MODELLING THE FOLDING OF TRANSMEMBRANE PROTEINS USING A NOVEL FRAGMENTAL APPROACH: THE HUMAN GHRELIN RECEPTOR AND THE GLUTAMATE TRANSPORTER EAAT1

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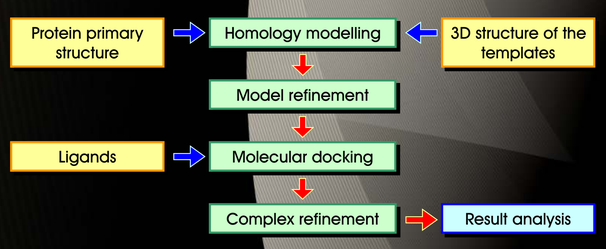
INTRODUCTION

The experimental structure of bovine rhodopsin has supported the building of GPCR models that were successfully exploited for virtual screening and ligand optimization [1], but the systematic use of this template can lead to repetitive GPCR "clones". In order to resolve this drawback, we here propose a fragmental approach and its soundness was verified by generating the models of both human ghrelin receptor (hGHS-R1a) in its open state [2] and the human glutamate transporter EAAT1.

The Human Ghrelin Receptor (hGHS-R1a)

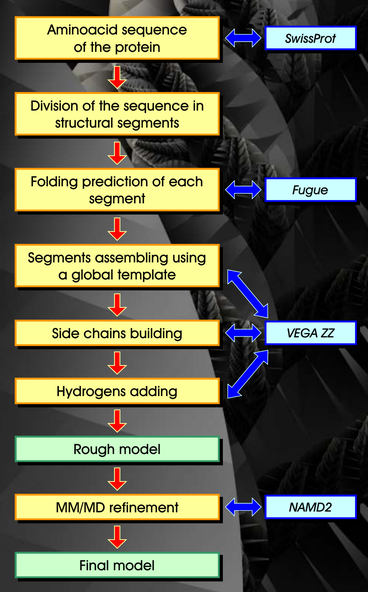
Ghrelin has been recognized as an important regulator of growth hormone (GH) secretion and energy homeostasis due to its orexigenic and adipogenic effects [3]. It's biosynthesized in several tissues having both endocrine and paracrine effects [4]. It has been shown to affect a number of different systems mainly including GH, ACTH, and prolactin release, feeding gastric secretion and mobility, metabolism, cardiac performances, and cell proliferation [5]. Therefore, peptidomimetic GH secretagogues find therapeutic applications in several pathological conditions [6]. A possible pharmacological target is the human ghrelin receptor (hGHS-R1a), a member of the GPCR family.



The Human Glutamate Transporter EAAT1

The L-glutamate amino acid represents the main excitatory neurotransmitter in the mammalian CNS [7]. Unfortunately, the physiological roles of L-glutamate are counteracted by its ability to over-activate the ionotropic receptors inducing the neuronal death [8]. Such a glutamate-mediated neuronal damage is involved in some neurological diseases, including amyotrophic lateral sclerosis, Alzheimer disease, epilepsy, and CNS ischemia [9]. In this context, the excitatory amino acid transporters (EAATs) have attracted remarkable attention also because the glutamate system is lacking in an extracellular enzyme which rapidly degrades the neurotransmitter, avoiding excitotoxic phenomena. On these grounds, several EAAT ligands (substrates and blockers) have been proposed as novel therapeutics against neurodegenerative diseases.The EAATs have a homotrimeric architecture in which each monomer appears functionally independent [10]. The EAAT monomer is characterized by a single amino acidic chain (500-600 residues) involving eight transmembrane helices (TM1-8) and two transmembrane helical hairpins (HP1-2). The transmembrane segments are connected by five extracellular loops (EL1-5) and four cytoplasmic loops (CL1-4) [11].

RECEPTOR MODELLING

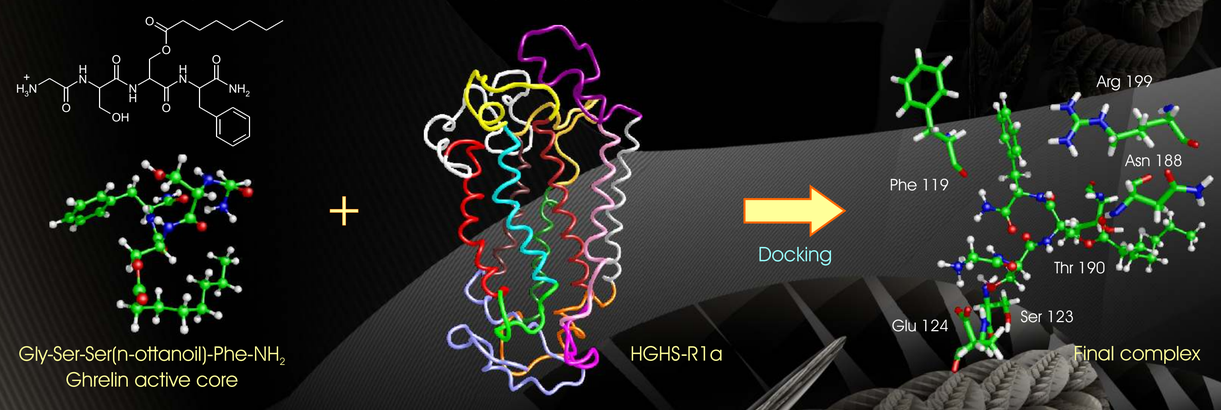


The amino acidic sequences of two receptors were retrieved from *Swiss-Prot* database and they were fragmented in structural domains. The folding of each segment was predicted using the *Fugue* approach [12]. It was able to generate several realistic models for each segment and the best structure has been chosen considering what result better fulfilled the following major conditions: a) the predicted secondary structure; b) the lack of not predicted gaps; c) the *Fugue* prediction score; d) the overall conformation of helix transmembrane segments; e) the global "U" shape for the loops; f) the possibility to make disulfide bridges where they are present. Finally, the assembly of predicted fragments was performed superimposing the backbone of a fragment with that of the correspondent segment in the bovin rhodopsin structure (PDB ID 1f88) [13] for hGHS-R1a and in the transporter homologue structure from *Pyrococcus horikoshii* for EAAT1 (PDB ID 1xfh) [14]. The assembly procedure and the model completion (side chains and hydrogens adding) were performed by *VEGA ZZ* [15].The obtained rough models were optimized in order to gain a better relaxation and a more correct arrangement, performing molecular dynamics equilibrations in vacuo. The simulations were carried out in 3 phases by *NAMD2* software [16]: 1) heating from 0 to 300 K (3.000 steps); 2) starting equilibration (2.500 ps, transmembrane backbone kept fixed); 3) equilibration (7.500 ps, transmembrane backbone was harmonically restrained with decreasing harmonic force constants). The last MD frames were used for the docking calculations after a final minimization with harmonic constraints.

hGHS-R1a sequence 1 - 366														
Domain	N-Ter	TM-1	CL-1	TM-2	EL-1	TM-3	CL-2	TM-4	EL-2	TM-5	CL-3	TM-6	EL-3	TM-7	C-Ter
Segment	1-40	41-66	67-72	73-96	97-117	118-139	140-162	163-183	184-211	212-235	236-263	264-285	286-302	303-326	327-366
PDB ID	1cee	1i7d	1f88	1uv7	1gzt	1f60	1fgg	1f0	1i15	1orv	1kcz	1f88	1ej6	1orv	1i26

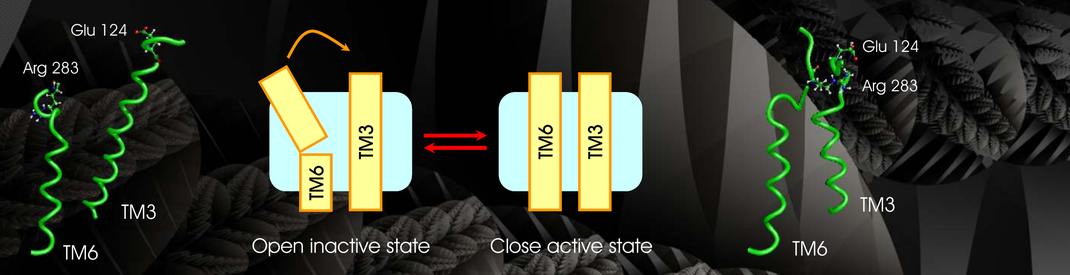
LIGANDS DOCKING

The three dimensional structures of the considered compounds were built by *VEGA ZZ* software and the resulting geometries were optimized applying the semi-empirical Hemiltonian Austin Model 1 (AM1) implemented in *Mopac 7.01* [16]. The conformational behaviour of the compounds was investigated by Monte Carlo procedure using the *Quanta/CHARMm* package [17]. It generated 1.000 conformers randomly rotating the rotors and the obtained geometries were stored and optimized to avoid high-energy rotamers. The 1.000 conformers were clustered according to similarity to discard redundant ones and only the lowest energy geometry was retained in each cluster. The *FlexX* [18] program was used to dock the ligands to the binding: it's a fast-automated docking software that considers the ligand conformational flexibility by an incremental fragment placing technique. For each molecule, 30 docking solutions (poses) were computed and scored. The best complexes were optimized by *NAMD2* (10.000 steps, conjugate gradients).

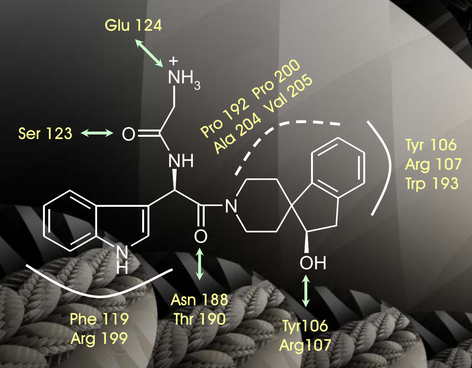


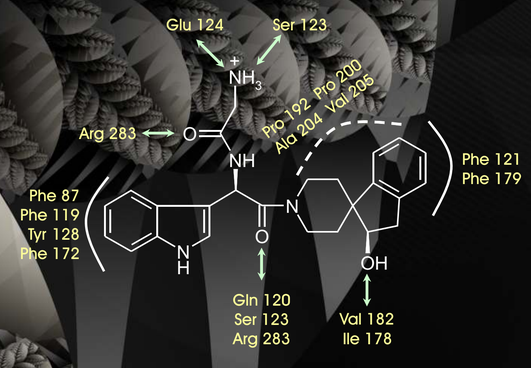
HGHS-R1a DOCKING RESULTS

Mutagenesis studies evidenced that the hGHS-R1a binding cavity can be considered as formed by two subpockets: a first polar cavity, lined by TM2, TM3 and a second aromatic cavity, lined by TM5 and TM6. Moreover, it is possible to consider two hGHS-R1a states: an open state, in which the two subpockets form two distinct binding sites, and a close state, in which the aromatic cluster approaches the polar subpocket, and Glu 124 (TM3) interacts with Arg 283 (TM6) [21].

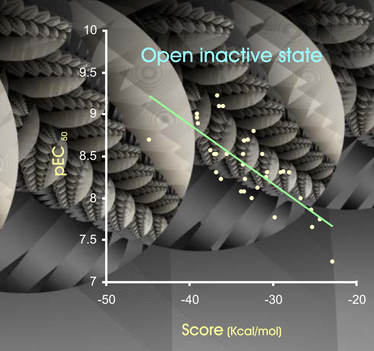


A dataset with 35 heterogeneous peptidomimetic GH secretagogues was compiled from literature, considering GHR-1a agonists for which the biological activity was evaluated through in vitro assay of GH release from rat pituitary cells. These compounds were docked in both open and close state structures. The following bi-dimensional schemes show the main iteractions of one considered compound in both open and close state hGHS-R1a models:

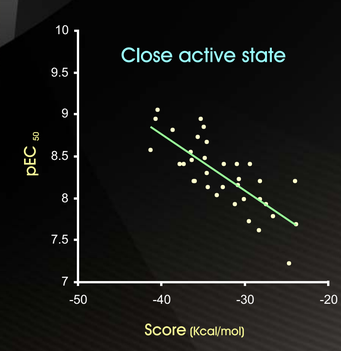




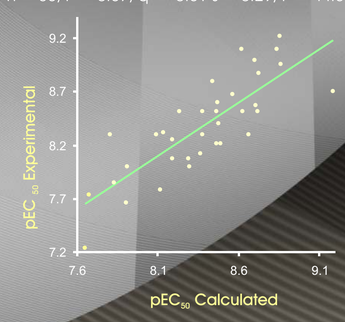
The experimental activity values of each considered compound (expressed in pEC_{50}) were related to the best docking scores (expressed in Kcal/mol), showing significant agreement between their trends.



pEC_{50} = 6.06 (±0.35) - 0.070 (±0.01) $Score_{Open}$
n = 35; r^2 = 0.57; q^2 = 0.51 s = 0.29; F = 44.30



pEC_{50} = 5.95 (± 0.37) - 0.074 (± 0.01) $Score_{Close}$
n = 35; r^2 = 0.57; q^2 = 0.55; s = 0.30; F = 44.42

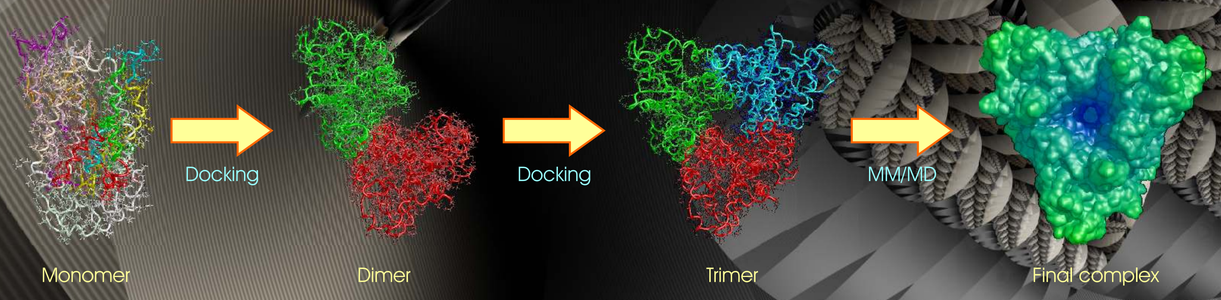


Since the cross-correlation between the scores obtained in close state and those computed in open state is modest (r^2 = 0.55), the correlative equation was recalculated inserting the docking scores obtained in both open and close state hGHS-R1a complexes. The resulting equation shows an interesting improvement of the statistical parameters (r^2 = 0.65 vs. 0.57) suggesting that a good ligand might successfully interact with both hGHS-R1a states.

pEC_{50} = 5.70 (± 0.33) -0.042 (± 0.01) $Score_{Close}$ - 0.042 (± 0.01) $Score_{Open}$
n = 35; r2 = 0.65; q2 = 0.62; s = 0.27; F = 29.63

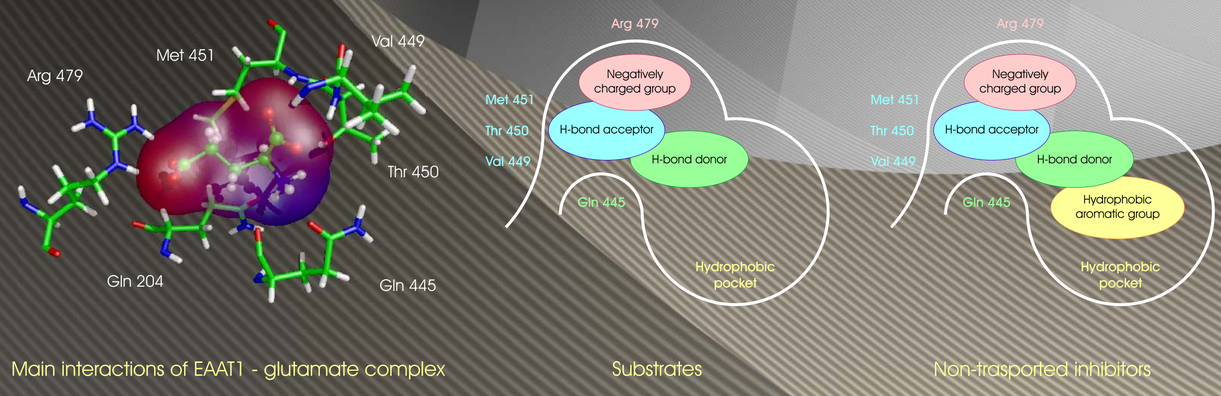
EAAT1 HOMOTRIMERIC COMPLEX

The obtained EAAT1 monomer was used to build the corresponding homotrimer through automatic docking using *Escher NG* program [19]. The trimer assembly is organized in two sequential steps: 1) two EAAT1 monomers are docked in order to obtain the corresponding homodimer; 2) the dimer was docked with a monomer to generate the final EAAT1 homotrimer. In both analyses, the best solution was selected considering: 1) the score of *Escher NG*, 2) the similarity with homotrimeric architecture of glutamate transporter homologue from *Pyrococcus horikoshii*, 3) the accessibility of regions, which constitute the solvent accessible extracellular basin extending halfway across the membrane segment. The obtained homotrimer underwent to a preliminary minimization followed by a 1 ns MD simulation with transmembrane segments harmonically restrained. The last frame was used for the docking calculations after a final minimization.



EAAT1 DOCKING RESULTS

Two sets of EAAT1 transported ligands were compiled from literature data: the first one comprises the substrates (7 compounds) and the second one includes the non-trasported inhibitors (28 compounds). For each ligand class, it was possible to build a pharmacophoric model in which the Val 449 and Arg 479 play a crucial role in the interaction. These residues were identified by mutagenesis studies as key in the substrate recognition [20] and the computational results confirm this data. Comparing the two models, it's possible to identify a hydrophobic pocket occupied by competitive substrate inhibitors only.



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