought.[15] Positive symptoms usually appear in late adolescence or early adulthood and are characteristically episodic. Negative symptoms involve a decrease or loss of normal function and include affective flattening, anhedonia, associality withdrawal and lack of motivation.[16,17] The pathophysiology of negative symptoms is poorly understood[18] and they remain a relatively treatment-refractory and debilitating component of schizophrenic pathology.[19,20] Cognitive impairments include deficits in attention, executive dysfunction and memory. These symptoms often begin before the presentation of any psychotic symptoms (during adolescence) and remain severe, with some progression, throughout the course of the disease.[21] The pool of the symptoms in schizophrenia has a large impact on the quality of life of the people who is afflicted (leading for instance to unemployment, social isolation and suicides), which makes it a very costly disease for families and society.[22,23]

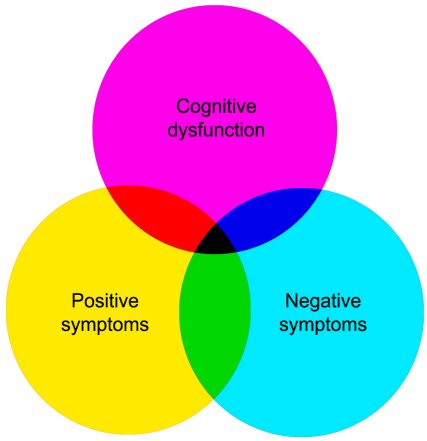


Figure 1. The complexity of symptoms in schizophrenia. Adapted from[24].

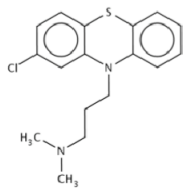
Treatment

Schizophrenia is a complex disease that depends on multiple genetic and environmental factors[11,25] and after nearly a century of research, its etiology and patophysiology remain largely unresolved. However, it is treatable and pharmacological therapy often produces satisfactory results. The first drugs for schizophrenia, the so-called typical antipsychotics (Chart 1, compounds 1-2), were serendipitously discovered in the late 1940s with chlorpromazine, and their mode of action (blockade of postsynaptic D_2 receptor in the mesolimbic area) was elucidated retrospectively.[26-28] However, treatments with these drugs were of little benefit for treating cognitive symptoms and were associated with the development of extrapyramidal side effects (EPS) such as dystonias, akathisias[29] and tardive dyskinesia (abnormal involuntary movements of tongue, facial muscles or limb muscles) in about 20% of patients.[30,31]

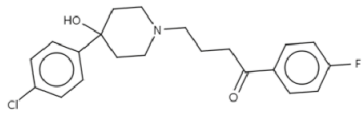
The first drug to have a significant effect on negative symptoms was clozapine, synthesized in the 1960s.[32] The clinical studies showed that clozapine was a significantly better drug in the treatment of schizophrenia regarding negative symptoms, aggression, social functioning, treatment resistance and suicide. Clozapine side effects did not include EPS[33] but in 1975 it was reported to produce eight deaths due to agranulocytosis.[34] Nevertheless, following psychotic relapses in many clozapine-treated patients after being switched to other compounds, clozapine remained available for prescription under specific safety rules in several countries. In 1988 the superiority of clozapine over chlorpromazine in patients with treatment-resistant schizophrenia was reported.[35] This led to the approval of clozapine in the US in 1989[32] as prototype of the second generation of drugs for schizophrenia, the so-called atypical antipsychotics, today also represented by drugs like olanzapine, risperidone, quetiapine, aripiprazole and ziprasidone (Chart 1, compounds 3-8). Clozapine is still considered to be the prototypic atypical antipsychotic[36,37] or "gold standard". In general, atypical antipsychotic drugs are characterised by improving psychotic symptoms of schizophrenia and preventing their recurrence[38,39] having a lower EPS liability.[40,41]

Many "clozapine-like" APDs have been reported to offer diverse benefits in schizophrenia treatment[36,42,37,43] providing in particular a satisfactory treatment for positive symptoms of psychosis. However, these drugs appear to be associated with varying degrees of metabolic adverse effects, such as weight gain, impaired glucose metabolism, dyslipidemia and cardiovascular diseases.[44,45] On the other hand, clozapine treatment is still associated to an increased risk of agranlulocytosis,[46] which strongly limits its therapeutic use

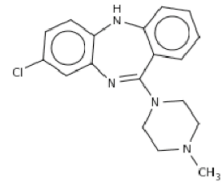
to treatment-resistant individuals. In addition, it is increasingly recognized that cognition is not appreciably affected by antipsychotic medication.



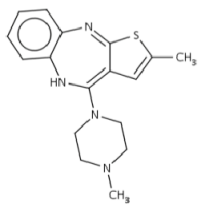
1. Chlorpromazine



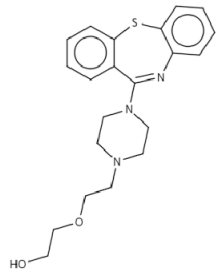
2. Haloperidol



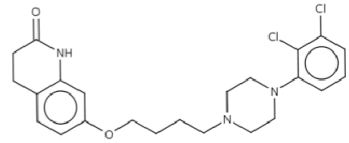
3. Clozapine



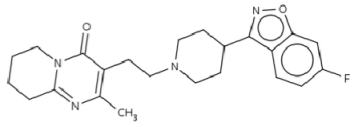
4. Olanzapine



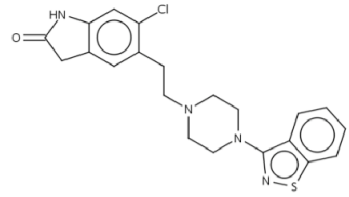
5. Quetiapine



6. Aripiprazole



7. Risperidone



8. Ziprasidone

Chart 1. Structures of some typical (compounds 1-2) and atypical antipsychotic drugs (compounds 3-8) used for the treatment of schizophrenia.

All in all, the discovery of novel antipsychotic agents, more effective, able to augment cognitive function of the illness and free of side effects remains today a challenging research goal.

1.3. Pharmacological Targets for Schizophrenia's Treatment

The 'classical' dopamine hypothesis of schizophrenia, proposed over 40 years ago, states that florid psychotic symptoms of schizophrenia are associated with exaggerated dopaminergic function in the central nervous system.[47,48] This hypothesis arose from the fact that all effective antipsychotic agents block dopamine D_2 receptor. In 1989 Meltzer *et al.*[49] suggested that not only D_2 is implicated in the effect of atypical APDs, but also 5-HT_{2A} receptor. They proposed that a higher ratio of a drug's affinity for serotonin 5-HT_{2A} receptor relative to dopamine D_2 receptor could predict 'atypicality' and would explain the enhanced efficacy and reduced EPS liability of the second generation of antipsychotic drugs. This "Meltzer index" continues being used to discriminate and classify new compounds as "typical" (pKi ratio< 1.09) or "atypical" (pKi ratio > 1.12) agents and can be regarded as a first rough attempt of characterizing the complex multireceptorial binding profile of APDs. Nowadays, it is widely accepted that additional neurotransmitter receptor subtypes have a putative role in the effect of APDs (Figure 2), including other serotonin (5-HT_{1A}, 5-HT_{2C}, 5-HT_6 and 5-HT_7) and dopamine receptors (D_1, D_3 and D_4), as well as the histamine receptor H_1, muscarinic receptors (M_1, M_2, M_3, M_4 and M_5) and adrenergic receptors (ADA_1 and ADA_2).[42] Emerging research in either academic and corporative environments has let the elucidation of other new target receptors for the psychosis treatment, such as metabotropic glutamate 5,[50] alpha7 neuronal nicotinic[51] and neurokinin-3[52] receptors.

The finding that antipsychotic medications interact with multiple neurotransmitter receptors is consistent with the fact that such drugs have clinical effects beyond their antipsychotic efficacy. Consequently, a number of attempts, largely unsuccessful, have been made to develop APDs that target a single receptor without raising the risk of severe side effects associated with such multiplereceptor drugs such as clozapine. For instance, D_4-selective compounds,[53] as well as compounds with 5-HT_{2A}/D_4 antagonism,[54] are ineffective in treating schizophrenia.

The antipsychotic drugs receptorome

Kroeze K et al.[55] coined the term 'receptorome' to describe that fraction of the genome that encodes receptors, transporters and ion channels that can potentially serve as drug receptors, which probably constitute about 5% of the human genome.[56] Among them, the G protein-coupled receptors

(GPCRs) comprise a significant proportion of (estimated at 3.7%).[57] In this thesis we will use the term APDs receptorome to refer to the set of receptors potentially involved in the pharmacologic effects (either therapeutic or side effects) of APDs. All the members of the so-called APDs receptorome have in common to be part of the GPCRs superfamily, and includes the ones mostly implicated in the binding profile of the gold standard APD clozapine: the serotonin, dopamine, histamine, muscarinic and adrenergic receptor subtypes listed above.

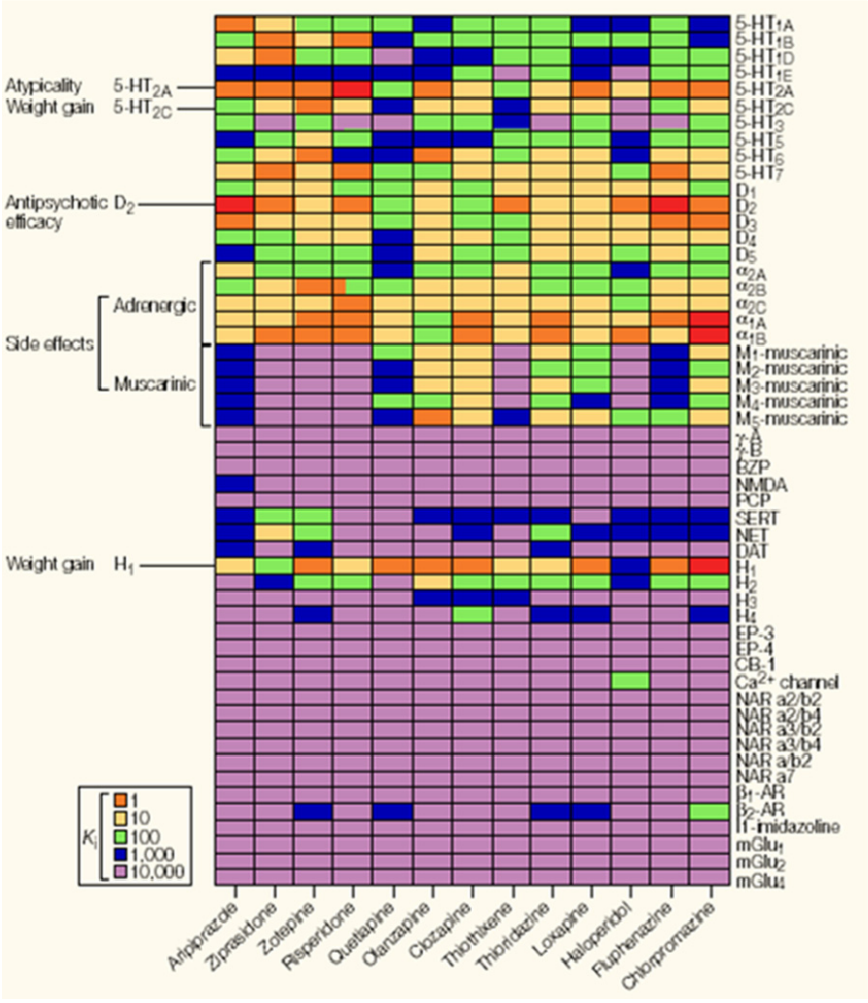


Figure 2. Multiple molecular targets implicated in APDs actions (extracted from[42]).

1.4. G Protein-Coupled Receptors (GPCRs)

Members of the GPCR superfamily are diverse in their primary structure, and this has been used by various research groups for their phylogenetic classification. The one done by Fredrikson *et al.* showed that most of the human GPCRs can be found in five main families, termed Glutamate, Rhodopsin, Adhesion, Frizzles/Taste2 and Secretin (shortened to the acronym GRAFS). [58] The Rhodopsin family is by far the largest and most diverse of these families, having around 600 members in humans, of which 65 are still orphans;[59] that is, their endogenous ligand is still unknown. The neurotransmitter receptors defined in the APDs receptorome are included among the Rhodopsin members.

General architecture of GPCRs

GPCRs are characterized by the presence of seven membrane spanning α-helical segments separated by alternating intracellular and extracellular loop regions and an eighth helix (H8) which lies approximately parallel to the intracellular membrane (Figure 3). This overall architecture is highly conserved among GPCRs, despite of the often-limited sequence homology.[60] Nonetheless, Rhodopsin family of GPCRs contains a pattern of highly conserved residues on each of the seven transmembrane (TM) helices.[61]

To facilitate comparison of residues between receptors belonging to Rhodopsin family, Ballesteros and Weinstein[62] proposed a generalized numbering scheme in which the number preceding the dot refers to the TM helix on which an amino acid resides. The second number designates the position relative to the most highly conserved residue among Rhodposin family, numbered 50. For example, 6.55 indicates a residue located in TM6, five residues carboxy terminal to Pro6.50, the most conserved residue in TM6 (Figure 3). All residues in this thesis will be indicated according to the Ballesteros/Weinstein numbering system.

The first insights into the structure of GPCRs came from two-dimensional (2D) crystals of bacteriorhodopsin,[63,64] from which the basic seven-TM linked by loops was highlighted (Figure 3). In 2000, Palczewski and colleagues published the first crystal structure of a GPCR, solved at 2.8 Å: the bovine rhodopsin (PDB ID 1F88).[65] The crystal structure was further refined to a resolution of 2.6 Å (PDB ID 1L9H)[66] and 2.2 Å (PDB ID 1U19, Figure 4).[67]

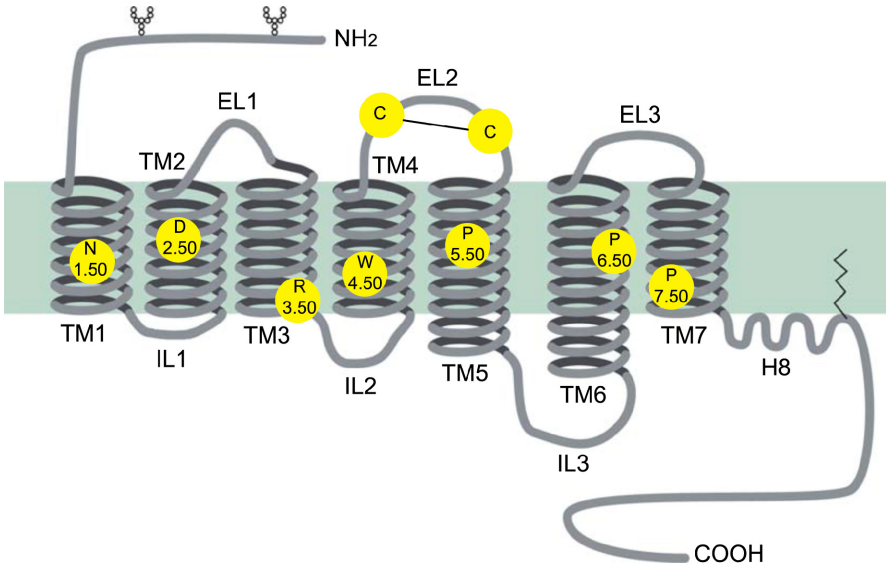


Figure 3. Secondary structure common to rhodopsin-like GPCRs. The conserved key residues (in yellow) are indicated according to the Ballesteros-Weinstein numbering.

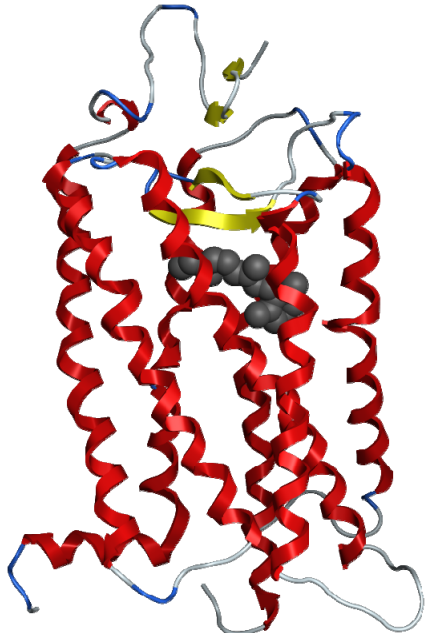


Figure 4. Crystal structure of the bovine rhodopsin receptor (PDB ID 1U19) in complex with 11-*cis*-retinal (depicted in spheres).

The release of the crystal structure of the GPCR rhodopsin allowed improving some assumption about the structure and the organization of the GPCRs helices.[65,68] The seven-TM helices have an anti-clockwise arrangement when viewed from the extracellular side, they have different lengths due to several kinks and bends and varying tilts with respect to the membrane surface. The conformation of bovine rhodopsin, as found in the crystal structure, is stabilized by a number of interhelical hydrophobic interactions and hydrogen bonds; most of them formed by highly conserved residues among rhodopsin-like GPCR receptors.

The difficulty of obtaining GPCR crystals made rhodopsin the only model for studying these receptors until 2007, when the development of a new technique for isolating GPCRs allowed the crystallization of the first human GPCR, the β_2-adrenergic receptor (β_2-AR).[69,70] At the time of writing this thesis, the crystal structures of four GPCRs are available (Figure 5): human β_2-AR bound to the high-affinity inverse agonist carazolol[69,70] (PDB ID 2RH1) and timolol[71] (PDB ID 3D4S), avian β_1-AR bound to the antagonist cyanopindolol[72] (PDB ID 2VT4), bovine rhosopsin containing the covalently bound inverse agonist 11-*cis*-retinal[65] (PDB ID 1U19) and the adenosine A_{2A} receptor, bound to the high subtype-selective antagonist ZM241385[73] (PDB ID 3EML).

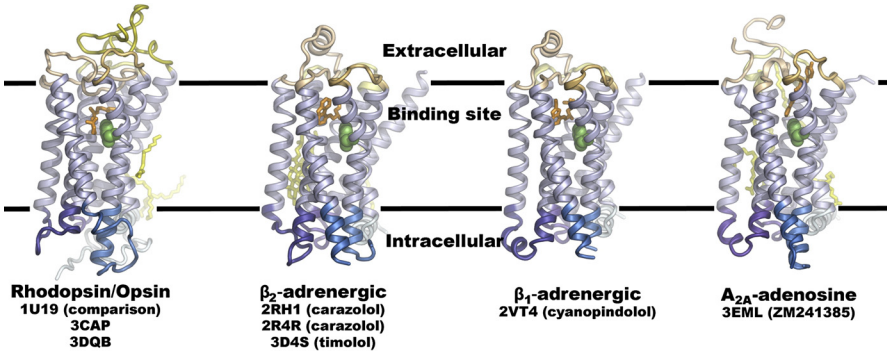


Figure 5. Representative GPCRs solved to date. Each ligand is colored in orange and in green is shown the conserved toggle switch tryptophan residue (extracted from[74]).

The four GPCRs structures have a similar overall architecture with an average Cα root-mean-square deviation (RMSD) lower than 3 Å,[74] providing a good basis for homology modeling.[75] The comparison of GPCR structures and sequences reveals some conserved functional microdomains, which suggests common structural features and activation mechanism. On the other hand, as might be expected from the functional differences between the

receptors, the most significant structural divergences lie in the extracellular loops (EL) and in the ligand binding regions (Figure 5).

Functional microdomains in GPCRs

The comparison of the crystal structures reveals common functional structural features in the cytoplasmatic surface of the receptors:

- *E/DR3.50Y motif:* these amino acids are part of a hydrogen bond network linking DR3.50Y motif on TM3 and a glutame residue in TM6 (Glu6.30). This interaction, called 'TM3-TM6 ionic lock' stabilizes the inactive-state conformation of GPCRs.[65,76] However, the analogous polar interactions in β_2-AR, β_1-AR and A_{2A} receptor are broken. In these structures, TM3 and TM6 helices are further apart and the salt bridge between Arg3.50 and Glu6.30 is absent, whereas polar contact between adjacent acidic and basic residues on TM3 (Glu/Asp3.49 followed by Arg3.50 of the E/DRY motif) is maintained in the four inactive-state structures (Figure 6a). This difference in the ionic lock interaction is a possible explanation to the fact that β_2-AR, β_1-AR and A_{2A} receptor generally have higher basal activity than rhodopsin.[77]
- *NP7.50xxY(x)$_{5,6}$F motif:* located at the cytoplasmatic end of TM7 (Figure 6b), it is functionally bipartite because provides two constraints:[78] Asn7.49 is part of a hydrogen bond network linking TM1, TM2 and TM7, whereas the Y(x)$_{5,6}$F submotif constrains TM7 with H8.
- *CWxP6.50 motif:* this motif is the basis of the so-called 'rotamer toggle switch' hypothesis. In GPCR activation, the rotamer states of Trp6.48 and Phe6.52 are coupled and changed during receptor activation, thus providing a link between the CWxP6.50 motif and shift of the cytoplasmatic part of TM6 helix[79] (Figure 6b).

Apart from the microdomains, there are also highly conserved residues such as Pro5.50, Pro6.50 and Pro7.50, which induce kinks in TMs, as well as a common disulfide bridge between Cys3.25 on TM3 and Cys187 of the EL2, connecting TM4 and TM5 helices.[65,80] Structural and functional studies show that many of the conserved residues have (often in concert with structurally conserved water molecules) a dual role: they constrain the seven-TM bundle in its inactive conformation and are main determinants of the structural changes that occur on receptor activation.

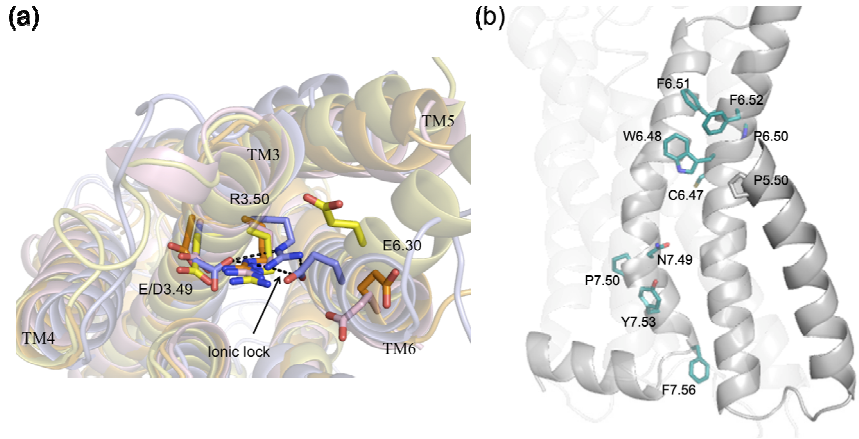


Figure 6. (a) The ionic lock residues are shown for rhodopsin (blue), avian β_1-AR (pink), human adenosine A_{2A} receptor (yellow) and human β_2-AR (orange); (**b**) Residues corresponding to the NP7.50xxY(x)$_{5,6}$F and CWxP6.50 *motifs*.

Extracellular surface of GPCRs

The analysis of the extracellular regions in the representative GPCRs reveals a great deal of topological divergence[74] (see Figure 8). For instance the EL2 of rhodopsin forms a four-stranded β-sheet with additional interactions between EL3 and EL1.[65] The extracellular region of the β-AR is very open in comparison to rhodopsin and is characterized by a short helical segment within EL2 that is supported by limited interactions with EL1 and two disulfide bridges.[69,70] The extracellular region of the adenosine receptor is highly constrained by four disulfide bridges and multiple polar and van der Waals interactions among the three loops.[73]

Ligand Binding Pocket of GPCRs

Since we aim to study the structural characteristics that justify the observed differences in binding activity of APDs for several receptors, the analysis of the ligand binding pocket structure is a central issue.

Mutagenesis data[81,82] and information extracted from the available GPCR X-ray structures indicate that ligand binding pocket is located between TM3, TM5, and TM7 helices. However, subtle differences can be found in the ligand-binding pockets of the crystallized receptors (see Figure 8). In rhodopsin, retinal extends deep into the binding pocket and is covalently bound to the TM5/TM6 interface, where it is sandwiched between Phe5.47

and Tyr6.51, and interacts with the highly conserved Trp6.48, which as stated before is suggested to undergo key rotamer conformational transitions in GPCR activation[79] (Figures 7a and 8a). On the other hand, carazolol, which bears structural similarity to antipsychotic drugs, does not interact directly with the toggle switch on TM6 (Figures 7b and 8b). However, it interacts with Phe6.51 and Phe6.52, which form an extended aromatic network surrounding the Trp6.48. As a result, Trp6.48 side chain adopts the rotamer associated with the inactive state.

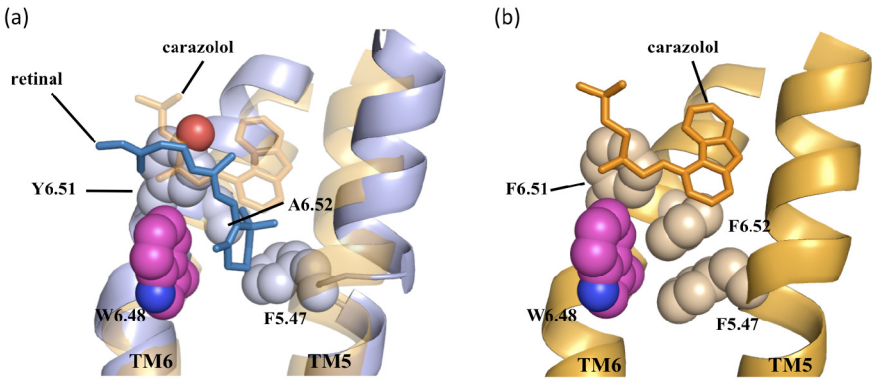


Figure 7. Ligand binding comparison between rhodopsin (purple) and β_2-AR (orange), showing the residues involved in the toggle switch. (a) Superimposition of retinal (blue) in rhodopsin and carazolol (orange) in β_2-AR; (b) Binding orientation of carazolol.

The recent elucidation of the adenosine A_{2A} receptor structure has shown that the ligand binding pocket can assume very different location to that of rhodopsin and β-AR. In addition to shifting to the interface of TM6 and TM7 helices, ligand ZM241385 binds to the A_{2A} receptor forming extensive interactions with EL2 and adapting a perpendicular position to the plane of the plasma membrane (Figure 8c). This shows that ligands of GPCRs may bind in quite distinct fashion, having different degrees of interaction with regions involved in known protein conformational switches.

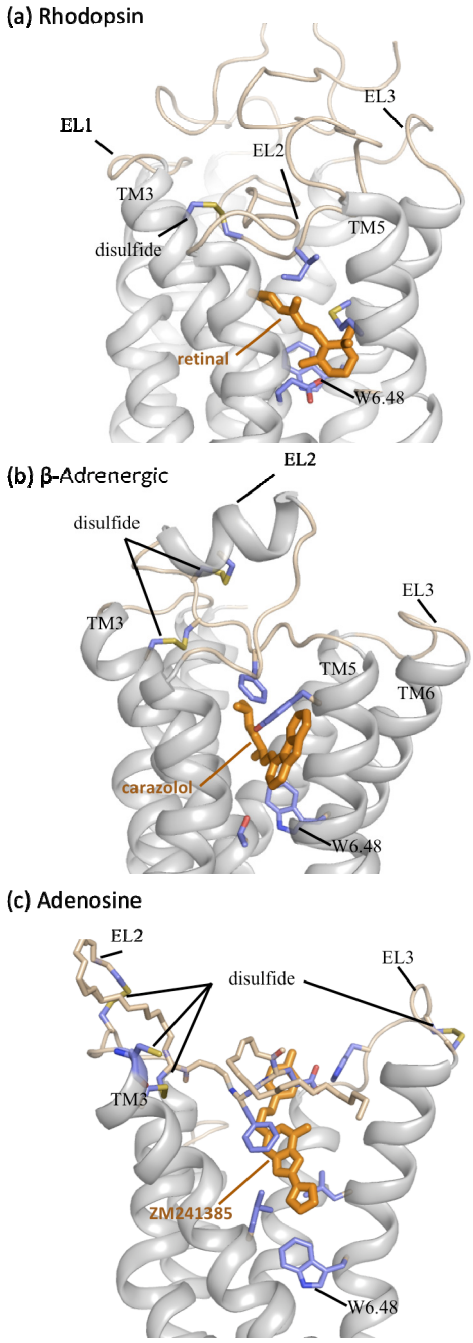


Figure 8. Extracellular view and locations of bound ligands (in orange) for the structures of (a) bovine rhodopsin receptor; (b) human β_2-AR and (c) human A_{2A} adenosine receptor. Trp6.48, the key residue of the rotamer toggle switch, is shown as reference.

Activation process of GPCRs

GPCRs respond to the binding of extracellular ligands (and other external stimulus) with a conformational change in the ligand binding site,[83] which extends via their seven-TM scaffold into the intracellular domain.[84,85] Here GPCRs activate one or more of the guanine-nucleotide-binding signal transducing proteins (G proteins) that carry the information received by the receptor to cellular effectors such as enzymes and ion channels.[86] G protein are heterotrimeric proteins consisting of three subunits, commonly denoted as α, β and γ.[87] After GPCR activation, the conformation changes in the associated G protein α-subunit and leads to release of guanosine diphosphate (GDP) followed by binding of guanosine triphosphate (GTP).

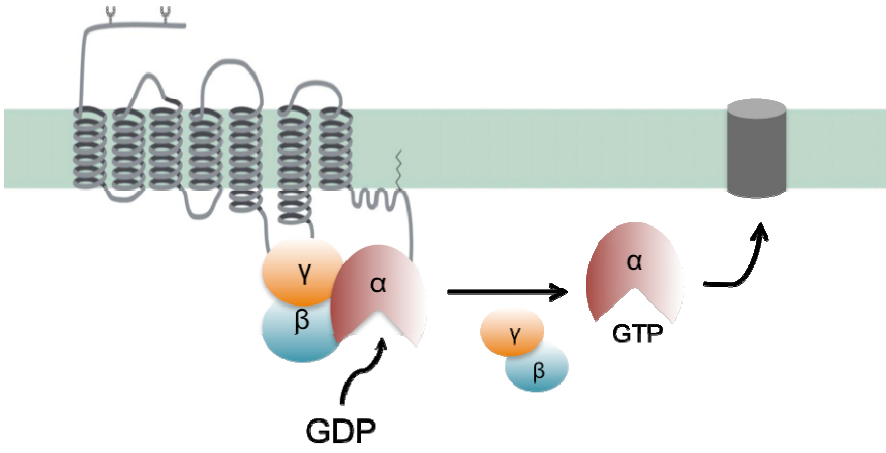


Figure 9. Representation of the heterotrimeric G protein activation by GPCRs.

GPCRs can activate more than one G protein isoform, and recent evidence suggests that they can also signal through G protein independent pathways.[88-90] Moreover, ligands for a given GPCR can show different efficacy profiles for coupling to distinct signaling pathways.[91] All this information accumulated during last years has changed the depiction of GPCRs as bimodal switches with inactive and active states[92] to highly dynamic structures that exist in equilibrium between active (R*) and inactive (R) conformations, able to sample a continuum of conformations with relatively closely spaced energies.[93] Thus, the binding of an agonist ligand shifts this equilibrium toward R* and leads to the formation of a high affinity agonist-receptor (R*) – G protein ternary complex. Neutral antagonists maintain the existent equilibrium between R and R*, while avoiding the activation by the

agonist. Instead, inverse agonist reduces the basal activity of the receptor population, shifting the equilibrium toward R.

Membrane environment of GPCRs

GPCRs are membrane embedded proteins, and the role of the membrane environment on the protein structure and functionality should not be neglected. Indeed, the membrane constitutes a complex environment in which the interactions of lipids with the membrane-bound proteins are responsible for large part of its function.

Biological and biophysical studies have indicated that there is selective confinement of proteins (including GPCRs) in discrete regions of the membrane, called lipid rafts.[94] These raft regions are composed mainly of sphingolipids and cholesterol in the outer leaflet, somehow connected to domains of unknown composition in the inner leaflet.[95] For membrane proteins such as the GPCRs, lipid rafts are important because they provide a platform for the assembly of signaling complexes[96] influencing also their potency and efficacy in the signal transmission after activation.[97]

1.5. Computational Methods in Drug Discovery

The process for obtaining a new drug is difficult, and consumes much time and resources.[98] Currently, computational methods are used in order to streamline drug discovery, design, development and optimization. In particular, computer aided drug discovery (CADD) is being utilized to identify active drug candidates, select leads (most likely candidates for further evaluation) and optimize them, i.e. transform biologically active compounds into suitable drugs by improving their physicochemical, pharmaceutical and pharmacokinetic properties. Strategies for CADD depend on the extent of available information regarding the target and the ligands. In this section we will describe most suitable computational methods for studying the APDs receptorome.

GPCR homology modeling

Even with the recent progress in GPCR crystallography, the structures available today represent only a small fraction of the GPCRs. In particular, there is still no crystal structure for most of the Rhodopsin family members and the GPCRs included in the APDs receptorome. Hence, one can infer their structure by computational methods, namely by building homology models.

Homology modeling has been extensively used to construct 3D models of Rhodopsin-like GPCRs, to examine receptor interactions with ligands, and to examine the effects of specific receptor mutations on receptor structure and/or ligand binding. Such receptor models play an essential role in providing hypotheses, which could be tested in protein engineering experiments.[99,100] Homology modeling relies on the similarity between the sequence of the target protein and at least one known structure (the template)[101] and is based on the general assumption that evolutionary related proteins conserved more of their 3D structure than their amino acid sequences. The modeling process is carried out in five sequential steps (Figure 10): (i) identification of known structures (templates) related to the sequences to be modeled; (ii) alignment of the sequences with the template and fold assignment; (iii) building of the model; (iv) optimization of the model (v) quality assessment and validation of the model.

The first crystal structure of a human GPCR, the β_2-AR (PDB ID 2RH1) was released in late 2007 in its inactive state.[69,70] The β_2-AR shares a high sequence identity within the receptors we are interested in (up to 60%),[102] and the available structure contains a non-covalently bound ligand, carazolol, which bears structural similarity to antipsychotic drugs. All these facts point to β_2-AR as a good template for modeling the binding site of the antipsychotic drugs receptorome. In fact, although other crystal GPCRs structures have been solved lately, the most successful prediction methods of GPCR structures have relied on homology modeling based on the structure of β-AR as the template.[103]

With respect to the first step (sequence alignment) diverse softwares, such as CLustal X,[104,105] can carry out automatically the multiple sequence alignment of the sequences to the template. Nevertheless, generating models for proteins with less than 30% overall homology to the template often means that the alignment can be unreliable.[106] In this sense, GPCRs are a unique case, as the low sequence identity is compensated by a high structural similarity, namely the 7TM helices pattern (see section 1.4). Therefore, the key GPCR residues conserved in each helix can be aligned manually to generate good quality homology models, particularly within the TM

region.[107]

The most widely applied software for homology modeling is MODELLER.[108] It uses the alignment of the sequence to be modeled with the template for calculating automatically a model containing all non-hydrogen atoms. The loop regions of GPCRs tend to be less conserved than the TM regions and are structurally diverse in the available GPCR structures, as described in section 1.4. Therefore, modeling of these loop regions constitues a more difficult task. The loops can be modeled with reasonable confidence if they bear similarity in length and conformation with the template. If not, it would be necessary to use fragment-search based or *ab initio* based methods[109] for predicting these loop conformations.

Geometrical optimization methods are then applied to refine the obtained models. These methods relax the structures and improve the side chains packing allowing to obtain a conformation corresponding to the nearest local minimum of a molecular energy function. The easiest geometrical optimizations (in term of simplicity of the model and time of the calculation) make use of molecular mechanical forcefield, which treats a molecule as a collection of atoms whose interactions could be described by Newtonian mechanics. Among the forcefields available, AMBER99[110] is parameterized for proteins and nucleic acids and is widely used for the study of biomolecules. Several programs, like MOE (Molecular Operating Envionment), allow the application of such validated forcefields for energy minimization and molecular dynamics simulations.

The quality of the final models can be assessed with diverse tools, like PROCHECK,[111] which provides a detailed check on the geometry and stereochemistry of a protein structure (such as covalent geometry, planarity, dihedral angles, chirality, non bonded interactions, etc.).

All the GPCR template structures available when we started this work were in their inactive state. Modeling activated GPCRs is still an open problem as the structures are suspected to undergo large helical movement upon activation.[112] In addition, obtaining crystal structures of active GPCRs is very difficult. Nevertheless, recently the structure of opsin has been solved (PDB ID 3CAP),[113] which correspond to one of the activated states in the rhodopsin activation cascade, and may open a door to activate state modeling.

Since the structures of the binding sites can be inferred by homology modeling, they can be used for prediciting the ligand-receptor interactions through docking simulations.

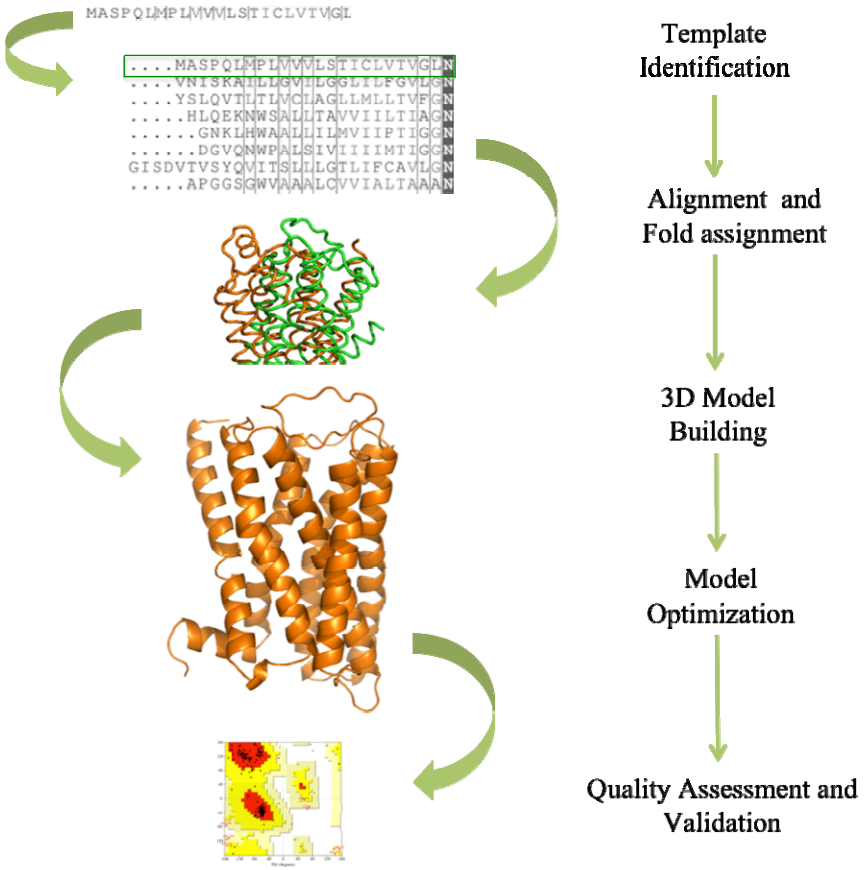


Figure 10. Overview of the GPCR homology modeling protocol.

Docking simulations

Docking, as a computational tool, allows the investigation of the binding between receptors and potential ligands to form non-covalent protein-ligand complexes. In general it can be considered as an energy optimization problem[114] with two components: the search and the score.[115] The "search" is performed by exploring the conformational space accessible for the interaction between the two molecules, with the goal of finding the orientation and conformation of the interacting molecules corresponding to the global minimum binding free energy. Scoring functions are applied to evaluate tightness of interaction i.e. estimate binding free energy. The success of a docking algorithm predicting a ligand-binding pose is normally measured in terms of structural discrepancy (quantified as RMSD) between

the experimentally observed heavy-atom positions of the ligands and the one(s) predicted by the algorithm. The flexibility of the system is a major challenge in the search for the correct pose. The number of degrees of freedom included in the conformational search is a crucial aspect that determines the searching efficiency.[116]

In this thesis we used GOLD3.1.1 program[117] for all docking simulations. It uses a genetic search algorithm and considers full ligand flexibility, as well as rotational flexibility for the protein-receptor polar hydrogen atoms. In the docking process, multiple conformations and ligand orientations are generated and the most appropriate ones selected using the scoring functions of the docking program. The obtained ligand-receptor complexes can be further refined using short molecular dynamics simulations; in this case a force field is necessary parameterized for small organic molecules, such as MMFF94x. The reliability of the docking results can be assessed by comparing key residues observed in the computed complexes with experimental results of site-directed mutagenesis.

Once models of ligand-receptor complexes are obtained, the description of their structures can be done in terms that allow a mathematical and statistical comparison. With this aim we needed to use suitable molecular descriptors.

Molecular descriptors

Molecular descriptors were developed in order to transform chemical information of the molecules into a numerical representation that can be manipulated mathematically. For the characterization of the potential interaction of a small compound with a receptor the most suitable molecular descriptors are the three dimensional (3D) descriptors called molecular interaction fields (MIFs).

Molecular interaction fields

Molecular interaction fields (MIFs) are continuous functions representing the energy of the interaction between a "molecular probe" and the compound studied in every point of the space. Even if analytic MIFs formulations are possible, they are often computed by sampling a closed space around the compound using a simple energy function. For MIFs calculation, a probe is moved at regular intervals within a 3D-box that surrounds the molecule or the regions to be studied, creating a grid of points, so-called nodes, at which the probe-compound energy of interaction is computed using a molecular mechanics energy function. As a result, the intrinsically continuous MIFs

function is transformed into a discrete number of energies associated to defined x, y, z cartesian coordinates.

MIFs can be used in two ways: on proteins, for identifying the regions where a ligand could bind or on ligands, for describing the kind of interaction which the ligand can establish at the receptor binding site. The first application of MIFs computation to ligand design was described by Goodford in his pioneering work of and implemented in his program GRID,[118] which was specifically designed to describe biomolecules for identifying energetically favorable regions where a ligand could bind (Figure 11a). Later on, MIFs have been applied for describing the ligands and their ability to interact with receptors, either individually (Figure 11b) or for series of compounds.

The rational for using these descriptors in the characterization of the receptorome binding site is based on the idea that the MIF are rich in information describing the ligand-receptor binding process. Therefore, the observed differences in the MIFs computed for the set of receptor binding sites, can be associated to changes observed in the binding affinities of the compounds. Similarly, the differences in the MIFs computed for active and inactive molecules can be associated to the changes observed in their biological activities. This is the underlying idea in the comparative molecular field analysis (CoMFA)[119] and GRID/GOLPE[120] methodologies. In both cases, the comparison of MIFs computed in diverse compounds requires that these structures must be first superimposed in the space, in such way that the energies computed at the same position of the space could be directly comparable. This structural superimposition or alignment is not an easy task. When the compounds share common scaffold or evident pharmacophoric elements, it is feasible, but when they are structurally diverse or such common features are not that clear, the procedure is difficult and the results are often arbitrary. Moreover, the procedure is difficult to perform in an automatic way and usually requires intensive human intervention that limits the applicability of the method and also the size of the series to be investigated. For these reasons, GRID INdependent Descriptors (GRIND) were suggested as a new generation of MIF-based alignment-independent molecular descriptors, specifically designed to characterize ligand-receptor interactions.[121,122] The GRIND method does not aim to capture all the information present in the MIF, but just to identify relevant regions of interaction and describe their relative positions. The latest generation of the GRIND, the so-called GRIND-2,[123] were developed for improving the quality, calculation speed and interpretability of the GRIND. GRIND-2 have been implemented in Pentacle software developed recently in our group,[124] which includes all the tools for their application in quantitative structure-activity relationship (QSAR) studies.

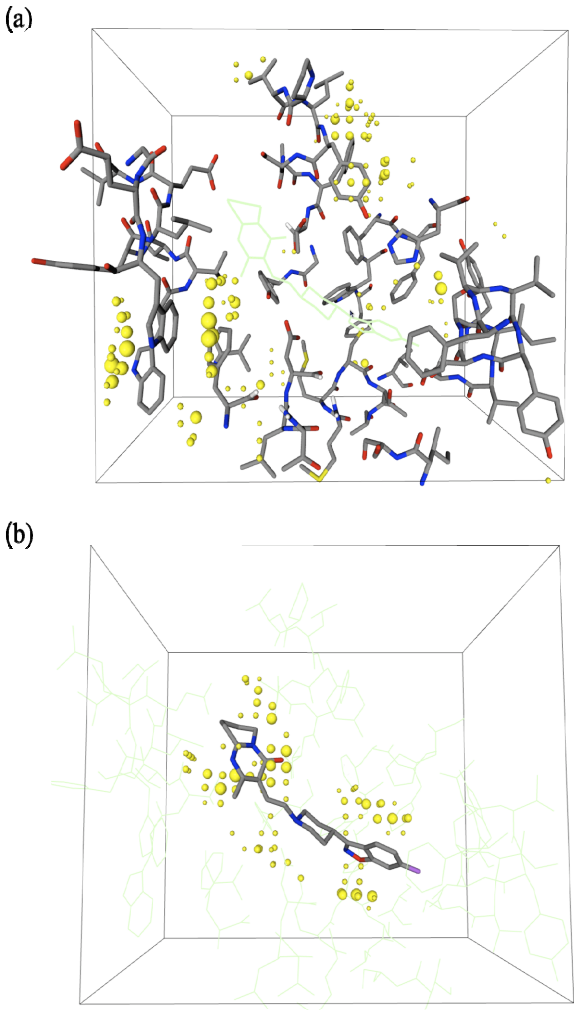


Figure 11. MIF calculation results for a receptor (D_2 receptor) (a) and for a ligand (risperidone) (b) using DRY probe.

3D-quantitative structure-activity relationship studies

Quantitative structure-activity relationship (QSAR) is a set of mathematical and statistical techniques trying to correlate quantitatively structural molecular properties (molecular descriptors) with biological activity for a set of similar compounds (Figure 12). The empirical model obtained with these

techniques describes the relationship between differences observed in the structure and the changes in the biological activity. It is important to emphasize that a QSAR model is not a mechanistic model, like the ones found in Physics or Chemistry. Such models are only possible for phenomena that could be described exhaustively, which is not the case in most drug discovery process. QSAR models belong to an inferior rank, the so-called empirical models that approximate the response of the system in a limited range of the variables involved.[125]

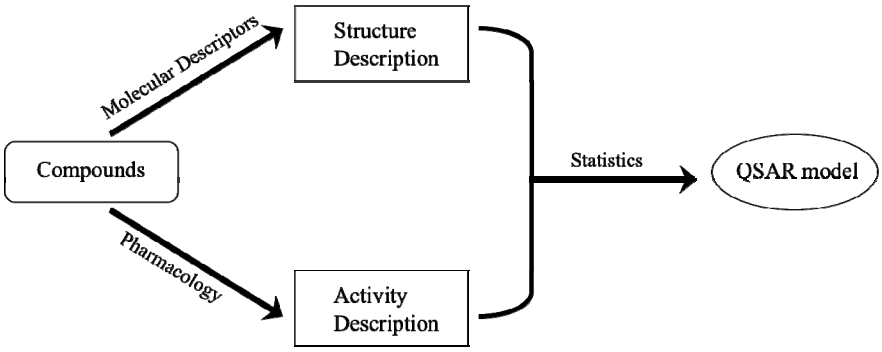


Figure 12. Scheme of the process to obtain a QSAR model.

3D-QSAR methods are a subfamily of QSAR which make use of molecular descriptors in which the variables are linked to 3D coordinates, like MIFs. Very popular methods like CoMFA and GRID/GOLPE cited above are examples of 3D-QSAR methodologies.

Even if the MIFs are highly relevant descriptors, their use in 3D-QSAR has the drawback of producing a large number of variables (between a 10^4 and 10^6), difficult to handle and to analyze. In this case, the application of multivariate analysis techniques for extracting information and building regression models is compulsory. Among the most popular methods are the principal component analysis (PCA) and partial least squares (PLS) regression.

Principal component analysis

Principal component analysis (PCA)[126,127] is a multivariate analysis tool for data supervision and dimensionality reduction that allows the discovery of trends in a set of objects defined by several variables. In few words, PCA is applied to a *X* matrix, where each row contains the variables (descriptors) representing an object (molecule). The result of the analysis is a summary of

the original matrix which can be used to describe the objects using a few, highly informative variables called Principal Components (PC). The underlying formula in PCA calculations is defined by equation 1.

$$X = 1 \cdot \overline{x}' + T \cdot P + E \qquad \text{eq.1}$$

where X is the object matrix, $1 \cdot \overline{x}'$ represents the variable averages, T is the scores matrix that contains information about the objects, P is the loading matrix that contains the weight of each variable in the model, and E is the residual matrix that contains the information not explained by the model.

PCs are extracted in such a way that the projection of the X matrix on the PC maximizes the sum of squares. Also, each PC extracted must be orthogonal to the previous ones, that is, each PC is completely independent to each other and there is no correlation between the information they contain. As a consequence, the first PCs condense much of the information present in the original X matrix and a 2D or 3D scatter plot of the first PCs clearly shows the types of objects, the presence of clusters, outliers, etc. On the other hand, the scatterplot or bar plots of loadings are useful to identify the variables that discriminate between the objects.

Partial least squares

Partial least squares (PLS)[128] is a regression analysis tool, which connects the information included in two blocks of variables, X and Y, to each other. It is used for building predictive models when the number of variables is much higher than the number of objects. In the context of 3D-QSAR the biological activity is used as Y variable. The function relating X with Y variables can be represented by equation 2.

$$Y = XB + G \qquad \text{eq.2}$$

, where B is the regression coefficient matrix and G a noise matrix. The B matrix can be split into three matrixes: the weights (W and C) and the loadings (P) of the model,[129] as the next equation shows:

$$B = W(P'W)^{-1}C' \qquad \text{eq.3}$$

One of the problems of PLS regression models is the possibility to overfit, that is, to explain the noise present in the model instead of its underlying relationship. In order to avoid overfitting, the determination of the suitable

number of Latent Values (LV) cannot be done based on the quality of the fitting but on the predictive ability of the model. Ideally, such predictive ability must be evaluated using an external set, however the selection of an external test is not an easy task and in practice, the most common way to assess the predictive ability of the model is to use cross-validation. There are different cross-validation methods depending on how many objects are used in each interaction. Two examples are: Leave One Out (LOO), where one object is extracted from the model and predicted by using the model obtained with the whole set without itself, and Random Groups (RG), where a number of *k* groups of *j* objects are extracted randomly and predicted in front of all the remaining objects. Then the predicted Y values (y') are compared with the real Y values (y) in order to obtain a quantification of the prediction. Two metrics used for assessing the prediction are Standard Deviation of Error of Prediction (SDEP) and the predictive correlation coefficient (q^2), defined by the following equations:

$$SDEP = \sqrt{\frac{\sum (y - y')^2}{N}} \qquad \text{eq.4}$$

$$q^2 = 1 - \left[\frac{\sum (y - y')^2}{\sum (y - \bar{y})^2} \right] \qquad \text{eq.5}$$

, where y is the real value, y' is the predicted value, $\bar{y}$ is the average Y value, and N is the number of objects.

GRID/CPCA approach

The GRID/CPCA approach is based on the application of multivariate statistical methods to the MIFs obtained using GRID for a large set of proteins, allowing the identification of residues or regions most involved in the interactions ligand-receptor.[130] The method implies the superimposition in the space of the MIFs computed on the binding site of the modeled complexes in such a way that the energies computed at the same position of the space could be directly comparable. Then, the computed MIFs can be analyzed using chemometrical tools such as GOLPE,[131] applying multivariate methods like PCA (described above) or consensus principal analysis (CPCA). The CPCA method uses exactly the same objective function as PCA providing information regarding the importance of the different probes in the analysis. However, CPCA is able to work with several protein targets allowing the generation of selectivity profiles between groups of targets. In the GRID/CPCA approach, the 3D structures representing the targets are

analyzed using GRID, but the MIFs obtained for the different probes are added, side-by-side, adding new variables to the same object. CPCA highlights then differences between the common features of the targets, which will conform obvious hot spots for the design of compounds with a unique profile.[132] This methodology has been used previously for the analysis of diverse sets of targets, like human and bacterial DHFR[130] and serine proteases.[132]

2. HYPOTHESIS AND OBJECTIVES

Hypothesis

The most relevant pharmacological properties of antipsychotic drugs depend on their affinity binding profile towards a set of receptors (the so-called antipsychotic drug receptorome). Therefore, the comparative analysis of the receptorome binding sites and of the molecular mechanisms involved in ligand binding can provide useful information for the design of more effective and safer compounds, characterized by showing an optimum binding profile.

Main Objective

Main objective of this thesis was to build suitable structural models of all the receptors putatively involved in the antipsychotic drug receptorome and to carry out a detailed comparative analysis of their binding sites. In more detail, we can distinguish the following concrete objectives.

Concrete objectives

1. Obtain structural models for all the receptors included in the antipsychotic drug receptorome using homology modeling methods, in order to obtain comparable structures of the ligand binding sites, alone and in complex with relevant antipsychotic drugs.

2. Carry out a detailed comparative analysis of the receptor binding sites and connect the differences observed with the binding affinity profiles and the pharmacological properties of relevant antipsychotic drugs.

3. Further exploit the structural information obtained with the models for rationalizing observed experimental results of particular interest.

3. RESULTS AND DISCUSSION

After the background and methodological introduction presented in the previous sections, here we will describe and discuss the results obtained in this thesis. A more detailed description of all outcome and the methods applied can be found in the publications attached in the next section.

Objective 1.

> *Obtain structural models for all the receptors included in the antipsychotic drug receptorome using homology modeling methods, in order to obtain comparable structures of the ligand binding sites, alone and in complex with relevant antipsychotic drugs.*

The first objective of this thesis was to generate 3D homology models of some of the GPCRs putatively implicated in psychosis treatment. My work started by defining the receptorome set to model and by selecting the template. The selection was based on the multireceptor profile of clozapine, widely considered as a gold standard (described in section 1.3), and on the pharmacological data available. The set of receptors to study included the following GPCRs: dopamine (D_1, D_2, D_3 and D_4), histamine (H_1), cholinergic muscarinic (M_1, M_2, M_3 and M_4), adrenergic (ADA_1 and ADA_2) and serotonin ($5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$, $5\text{-}HT_{2B}$, $5\text{-}HT_{2C}$, $5\text{-}HT_6$ and $5\text{-}HT_7$) receptors.

The homology modeling of all these receptors was carried out using the crystal structure of the human β_2-AR (PDB entry 2RH1) as the template. The receptor sequences were aligned with ClustalX[104,105] introducing secondary structure information derived from the crystal structure and ensuring a perfect alignment of the highly conserved residues of the family A GPCRs. 3D models were then built using MODELLER[108] and optimized using AMBER99 force field[110]. The quality of the models was checked with PROCHECK software,[111] resulting in high quality parameters. The obtained models reproduced the correct orientation of the side chains for the set of highly conserved amino acids in the GPCR superfamily, in more detail the residues involved in the toggle switch (described in section 1.4). These residues were set to the "inactive state", being more consistent with the inactive state of the template structure (2RH1). Regarding the loops, we modeled them based on the conformation of the template.

We further validated our models through the construction of complexes with relevant antipsychotic drugs for the different studies. The drugs were docked using GOLD3.1.1 program[117] into the binding sites of the modeled receptorome, defined by the conserved residue Asp 3.32, known to be important for ligand interaction.[133,134] Afterwards, the complexes were refined by short molecular dynamics simulations and validated with

experimental mutagenesis data. Details of this part of work are described in **publications 1, 2, 3, 4, 5 and 6**

Objective 2.

> *Carry out a detailed comparative analysis of the receptor binding sites and connect the differences observed with the binding affinity profiles and the pharmacological properties of relevant antipsychotic drugs.*

The models I generated were subjected to structural analysis and their differences were connected with the binding affinity profiles shown by relevant antipsychotic drugs. Carrying out such analysis was far from being simple and required to develop a novel methodology for describing at diverse levels the drug-receptor interactions using multivariate analysis methods. In collaboration with other authors, such methodology was proposed and applied in **publication 1 (A Novel Multilevel Statistical Method for the Study of the Relationships between Multireceptorial Binding Affinity Profiles and *in vivo* Endpoints)**. The method was based on the sequential building of multivariate statistical methods, such as principal component analysis (PCA) and partial least square (PLS) to describe the connection between *in vivo* observations, profiles of binding affinities for multiple receptors and structural information. The method was applied for studying the metabolic side effects of 25 APDs, detecting clustered receptors confirmed by experimental evidence found in literature. My contribution in the structural part of the study required the building and analysis of complexes of clozapine with the receptor binding site models interesting for this work (dopamine D_2, D_3 and D_4, histamine H_1, cholinergic muscarinic (M_1, M_2 and M_3 and serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_6 and 5-HT_7 receptors). This analysis suggested that polar regions in TM5 and drug-receptor interactions involving hydrophobic residues in TM3, TM5 and TM6 were important discriminators between receptors associated to high metabolic side effects and those with low side effects. The results obtained emphasized the importance of a multireceptorial treatment and provided useful hints in our understanding of the therapeutic effect of APDs at a molecular level.

Most of the APDs studied in the previous work were characterized by a long extended structure, which differentiates from the tricyclic scaffold of clozapine-like compounds. Thus, I choose risperidone as representative of the extended architecture for doing an extensive analysis of its binding profile. In this case the GRID/CPCA methodology[130] was used for carrying out a comparative structural analysis of the whole APDs receptorome

modeled in objective 1. The study started with obtaining a consistent superimposition of the binding sites of risperidone-receptor complexes, allowing the comparison of the MIFs computed on their binding pocket. The preliminary results obtained, detailed in **publication 2** (**Comparative Structural Analysis of the Risperidone Receptorome Using GRID/CPCA Methodology**, manuscript draft), unveiled a receptor clustering related to metabolic side effects that was in agreement with the outcome of the previous work. Furthermore, the analysis based on the MIFs suggested that hydrogen bond region at the extrem of the pocket comprising TM2 and TM7 was important for discriminating receptors associated to metabolic side effects from the ones that do not. Moreover, the obtained data indicate that the change in the pocket size due to positions 6.52, 3.37 and 3.33 was of relevance. All these findings complement the distinctive features suggested in the previous work, which are susceptible to being exploited for designing new compounds that lack features related to undesired side effects.

Objective 3.

Further exploit the structural information obtained for rationalyzing observed experimental results of particular interest.

The set of modeled receptors obtained in this thesis were a valuable resource on its own. During the thesis, and in collaboration with other groups, we exploited them for rationalizing the observed experimental data available for different compounds.

In a first application we focused on clozapine and olanzapine. Both are good examples of efficient APDs with a complex multireceptor profile, having affinities toward serotonin, dopamine, α-adrenergic, muscarinic, and histamine receptors, among others. In **publication 3 (Multireceptorial Binding Profile of Clozapine and Olanzapine. A Structural Study Based on the new β_2-Adrenergic Receptor Template)** such modeled receptors were used for identifying characteristics of the complexes with clozapine and olanzapine that could explain their clinical behavior. In this work the models of the binding site of 5-HT_{2A} and D_2 receptors were submitted to a punctual comparative analysis for explaining the differences in affinity of both ligands for the same receptor, which pointed to position 3.36. The models were also used to explain the differences in affinity on both compounds for different receptors, which were described to be related to structural differences between clozapine and olanzapine together the diversity in both TM5 and TM6 helices.

We further applied the modeled receptors for studying experimental data available for new synthesized compounds as putative APDs. This part of work was made in collaboration with the Chemical and Pharmacological departments of Universidad de Santiago de Compostela, who provided the new compounds and their pharmacological activity evaluation.

The Pharmacology and Chemistry group synthesized a series of aminomethylbenzofuranones based on the idea to find a single molecule with balanced affinities for 5-HT_2 and the D_2 receptor families for the potential use as treatment for schizophrenia. With this aim, they obtained the binding activity of the compounds for D_2, 5-HT_{2A} and 5-HT_{2C} receptors and we were involved in the computational studies, as described in **publication 4 (Synthesis, Binding Affinity, and Molecular Docking Analysis of New Benzofuranone Derivatives as Potential Antipsychotics)**. My contribution in this work, in collaboration with other authors, consisted on rationalizing the differences in the observed affinities. We analyzed the 5-HT_{2A} and D_2 receptor complexes with representative compounds, suggesting the serine residues Ser3.36 and Ser5.46 as explanation for these differences. The results obtained provided new insights into the binding mode of ligands to the D_2 and 5-HT-$_{2A}$ receptors and thereby contributed to this very active research area.

Besides 5-HT_{2A} and D_2, other receptors are included in the multireceptor profile of the antipsychotic drugs, as stated in section 1.3. Among them D_3 receptor seems to be another promising target. Following this idea, the Pharmacology and Chemistry group synthetized a series of new benzolactam derivatives as putative D_3 antagonists and evaluated their affinities at the dopamine D_1, D_2, and D_3 receptors. As in the previous collaboration, I contributed on providing validated complexes between some of the most representative compounds of this series and the D_2 and D_3 receptors in order to rationalize the experimental data obtained. The details of this work are fully described in **publication 5 (Synthesis, Binding Affinity and SAR of new Benzolactam Derivatives as Dopamine D_3 Receptor Ligands).** The structural analysis suggested that polar residues in TM7 and hydrophobic residues in TM2 and EL2 act as modulators in binding affinity for D_2 and D_3 receptors. The analysis also revealed structural features of the ligands that seem to modulate D_3 selectivity versus D_2 receptor.

With the aim of studying more in deep structural properties linked to the observed selectivity of the benzolactam derivatives in the preceding results, I proceed to investigate the relationships between their structure and their binding affinities in D_2 and D_3 receptors using both ligand-based (3D-QSAR) and receptor-based methods. All the compounds (the ones from the previous series and 12 new ones provided by the Pharmacology and Chemistry group)

were submitted to docking simulations into homology models of D_2 and D_3 receptors and the docked ligand structures were used to build 3D-QSAR. An extended description of this analysis can be found in **publication 6 (Synthesis, 3D-QSAR and Structural Modeling of Benzolactam Derivatives with Binding Affinity for the D_2 and D_3 Receptors)**. The 3D-QSAR results suggested some of the most influencing ligand features for D_3 selectivity, such as the adequate location of the benzolactam carbonyl oxygen. The rational analysis of the ligand-receptor complexes pointed to the presence of a hydrogen bond network in D_2 receptor, absent in D_3 receptor, as the explanation for the differences observed in binding. The data obtained improved significantly our knowledge of the structural diversity of the D_3 and D_2 binding sites that can be exploited for the designing of novel compounds for the treatment of schizophrenia.

4. PUBLICATIONS

PUBLICATION 1

A Novel Multilevel Statistical Method for the Study of the Relationships between Multireceptorial Binding Affinity Profiles and *in vivo* Endpoints

Jana Selent, Anna Bauer-Mehren, Laura López, María Isabel Loza, Ferran Sanz, and Manuel Pastor

Mol. Pharmacol. **2010**; 77: 149-58

Selent J, Bauer-Mehren A, López L, Loza MI, Sanz F, Pastor M. A novel multilevel statistical method for the study of the relationships between multireceptorial binding affinity profiles and in vivo endpoints. Mol Pharmacol. 2010; 77(2): 149-58.

PUBLICATION 2

Comparative Structural Analysis of the Risperidone Receptorome Using GRID/CPCA Methodology

Laura López, Anna Bauer-Mehren, Jana Selent and Manuel Pastor

(Manuscript draft)

Comparative structural analysis of the risperidone receptorome using GRID/CPCA methodology

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We present a comparative structural analysis aimed to describe the pharmacological profile of risperidone, with potential application for guiding the design of antipsychotic drugs. It starts from a set of consistent structural models of the receptors implicated in risperidone activity. Then, the sequence and molecular interaction fields analysis computed at the binding sites are compared using multivariate statistical methods. The results show an interesting receptor clustering which can be related with pharmacological effects, and which can be interpreted in structural terms by the presence of different hydrogen bond regions comprising TM2 and TM3-TM5.

Introduction

Attempts to develop selective drugs for the treatment of schizophrenia have been frustrated by the complex etiology of the disease. Indeed, currently available antipsychotics, such as risperidone or clozapine, exhibit nanomolar affinities for several receptors, (mainly G protein-coupled receptors, GPCR) which would account for the particular efficacy/side effects of each of these drugs.[1] This new paradigm in drug discovery expands the classical concept single target, by incorporating a whole collection of biomolecules the function of which must be modulated simultaneously.[2]

Given the unknown etiology of schizophrenia, and the fact that gene-based methodologies to target identification and drug discovery have not resulted in the discovery of any of the currently used antipsychotics,[3] the detailed study of the mechanisms of action of the current drugs in order to define their optimum binding profile constitutes an interesting alternative in antipsychotic drug discovery. In this respect, our group introduced recently a novel multilevel methodology[4] which could be applied for the identification of the binding affinity profiles linked to relevant *in vivo* effects and which allowed to translate these findings in structural terms. In the first published example, the binding affinity profile linked to antipsychotic metabolic side effects was associated with sequence changes in the binding site, using as a reference structures of the receptors with clozapine.

In the present work we are extending this method by applying a more sophisticated description of the receptor binding site based of Molecular Interaction Fields, and using as reference complexes of the receptor set with risperidone. Risperidone is a clinically useful antipsychotic, associated to weight gain side effects,[5] which is probably more representative than clozapine of the chemotype shared by antipsychotics. Unlike the tryciclic scaffold of clozapine-like compounds, risperidone is structurally characterized by two bi-aromatic moieties (fluorobenzisoxazolyl, FB, and benzouracil, BU) connected by a flexible linker (Chart 1).

Herein, the aim of this work is to identify the structural differences between the binding sites of a set of receptors putatively involved in the antipsychotic effect of risperidone and link them to the binding affinity profiles identified in previous works and, indirectly, to observed pharmacological effects, including both side effects and characteristics of the pharmacological effects.

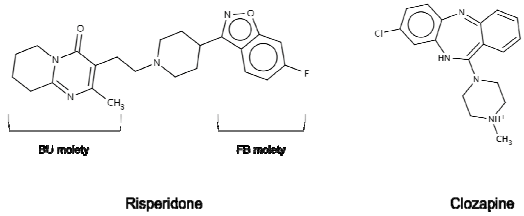

Chart 1. Chemical structures of risperidone and clozapine.

Results and Discussion

Risperidone docking into the receptorome binding site

The set of receptors putatively implicated in antipsychotic effects was selected as described in[4], adding three receptor subtypes to the published set, 5-HT_{2B}, D_1 and M_4 (Table 1). Homology models for all of them were obtained based on β_2-adrenergic structure[6][7] and following a standardized protocol that was previously published in our group.[8]

A first docking of risperidone into each receptor was carried out, hinting the binding pocked by the position of the Asp3.32, which is known to establish a charge-reinforced hydrogen bond with the protonated nitrogen present in aminergic ligands (like risperidone). In order to obtain a consistent arrangement of the side chains lining the binding site, we adjusted them to the ones in the template structure (PDB ID 2RH1) [6,7] and then re-docked the ligand in the refined pocket (see details in the experimental section). The first docking results showed that risperidone can adopt two major modes upon binding, which differ mainly in the 3D orientation of the fluorobenzisoxazolyl ring (FB): (1) directed towards transmembrane helix (TM) 5; (2) directed towards the extracellular loop 2 (ECL2). In both modes, the ligand occupies the same pocket on the GPCRs, located in a region comprising TM2, TM5, TM6 and TM7 (Figure 1).

Table 1. Receptors used in this study	
Receptor	pK_i risperidone
5-HT_{1A}	6.4
5-HT_{2A}	9.4
5-HT_{2B}[9]	7.5
5-HT_{2C}	7.5
5-HT_6	5.8
5-HT_7	8.2
D_1[9]	6.4
D_2	8.4
D_3	8.0
D_4	7.9
ADA_1	7.9
ADA_2	7.5
H_1	7.9
M_1	5.2
M_2	5.2
M_3	5.1
M_4[9]	5.3

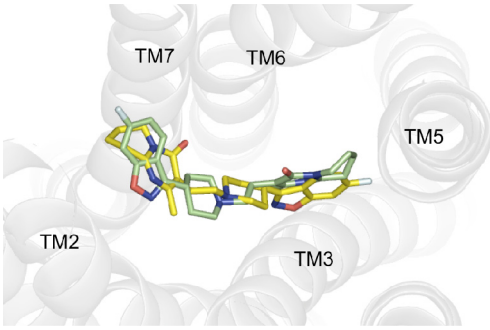

Figure 1. Two possible binding modes of risperidone. Mode (1) is shown in yellow; mode (2) in green.

Multiple ligand binding modes have been reported previously.[10] In the works of Runyon *et al*.[11] and Dezi *et al*.[12], for example, they obtained ketanserin binding modes with different orientation in 5-HT_{2A} receptor which are likely to contribute to the observed affinity for the ligands. Thus, valid docked solutions with different orientation for risperidone could exist. Nevertheless, after adjusting the binding site sidechains, the most preferred pose was the one with the FB group oriented toward the inner part of the binding pocket (TM5-TM3), and it was selected as the most representative for our study.

Risperidone docks into the binding site defined by two subsites (Figure 2): the FB moiety is directed towards the region flanked by TM6 and TM7, marked with a strong aromatic character and the hydrophilic region between TM5 and TM3; the benzouracil moiety (BU) is stabilized mainly by aliphatic and polar residues in TM1, TM2 and TM7. This position is in agreement with previous docking results of ligands structurally similar to risperidone[12,13] and mutagenesis data.[14-16]

The consistency of the refined risperidone-receptor complexes obtained was quantified in terms of RMSD for the C-alfa of the conserved residues located within a radius of 8 Å from the ligand. The average of RMSD was 0.29 (Figure 3), showing a good uniformity of the side chains lining the binding site of the receptorome. The residues with the higher deviations are those situated in the loops, as it could be expected due to the variability of these regions between receptors.

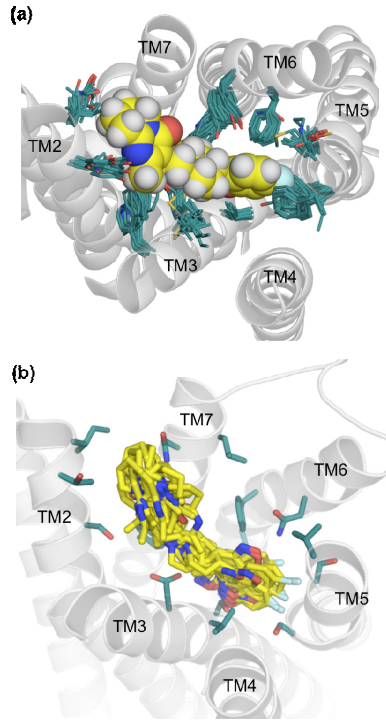

Figure 2. (a) Side chains lining the binding site of the 17 GPCR homology models. Risperidone in the 5-HT_{2A} complex is shown as reference in yellow. (b) final docking positions of risperidone obtained for the 17 receptors. The structure of 5-HT_{2A} is shown as reference.

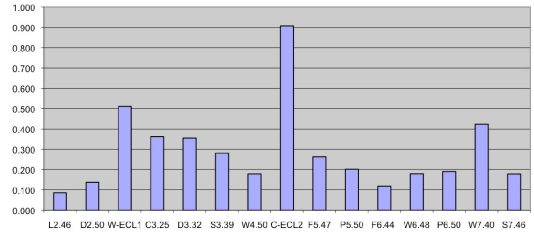

Figure 3. RMSD values for the C-alfa of the conserved residues lining the binding site of risperidone.

Sequence analysis: Principal properties analysis

The analysis of the principal properties of the residues lining the binding site of risperidone was performed using the same methodology described in Selent *et al*.[4] The residues aligned around 8 Å of the ligand in the binding site, were extracted and described by their principal properties using 5 different descriptors[17] representing charge, molecular weight, lipophilicity, as well as rigidity and flexibility. Subsequently, Principal

Component Analysis (PCA) was applied in the resulting matrix in order to discriminate the receptors in base on the properties of the residues.

The PCA scores plot reveals two clusters at both ends of the PC1 (Figure 4a): (1) muscarinic receptors and (2) the rest of receptors. According to the findings reported in[4] the first PC discriminates between receptors related to metabolic side effects of risperidone (cluster 1) and the ones which not (cluster 2). Regarding to PC2, the scores discriminates a cluster formed by 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} (on top) from the rest of receptors. Interestingly, PC2 discriminates 5-HT_{2A} from D_2, two receptors included in the Meltzer index, which is used even nowadays for measuring the "atipicality" of the antipsychotic drugs.[18]

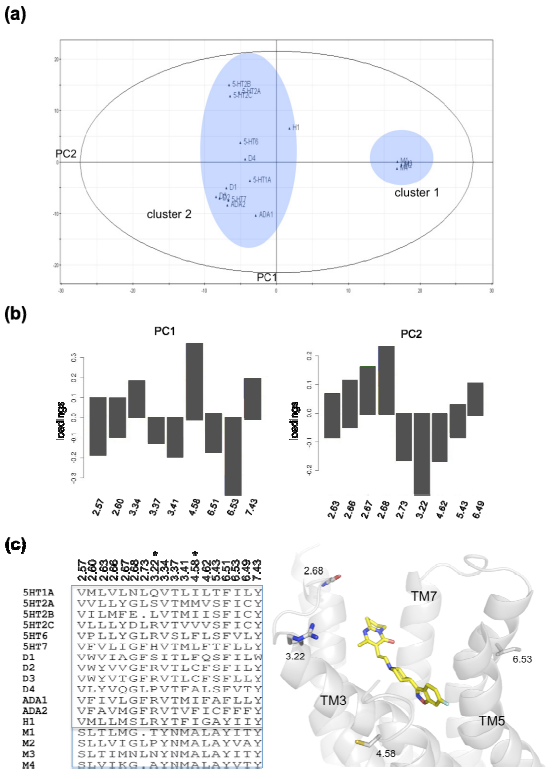

Figure 4. PCA outcome of the principal properties analysis. (a) Scores plot results (b) Coefficient plots of PC1 and PC2; (c) Sequence alignment of the sequence positions highlighted in the analysis (left) and the depiction of some of the most representative in the risperidone-D2 receptor complex (right).

The loadings plot in Figure 4b shows the sequence positions contributing most to both PC. Of particular importance are positions 4.58 in PC1 and 3.22 in PC2. The residue 4.58 is situated in the outer part of TM4, quite far from the binding site crevice, and the residue 3.22 is situated at the extracellular surface of TM3 helix, also far from the binding site (Figure 4c). Thus, the description based on the principal properties analysis has highlighted residues that probably are implicated only indirectly in the discrimination between the receptors but not involved in direct interactions with the ligand. This is not surprising since the degree of sequence homology in the binding site is rather high and the description based in principal properties does not account for changes in the residues locations, only for sequence changes. Nevertheless, it is worth to mention that residue 4.58 has been reported to be implicated in the dimerization process of the receptors[18] while 3.22 seems to be important for the coupling selectivity (binding of Gi/o protein)[19], so both residues are potential hotspots for further studies. Therefore, in order to introduce a more detailed description of the structural differences present at the binding sites, we applied the GRID/CPCA methodology to the modeled complexes.

GRID/CPCA methodology

GRID/CPCA methodology[20] describes potential interactions of the receptors by computing molecular interaction fields (MIF) at the ligand binding site and analyzing the differences observed for the diverse receptors.[20,21] The MIFs were computed applying GRID[22] using three probes, DRY, O, and N1, representing, respectively, a hydrophobic, a hydrogen bond acceptor, and a hydrogen bond donor group, on a grid cage which encloses completely the risperidone binding site. The so obtained MIFs were imported into the program GOLPE,[23] pretreated and scaled as described in the Methods section, before using the consensus principal component analysis (CPCA).

The scores plot show two clusters, muscarinic receptors (cluster 1, associated to metabolic side effects) and the rest of receptors (cluster 2) separated by the loadings of PC1, whereas PC2 discriminates D_2 and D_3 receptors from the rest, mainly from 5-HT_{2A} and D_1 (Figure 5). It must be noticed that these results are rather similar to the ones obtained using the principal properties (Figure 4b). The CPCA results also signal the probe O explains 42.3 % SS and is the one which explains more variability in PC1 (Table 2).

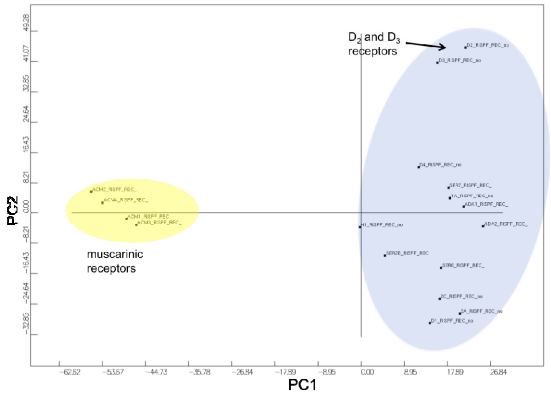

Figure 6. CPCA Score plot result. PC1 can be used for discriminate between muscarinic receptors and the other ones, whereas PC2 discriminates between D_2 and D_3 receptors and the rest.

In this work we will focus on the analysis of the PC1, which seems to be clearly related with the receptors more linked to the metabolic side effects. Before starting the interpretation of the coefficient plots, the reader must be aware that the MIFs assign negative energy values to favorable probe-ligand interactions and positive values to unfavorable (repulsive) probe-ligand interactions. Positive field values represent mainly the molecular shape, while negative values represent regions where the ligand

can make energetically favorable interactions with the binding site. For the interpretation of the results, we have chosen risperidone-M_2 and risperidone-D_2 complexes as representative cases for the clustering of receptors.

Table 2. GRID/CPCA results

	Component		
%VarAccum[a]	1	2	3
Total	16.71	22.11	26.35
DRY	9.98	12.11	16.36
N1	18.01	24.26	28.98
O	20.55	26.31	30.45

[a] Accumulated percentage of X variance explain by the model

DRY probe: The most negative values correspond to favorable interactions with muscarinic receptors in cluster 1. These are located in two small areas, one between the FB moiety and the piperazine ring, and a smallest one in the BU moiety (figure 7a). The superimposition of these regions in the binding site of M_2 receptors (Figure 7a, right) show that the residues implicated in the hydrophobic interactions are diverse tyrosine situated in TM3, TM6 and TM7 helices. Y7.39 and Y6.51 close the binding site allowing the FB moiety to make π interaction with Y3.33 and Y7.39 to make π interaction with the piperazine ring. Besides, Y2.61, situated in TM2, interacts with W7.40 and the backbone of T7.36, limiting the size of the binding pocket (Figure 8a). In cluster 2, the highest positive values for the hydrophobic interactions signal slightly different regions in the receptors. In this case, in position 3.33 there is a Val instead of Tyr, allowing a hydrophobic sandwich of FB moiety between V3.33 and F6.52. On the other hand, the BU moiety is stabilized by hydrophobic interactions with V2.61 and the residue in the ECL2 Ile182, giving the space necessary to the ligand for accommodating the carboxyl of the BU moiety towards TM7 where it can establish polar contacts (Figure 8b). Thus, the hydrophobic regions surrounding risperidone are important for discriminating between receptors, which is in agreement with previous work,[4] and the change in binding pocket due to the presence of big aromatic residues (tyrosines in concrete) in TM2 + ECL2 + TM7 can be associated to metabolic effects of the antipsychotics.

O probe: Not surprisingly, the O probe overlaps some of the residues able to act as hydrogen bond donors in the receptors. Regarding to the negative loadings, a large region is found between TM6 (Y6.51, N6.52) and TM3 (S3.36, N3.37, Y3.33) in the receptors of cluster 1 (Figure 7b). According to the structural models, the FB moiety of the risperidone accommodates between Y3.33, Y6.51 and N6.52 residues, positioning the oxygen of the FB moiety towards residues N3.37 and S3.36, which can establish hydrogen bond interactions with the ligand. Interestingly, receptors in cluster 2 have at most one threonine in position 3.37 and a cysteine in 3.36, less suitable to establish hydrogen bond interaction with the FB moiety. Mutagenesis data[24] corroborates the importance of these residues in ligand binding. With respect to the positive loadings corresponding to favorable interactions with receptors in cluster 2, there are two main positive regions located on the extremes of the ligand.

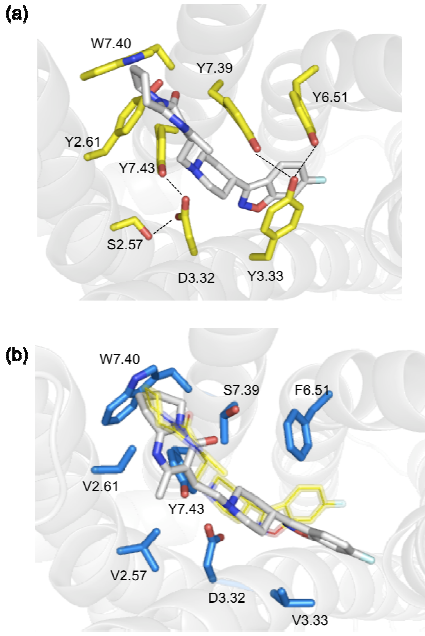

Figure 8. (a) Hydrophobic residues limiting the binding site of M_2 receptor and (b) the corresponding ones in D_2 receptor; for comparison, the position of risperidone in M_2, is depicted in yellow stick.

Their superimposition to the complex of risperidone into D_2 receptor (Figure 7b, right) shows that one region is located near the positions 5.42, 5.43 and 5.46 in TM5 and 6.55 in TM6. Interestingly, most of the receptors in cluster 2 have a serine in 5.42, 5.43 and 5.46 positions, allowing more polar interactions with the FB moiety than muscarinic receptors, which have threonine in 5.42 position and an alanine in 5.43 and 5.46 (Figure 8d). This structural difference due to the nature of three residues on top of TM5 helix (5.42, 5.43, 5.46) was already described in the study of the binding profile of clozapine and olanzapine.[8] Regarding position 6.55, most of the receptors have bulky residues that can act as hydrogen bond donors (eg. histidine, tyrosine, Figure 7d), whereas muscarinic receptors have a conserved valine. The 6.55 position has been previously reported to be important for the binding of agonists and antagonists at serotonergic and other closely related aminergic GPCR subtypes.[25,26] The other region highlighted by the positive loadings is located around the BU moiety. This region does not appear in the negative loadings corresponding to the favorable interactions in receptors in cluster 1, being a clear difference between clusters. This region corresponds to residues W7.40, T7.39 and T7.36 in D_2 receptor. As described before, in receptors of cluster 2 the BU moiety accommodates between residues V2.61 and Ile182, stabilized also by W7.40. In this position, the carboxyl oxygen of the BU moiety can interact with T7.39 and T7.36. Previous work has shown the importance of these residues in modulating ligand binding[27] corroborating the importance of these hydrogen bonds for discriminating receptors associated to side effects.

With respect to the **N1 probe,** overall regions of the N1 probe overlap with the ones of O probe since polar residues can act as hydrogen bond donors and acceptors, and no distinctive features is worth to be reported here.

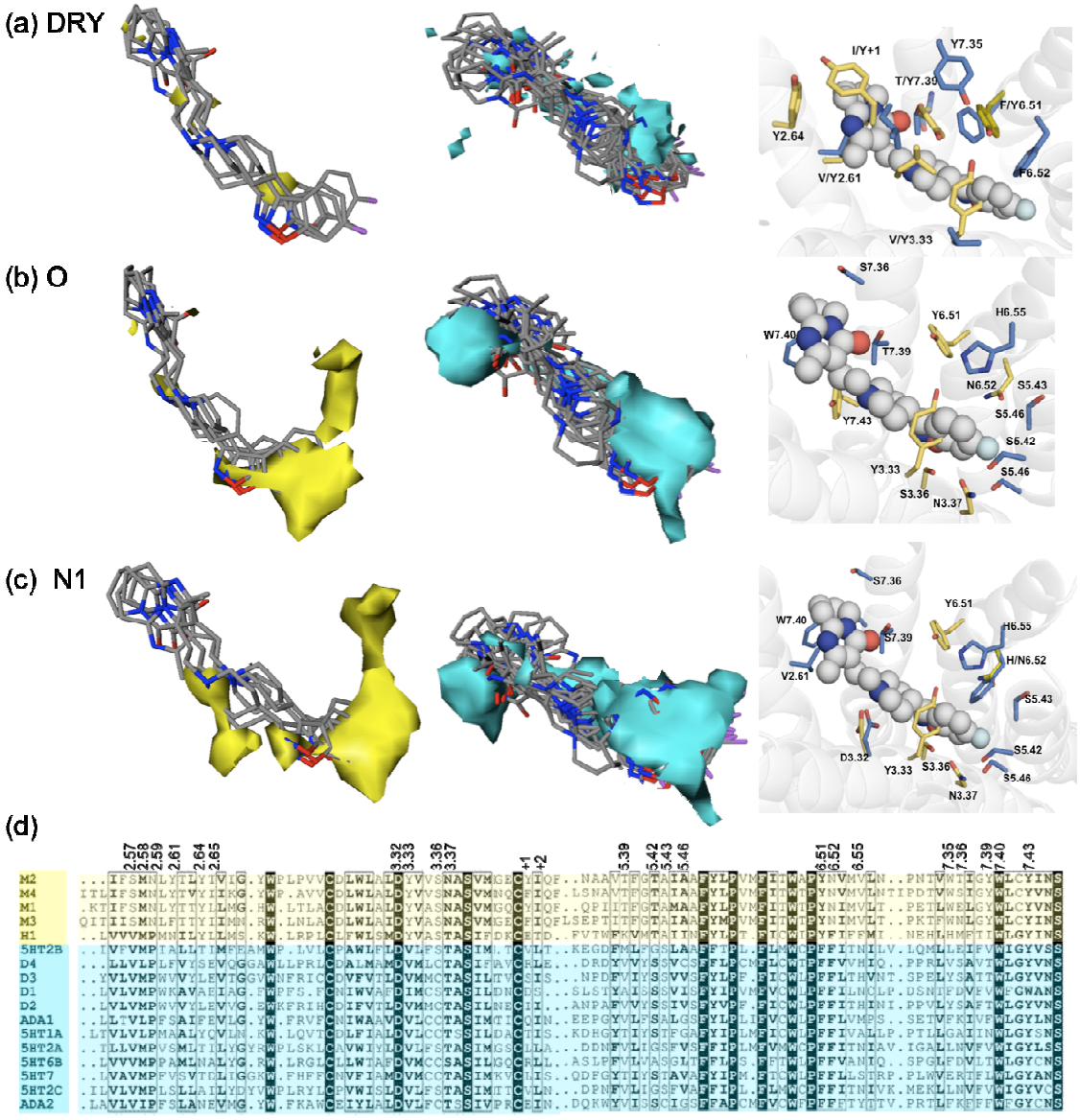

Figure 7. Left, loading plots corresponding to the negative values (yellow fields) and positive values (blue field) corresponding to DRY probe (a) O probe (b) and N1 probe (c); Right, relevant residues. In blue, residues corresponding to D_2. In yellow, residues corresponding to M_2; (d) Alignment of the residues in the binding site of the receptors studied.

To summarize, the model stresses the importance of hydrophobic and polar interactions already described for clozapine[4], like Y3.33, Y6.51, S5.42, S5.43 and S5.46, but adding some original information: (i) highlights the importance of the hydrogen bond donor regions at the extremes of the pocket, mostly the one comprising 2.65, 7.40, 7.39 and 7.36 positions, for discriminating receptors associated to metabolic side effects from the rest; (ii) the change in the pocket size due to the presence of asparagine in 6.52, 3.37 and 3.33 position would be related to side effects.

Conclusion

In this work we described diverse molecular characteristics discriminating the binding for different clusters of receptors involved in the effect of antipsychotics. The use of the principal properties identifies mainly sequence differences. On the other hand, the GRID/CPCA methodology provides further insight, including in the description the changes in the relative position of the residues and demonstrating to be a powerful method for highlighting the most relevant ligand-receptor interactions that discriminate between receptors associated to metabolic side effects and receptors that do not. Then, the simultaneous use of different approaches for describing antipsychotic drugs is essential to define their optimum binding profile, and the information extracted can finally be exploited for the design of compounds with less side effects.

Experimental Section

Numbering of residues:

For residues belonging to helix regions of the G-protein-coupled receptors (GPCRs), the generalized numbering scheme proposed by Ballesteros and Weinstein[28] was used. For simplicity, the residues corresponding to the ECL2 have been numerated according to its position from the conserved cystein.

GPCR modeling and risperidone docking.

The structural models of the 17 GPCR used in this study were built using a standard homology modeling protocol based on the novel template of the β_2-adrenergic receptor, which was previously published by our group.[8] A molecule of risperidone was inserted in the binding site of all the receptors, using the docking software GOLD.[29] Risperidone was docked into the active site of each receptor by defining a 15-Å region centered on the Asp3.32, a residue conserved in all aminergic receptors and known to be crucial for ligand interaction. A distance constraint between the Asp3.32 and the charged nitrogen was also defined. For adjusting the side chains of the binding site, we manually arranged them to be consistent with the ones in the template 2RH1. The resulting structures were subjected to energy minimization using the MMF94x force field in the molecular modeling program MOE (Molecular Operating Environment; Chemical Computing Group) and risperidone was docked again into the refined pockets using the same settings as before. To simulate an induced fit mechanism, the best docking solution was subjected to an optimization protocol. In a first step, a brief energy minimization of the complexes was carried out considering the receptor residues in an 8-Å radius around risperidone. The complex was further refined by means of a 200-ps molecular dynamics simulation (force field MMF94x, 300 K, time step 2 fs) and was subsequently energy minimized by applying gradient minimization until the root-mean-square gradient was lower than 0.001 kcal/mol Å. For the RMSD calculation, we used an special script provided by MOE's team that calulates the rmsd of all heavy atoms of the side chains for a set of superimposed structures.

Prinicipal properties analysis.

The aligned residues within a radius of 8 Å from the ligand were extracted and described using five amino acid descriptors:[17] C7.4: charge as ionizaton state of AA side chain at pH~7.4; MW: molecular weight; CLP: lipophilicity; t1-Rig:rigidity; t2-Flex:flexibility). Subsequently, a principal component analysis (PCA) is applied in order to discriminate the receptors by the properties of these residues.

Principal Component Analysis

PCA is a multivariate analysis tool for data supervision and dimensionality reduction. The method has been described elsewhere.[30] In brief, it computes an approximated lower dimension representation of the original data matrix X, in terms of the product of two matrices: the matrix of objects T (scores) and the matrix of variables P (loadings). In the matrix T, every object is represented by a small number of new variables (principal components), which are orthogonal linear combinations of the original variables, chosen to explain as best as possible the variance present in X. In this work we also applied Consensus PCA (CPCA), a hierarchical variant of PCA which uses the same objective function, but the analysis is made at two levels: the block level, which expresses the contribution of each of the blocks, and the superlevel, which expresses the consensus of all blocks.

GRID/CPCA methodology:

The structures corresponding to the best docking solutions were imported into GRID[22] where MIF were computed for all of them using three probes (DRY, O, N1). All the MIFs were computed using a grid spacing of 1 Å. The analysis was made in a box of 27 x 25 x 25 nodes, containing 2148 energy measures per structure. The resulting MIF were then imported into program GOLPE 4.6.0[23], and the X matrix of variables obtained was pre-treated before submitting to the CPCA analysis, first applying a zeroing of very small values (under 0.01), removing variables with small standard deviation (under 0.05), and ill-conditioned variables that take only two or three different values, one of which is assigned to a single compound. Afterwards a Block Unscaled Weight scaling was applied to the whole matrix to equalize the importance of the different blocks.

Acknowledgements

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Keywords: risperidone · multireceptor profile · GRID/CPCA analysis · side effects · antipsychotic

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PUBLICATION 3

Multi-Receptor Binding Profile of Clozapine and Olanzapine: A Structural Study Based on the New beta(2) Adrenergic Receptor Template

Jana Selent, Laura López, Ferran Sanz, and Manuel Pastor

ChemMedChem **2008;** 3**:** 1194-8

Selent J, López L, Sanz F, Pastor M. Multi-receptor binding profile of clozapine and olanzapine: a structural study based on the new beta(2) adrenergic receptor template. ChemMedChem. 2008; 3 (8): 1194-8.

Supporting information to

Multireceptorial binding profile of clozapine and olanzapine. A structural study based on the new ß$_2$ adrenergic receptor template.

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Table of contents:

1. Numbering of Residues.

For residues belonging to helix regions of the G-protein-coupled receptors (GPCRs), the generalized numbering scheme proposed by Ballesteros and Weinstein[1] was used.

2. GPCR Modeling.

In order to obtain comparable GPCRs models, we developed a standardized modeling protocol to ensure that the structural differences in our receptor models were not consequence of differences in the modeling methodology applied.

The sequence of the 14 GPCRs reported in Table 1 were retrieved from the Swiss-Prot database[2] and aligned with the ClustalX software[3, 4], using the PAM250 matrix and "gap open" and "gap elongation" penalties of 10 and 0.05, respectively. The resulting multiple sequence alignment was realigned with the crystal structure of the human ß2 adrenergic G-protein-coupled receptor (PDB entry 2RH1) [5, 6] introducing secondary structure information derived from the crystal structure to avoid gaps within the seven helical segments. The alignment was then manually refined to ensure a perfect alignment of the highly conserved residues of GPCR superfamily, according to Baldwin et al..[7]

Extension of each helix was contemplated by taking into account the experimental length of the 2RH1 helices and the sequence conservation. The conserved disulfide bond between residues C3.25 at the beginning of TM3 and the cysteine in the middle of the extracellular loop 2 (a feature highly common among GPCR receptors) was also created and kept as a constraint in the geometric optimization.

3D models were then built using the MODELLER suite of programs[8], which yielded 15 candidate models for each receptor final structure (Table 1). From these candidates, the best structures according to the MODELLER objective function and to visual inspection were selected. Models with interruptions or gaps in the transmembrane regions, as identified by visual inspection, were discarded. The resultant structures of the receptors were optimized using the Amber99 force field[9] using the molecular modeling program MOE (Molecular Operating Environment; Chemical Computing Group). PROCHECK software[10] was used to assess the stereochemical quality of the minimized structures resulting in good quality parameters with an excellent distribution of Psi and Phi angles in the Ramachandran plot (over 90% of the residues are in the most favored regions). Also, the resulting models must reproduce the correct orientation of the side chains for the set of highly conserved amino acids in the GPCR superfamily[11-14], taking special care to the side chains of the high conserved residues F6.51, F6.52 and W6.48 which according to some authors[15] are involved in the activation process. In the recent data published for 2RH1[5, 6], the co-crystallized partial inverse agonist carazolol

interacts with F6.51 and F6.52, which form an extended aromatic network surrounding the W6.48. As a result, W6.48 side chain adopts the rotamer associated with the inactive state. Consequently, the conformation of these residues was set to the "inactive state", which probably is more appropriate for modeling the docking of antagonists and more consistent with the inactive state of the main template structure (2RH1).

3. Docking simulation.

Based on the 14 structural GPCR models obtained in the present study, the binding mode of clozapine and olanzapine with these receptors was explored using docking simulations with the GOLD3.1.1 program.[16] The ligands were docked into the active site of 5-HT_{2A}/D_2 by defining a 15 Å region centered on the CG of D3.32, a residue conserved in all aminergic receptors and known to be important for ligand interaction.[17, 18] The best docking solution, according to the scoring function of GOLD and mutagenesis data, was subjected to energy minimization using MOE. The complex was further refined in a 200 ps molecular dynamics simulations (force field MMF94x, 300 ºK, time step 2 fs) and subsequently energy minimized by applying gradient minimization until the RMS gradient was lower than 0.001 kcal/molÅ.

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PUBLICATION 4

Synthesis, Binding Affinity, and Molecular Docking Analysis of New Benzofuranone Derivatives as Potential Antipsychotics

Reyes Aranda, Karen Villalba, Enrique Raviña, Christian F. Masaguer, José Brea, Filipe Areias, Eduardo Domínguez, Jana Selent, Laura López, Ferran Sanz, Manuel Pastor and María I. Loza

J. Med. Chem. **2008**; 51: 6085-94

Aranda R, Villalba K, Raviña E, Masaguer CF, Brea J, Areias F, et al. Synthesis, binding affinity and molecular docking analysis of new benzofuranone derivates as potential antipsychotics. J Med Chem. 2008; 51(19): 6085-94.

PUBLICATION 5

Synthesis, Binding Affinity and SAR of New Benzolactam Derivatives as Dopamine D_3 Receptor Ligands.

Raquel Ortega, Enrique Raviña, Christian F. Masaguer, Filipe Areias, José Brea, María I. Loza, Laura López, Jana Selent, Manuel Pastor and Ferran Sanz

Biorg. Med. Chem. Lett. **2009**; 19: 1773-8

Ortega R, Raviña E, Masaguer CF, Areias F, Brea J, Loza MI, et al. Synthesis, binding affinity and SAR of new benzolactam derivates as dopamine D(3) receptor ligands. Bioorg Med Chem Lett. 2009; 19(6): 1773-8.

PUBLICATION 6

Synthesis, 3D-QSAR and Structural Modeling of Benzolactam Derivatives with Binding Affinity for the D_2 and D_3 Receptors

Laura López, Jana Selent, Raquel Ortega, Christian F. Masaguer, Eduardo Domínguez, Filipe Areias

ChemMedChem. **2010**; accepted

López L, Selent J, Ortega R, Masaguer CF, Domínguez E, Areias F. Synthesis, 3D-QSAR and structural modeling of benzolactam derivatives with binding affinity for the D2 and D3 receptors. ChemMedChem. 2010; 5(8): 1300-17.

5. FUTURE WORK

In this work we built 3D homology models for a set of receptors putatively implicated in the pharmacological profile of the APDs. It would be important for accurately predicting the key ligand interactions in GPCRs to refine the modeling of their structurally divergent regions, such as the extracellular loops. In addition, the static structural models obtained cannot help in understanding the growing body of experimental evidence on dynamic ligand-specific structural rearrangements at the receptor level. Therefore, a further step would be the use of molecular simulation methods including explicit representations of membrane and water, in order to gain information about the different receptor conformations stabilized by ligands. Furthermore, increasing evidence indicates that functional selectivity and conformational selection by small molecules at GPCRs is often associated with dimeric and oligomeric forms of the receptors. Thus, it would be interesting to extend the modeling process to receptor dimmers and oligomers.

Since the multireceptor profile of clozapine and other atypical antipsychotic drugs were described, new putative receptor targets have been elucidated, such as the metabotropic glutamate and neurikinine receptors, as stated in section 1.3. Thus, it will be interesting to expand the set of receptors putatively involved in the APDs receptorome and to apply to the new targets the homology modeling protocol and the comparative analysis approach.

Related to the multilevel statistical method developed, it could be improved including information from other approaches such as molecular interaction fields and mutagenesis data. Its applicability can be further extended for studying other *in vivo* effects than metabolic side effects, such as the cardiovascular effects or the therapeutic effects, in order to have a more complete view of the multireceptorial binding affinity profile of the APDs.

The rationalization of the observed experimental data for relevant APDs and compounds synthesized *de novo* have suggested some features in their chemical structure as possible modulators of their binding affinity to the receptors, as described in section 3. Further work is needed for checking if the proposals are correct. In the case of current APDs, scaffold-hopping tools can be applied to replace the significant parts of the structure with other fragments or scaffolds; regarding the benzolactam and butyrophenones derivatives, the compounds could be optimized with the suggested structural modifications and then evaluated experimentally their affinity. In addition, we plan to do, in collaboration with the Pharmacology and Chemistry group, mutagenesis experiments for validating the residues suggested to be involved in the differences of binding affinity in 5-HT_{2A}, D_2 and D_3 receptors.

6. CONCLUSIONS

1. Homology models of the receptors putatively implicated in psychosis treatment (dopamine D_1, D_2, D_3 and D_4, histamine H_1, cholinergic muscarinic M_1, M_2, M_3 and M_4, adrenergic ADA_1 and ADA_2 and serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_6 and 5-HT_7 receptors) have been built and validated. The consistency of the modeling protocol makes the models suitable for comparative studies.

2. Complexes of the modeled receptors with diverse antipsychotic drugs (clozapine, olanzapine, risperidone) and with series of newly synthesized compounds with antipsychotic drug-like binding properties (benzofuranone and benzolactam derivatives) were built and validated.

3. A multilevel statistical method for identifying the multireceptorial profile of antipsychotic drugs associated to *in vivo* effects has been developed. At the structural level, the practical application of the method allowed the identification of regions and ligand-receptor interactions implicated in the discrimination between antipsychotic drugs that produce certain *in vivo* effects and those that do not.

4. The comparative structural analysis of 5-HT_{2A} and D_2 receptor models in complex with clozapine and olanzapine allowed proposing hypothesis about the binding site regions and interactions more likely to be responsible of the observed differences in the binding profile of the compounds.

5. The comparative structural analysis of 5-HT_{2A} and D_2 receptor models in complex with bensofuranone derivatives allowed proposing hypothesis about the residues which are likely to act as key modulators of their binding activity.

6. The 3D-QSAR models and the comparative structural analysis of D_2 and D_3 receptor models in complex with benzolactam derivatives allowed proposing hypothesis about the more important structural features modulating the D_2/D_3 binding selectivity.

7. The results obtained in this thesis emphasize the importance of a multilevel approach for defining the optimum binding profile of antipsychotic drugs and provide useful hints for the design of improved drugs.

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