

**Mapping the binding pocket of dual antagonist almorexant to human
orexin 1 and orexin 2 receptors: Comparison with the selective
OX₁ (SB-674042) and OX₂ (EMPA) antagonists**

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Abbreviations: OX, orexin receptor; OX₁, orexin 1 receptor; OX₂, orexin 2 receptor; almorexant, (2*R*)-2-[(1*S*)-6,7-Dimethoxy-1-[2-(4-trifluoromethyl-phenyl)-ethyl]-3,4-dihydro-1*H*-isoquinolin-2-yl]-*N*-methyl-2-phenyl-acetamide; EMPA, *N*-Ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-*N*-pyridin-3-ylmethyl-acetamide; SB-674042, 1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((*S*)-2-(5-phenyl-(1,3,4)oxadiazol-2-ylmethyl)-pyrrolidin-1-yl)-methanone; GPCRs, G-protein coupled receptors; 3D, three-dimensional; 7TMD, seven-transmembrane domain; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; FLIPR, Fluorometric Imaging Plate Reader; OPSD, bovine rhodopsin; β₂AR, β₂-adrenergic receptor; A_{2A}, A_{2A} adenosine receptor; WT, wild type; NK, neurokinin; NK₃, neurokinin 3 receptor; V₁, vasopressin 1 receptor

Abstract

The orexins and their receptors are involved in the regulation of arousal and sleep–wake cycle. Clinical investigation with almorexant has indicated that this dual OX antagonist is efficacious in inducing and maintaining sleep. Using site-directed mutagenesis, β_2 -adrenergic-based OX₁ and OX₂ modeling, we have determined important molecular determinants of the ligand-binding pocket of OX₁ and OX₂. The conserved residues D^{45.51}, W^{45.54}, Y^{5.38}, F^{5.42}, Y^{5.47}, Y^{6.48} and H^{7.39} were found to be contributing to both orexin-A-binding sites at OX₁ and OX₂. Among these critical residues, five (positions 45.51, 45.54, 5.38, 5.42 and 7.39) were located on the ECL2b and in the top of TM domains at the interface to the main binding crevice, thereby suggesting superficial OX receptor interactions of orexin-A. We found that the mutations W214A^{45.54}, Y223A^{5.38}, F227A^{5.42}, Y317A^{6.48} and H350A^{7.39} resulted in the complete loss of both [³H]almorexant and [³H]EMPA binding affinities and also blocked their inhibition of orexin-A-evoked [Ca²⁺]_i response at OX₂. The crucial residues Q126^{3.32}, A127^{3.33}, W206^{45.54}, Y215^{5.38}, F219^{5.42} and H344^{7.39} are shared between almorexant and SB-674042 binding sites in OX₁. The non-conserved residue at position 3.33 of orexin receptors was identified as occupying a critical position that must be involved in subtype selectivity and also in differentiating two different antagonists for the same receptor. In summary, despite high similarities in the ligand-binding pockets of OX₁ and OX₂ and numerous aromatic/hydrophobic interactions, the local conformation of helix positions 3.32, 3.33 and 3.36 in TM3 and 45.51 in ECL2b provide the structural basis for pharmacologic selectivity between OX₁ and OX₂.

Introduction

Hypothalamic neuropeptides, orexin-A/hypocretin-1 (33 amino acids) and orexin-B/hypocretin-2 (28 amino acids), are derived from the proteolytic processing of 130 amino acids prepro-orexin (de Lecea et al., 1998; Sakurai et al., 1998). Orexin peptides elicit their effect through two G-protein-coupled receptors (GPCRs) called OX₁ and OX₂ (nomenclature follows Alexander et al., 2008) that couple to G_{q/11} and contribute to the activation of phospholipase C, leading to the elevation of intracellular Ca²⁺ concentrations (Sakurai et al., 1998). However, a detailed signaling profile of the hOX₂ has recently shown that OX₂ could couple to G_s as well as G_{q/11} and G_i pathways (Tang et al., 2008). The binding and functional characterization demonstrated that orexin-B has a 10-fold lower affinity for the OX₁ over the OX₂, while both orexin-A and orexin-B bind with similar affinities to OX₂ (Sakurai et al., 1998).

Distribution studies in rat brain using in situ hybridization and immunohistochemistry have shown that orexin neurons are found exclusively in the lateral hypothalamic area, having projections to the entire CNS (Nambu et al., 1999; Peyron et al., 1998). OX₁ and OX₂ receptors are differentially expressed in the CNS. Although both receptors are present in most brain regions such as neocortex L6, ventral tegmental area, preoptic area, dorsal and medial raphe nuclei, periaqueductal area and hypothalamus, OX₁ is most abundantly expressed in the locus coeruleus while OX₂ is expressed in regions controlling arousal, especially in the tuberomammillary nucleus, an important site for the regulation of sleep/wakefulness (Marcus et al., 2001; Trivedi et al., 1998).

Orexin system has been implicated in numerous physiological functions including energy homeostasis, feeding and reward, also regulation of arousal and the sleep–wake cycle (Kilduff and Peyron, 2000; Ohno and Sakurai, 2008). Preclinical (canine and rat) and Clinical (healthy male subjects; single dose) investigations have shown that almorexant, when

administered orally during the active period, promoted sleep in animals and humans without disrupting the sleep architecture or inducing cataplexy (Brisbare-Roch et al., 2007; Neubauer, 2010), thereby further validating the involvement of orexin system in the regulation of alertness and sleep. Thus, OX antagonists represent an alternative therapeutical approach for the treatment of insomnia (Boss et al., 2009; Nishino, 2007; Roecker and Coleman, 2008). Recently, the biochemical characterization of a high-affinity, reversible and *in vivo* active OX₂ antagonist, EMPA, with 900-fold selectivity in binding for OX₂ over OX₁ has been reported (Malherbe et al., 2009a). Furthermore, the biochemical characterization of almorexant (a high-affinity dual OX₁/OX₂) has demonstrated that it had an apparent non-competitive and long-lasting pseudo-irreversible mode of antagonism due to its very slow rate of dissociation from OX₂, whereas it displayed a competitive mode of antagonism at OX₁ (Malherbe et al., 2009b). SB-674042, which is a high-affinity and selective OX₁ antagonist (Langmead et al., 2004), has been also shown to behave in an apparent non-competitive manner at hOX₁, similarly to that of almorexant at hOX₂ (Malherbe et al., 2009b).

Several researchers, who have investigated the determinants of orexin-A required to activate OX₁ and OX₂ using truncated peptides and alanine-scanned peptides (systematic replacement of the natural amino acids with L-alanine) (Ammoun et al., 2003; Lang et al., 2006; Lang et al., 2004; Takai et al., 2006) have indicated that: (1) a minimal 19 amino acids of C-terminal segment of orexin-A (R15-L33) is required for OX activation, though functional activity of this peptide is reduced; (2) the replacement of orexin-A (R15-L33) truncated peptide residues, Leu16, Leu19, Leu20, His26, Gly29, Ile30, Leu31, Thr32, and Leu33 with alanine led to a significant reduction in the functional potency at the OX₁ (Darker et al., 2001); and (3) orexin-A distinctly recognized OX₁ from OX₂ and its binding to OX₁ required more molecular determinants than binding to OX₂. Thus far, little is known about OX ligand-binding pocket. The current research utilized a combination of β 2AR-based OX₁

and OX₂ modeling, site-directed mutagenesis, [³H]almorxant, [³H]EMPA and [³H]SB-674042 bindings and orexin-A evoked intracellular calcium ([Ca²⁺]_i) mobilization (FLIPR) assay to probe the antagonist-binding site of OX₁ and OX₂. Amino acid residues in the TM3, -5, -6 and -7 and ECL2b regions, initially identified from an alignment of the 7TMD of OX₁ and OX₂ with hβ₂AR were demonstrated by mutational analysis to be important determinants of the high-affinity antagonist-binding pocket of the OX₁ and OX₂. Furthermore, these experimental findings allow the construction of homology models of OX₁- and OX₂-7TMD based on the X-ray crystal of hβAR (2RH1) (Cherezov et al., 2007; Rosenbaum et al., 2007) and suggest possible binding modes for EMPA-OX₂, SB-674042-OX₁, and almorexant-OX₁ and -OX₂ complexes.

Materials and Methods

Materials. Almorexant (ACT-078573) (Brisbare-Roch et al., 2007), EMPA (Malherbe et al., 2009a), and SB-674042 (Langmead et al., 2004) were synthesized in the Chemistry Department of F. Hoffmann-La Roche. [³H]almorexant (specific activity: 42.7 Ci/mmol), [³H]EMPA (specific activity: 94.3 Ci/mmol) and [³H]SB-674042 (specific activity: 24.4 Ci/mmol) (**Fig. 1A**) were synthesized by Drs. Philipp Huguenin and Thomas Hartung at the Roche chemical and isotope laboratories, Basel, Switzerland. orexin-A (**Fig. 1B**) was purchased from Tocris Bioscience (Bristol, UK).

Construction of point-mutated hOX₁ and hOX₂ receptors. cDNA encoding human OX₁ (Accession No. O43613) and human OX₂ (Accession No. O43614) were subcloned into pCI-Neo expression vectors (Promega, Madison, WI). All point-mutants were constructed using the QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and using pCI-Neo-hOX₁ or pCI-Neo-hOX₂ as a DNA template. Complementary oligonucleotide primers (sense and antisense) containing the single site of mutation was synthesized by Microsynth AG (Balgach, Switzerland). The following PCR conditions were used for repeated extensions of the plasmid template: 95°C for 1 min and 20 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 8 min using 50 ng plasmid DNA, 100 ng each of primers and 2.5 units Pfu Turbo DNA polymerase (Stratagene). The entire coding regions of all positive point-mutants were sequenced from both strands using an automated cycle sequencer (Applied Biosystems).

Cell culture and membrane preparation. HEK293 cells were transfected as previously described (Malherbe et al., 2009a). Forty-eight hours after transfection, cells were harvested and washed three times with cold phosphate-buffered saline and frozen at -80°C. The pellet was suspended in ice-cold buffer containing 15 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.3 mM EDTA, 1 mM EGTA, protease inhibitor cocktail EDTA-free (Roche Applied

Science, RAS, Rotkreuz, Switzerland) and homogenized with a polytron (Kinematica AG, Basel, Switzerland) for 30 s at 16 000 rpm. After centrifugation at 48 000 X g for 30 min at 4°C, the pellet was suspended in ice-cold buffer containing 75 mM Tris-HCl, pH 7.5, 12.5 mM MgCl₂, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose, protease inhibitor cocktail EDTA-free. The membrane homogenate was frozen at -80°C before use.

[³H]almorexant, [³H]EMPA and [³H]SB-674042 bindings. After thawing, membrane homogenates were centrifuged at 48 000 X g for 10 min at 4°C, the pellets were resuspended in the binding buffer (1 X HBSS, 20 mM HEPES, pH 7.4, 0.1% BSA) to a final assay concentration of 5 µg protein/well. Saturation isotherms were determined by addition of various concentrations of [³H]almorexant (0.02-20 nM at OX₁, 0.03-15 nM at OX₂R), [³H]EMPA (0.01 to 12 nM at OX₂) or [³H]SB-674042 (0.03-15 nM at OX₁) to these membranes (in a total reaction volume of 500 µl). The incubation time for [³H]almorexant and [³H]SB-674042 on OX₁ membranes was 90 min at 23°C. The incubation for [³H]almorexant and [³H]EMPA on OX₂ expressing membrane was 120 min and 60 min at 23°C, respectively. At the end of incubation, membranes were filtered onto unitfilter (96-well white microplate with bonded GF/C filter pre-incubated for 1 h in wash buffer plus 0.5% polyethylenimine and 0.1% BSA) with a Filtermate 196 harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA) and washed four times with ice-cold wash buffer (1 X HBSS, 20 mM HEPES, pH 7.4). Nonspecific bindings for [³H]almorexant, [³H]EMPA and [³H]SB-674042 were measured in the presence of 10 µM almorexant, EMPA and SB-674042, respectively. Radioactivity on the filter was counted (5 min) on a Top-Count microplate scintillation counter (PerkinElmer Life and Analytical Sciences) with quenching correction after addition of 45 µl of microscint 40 (PerkinElmer Life and Analytical Sciences) and shaking for 1 h. Saturation experiments were analyzed by Prism 5.0 (GraphPad software, San Diego, CA) using the rectangular hyperbolic equation derived from the equation of a

bimolecular reaction and the law of mass action, $B = (B_{\max} * [F]) / (K_d + [F])$, where B is the amount of ligand bound at equilibrium, B_{\max} is the maximum number of binding sites, [F] is the concentration of free ligand and K_d is the ligand dissociation constant. For all mutants, the experiments were performed three to five times in triplicate and the mean \pm SE of the individual K_d and B_{\max} values were calculated and are reported. Statistical significance was determined using the Two-tailed t-test (Prism 5.0, GraphPad software).

Intracellular Ca^{2+} mobilization, $[Ca^{2+}]_i$ assay. HEK293 cells, which were grown to 80% confluency in growth medium (DMEM high glucose supplemented with 10% fetal calf serum and 100 μ g/ μ l penicillin/streptomycin), were transfected with the wild type or mutant orexin receptor cDNAs in pCI-Neo using Lipofectamine PlusTM reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Six hours after transfection, the DNA-transfection mixture was removed and the cells were maintained in growth medium. Twenty four hours after transfection,, the cells were harvested and seeded at 6×10^4 cells/well in the poly-D-lysine treated, 96-well, black/clear-bottomed plates (BD Biosciences, Palo Alto, CA). Forty-eight hours after transfection, the cells were loaded for 1h at 37°C with 4 μ M Flou-4AM (Molecular Probes, Eugene, OR) in loading buffer (1xHBSS, 20 mM HEPES). The cells were washed five times with loading buffer to remove excess dye and intracellular calcium mobilization, $[Ca^{2+}]_i$ were measured using a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Menlo Park, CA) as described previously (Malherbe et al., 2009b). Orexin-A (50 mM stock solution in DMSO) was diluted in FLIPR buffer plus 0.1% BSA. The EC_{50} and EC_{80} values of orexin-A were measured daily from standard agonist concentration-response curves in HEK293 cells transiently transfected with the WT or mutant orexin receptors. Inhibition curves were determined by addition of 11 concentrations (0.0001-10 μ M in FILPR buffer) of inhibitory compounds and using EC_{80} value of orexin-A as agonist (a concentration which gave 80% of maximum agonist response, determined daily). The

antagonists were applied 25 min (incubation at 37°C) before the application of the agonist. Responses were measured as peak increase in fluorescence minus basal, normalized to the maximal stimulatory effect induced by EC₈₀ value of orexin-A. Inhibition curves were fitted according to the Hill equation: $y = 100/(1+(x/IC_{50})^{n_H})$, where n_H = slope factor using Prism 5.0 (GraphPad software). K_b values were calculated according to the following equation $K_b = IC_{50}/(1+[A]/EC_{50})$, where A is the concentration of agonist added that is very close to agonist EC₈₀ value, and IC₅₀ and EC₅₀ values were derived from the antagonist inhibition and orexin agonist curves, respectively. The relative efficacy (E_{max}) values of orexin-A was calculated as fitted maximum of the dose-response curve of each mutated receptors expressed as a percentage of fitted maximum of the wild type dose-response curve from cells transfected and assayed on the same day.

Residue Numbering Scheme

The position of each amino acid residue in the seven membrane domain (7TMD) was identified both by its sequence number and by its generic number proposed by Ballesteros and Weinstein (Ballesteros and Weinstein, 1995). In this numbering system, amino acid residues in the 7TMD are given two numbers; the first one refers to the transmembrane (TM) number (1-7), the second one indicates the relative position relative to a highly conserved residue in class A GPCRs in that TM, which is arbitrarily assigned 50. The second extracellular loop (ECL2) is labeled 45 to indicate its location between helices 4 and 5 and the conserved cysteine thought to be disulfide bonded, is given index number 45.50. The residues within ECL2 loop are then indexed relative to this position. ECL2b is the C-terminal strand of ECL2, which connects C^{3.25} with C^{45.50}.

Alignment and model building. The amino acid sequences of the human OX₁ (O43613), human OX₂ (O43614) were retrieved from the Swiss-Prot database. A model was built by aligning the 7 TMs and ECL2 of orexin receptor sequences on the β 2-adrenergic

receptor (P07550) sequence to use the 2.4 Å high resolution crystal structure determined by Cherezov *et al* as a template (the PDB structure 2RH1, Cherezov et al., 2007; Rosenbaum et al., 2007). The initial alignment was generated with the clustalW multiple alignment program using the BLOSUM matrix and then manual inspection was performed to ensure that conserved residues were aligned. Then, the software package MOE (MOE version 2005.05; Chemical Computing Group, Montreal, Quebec, QC, Canada) was used to create 3D models of human OX₁ and OX₂ either based on β₂-adrenergic (2RH1). Ten intermediates were generated and the best model was selected. No minimization was performed to keep the backbone coordinates of the crystallographic structure. The three molecules (SB-674042, EMPA and almorexant) were then manually docked into the membrane cavity. The binding site was defined as the set of amino acids found at 6.0Å away from the carazolol in the X-ray structure of β₂-adrenergic receptor.

Results

Binding characteristics of [³H]SB-674042, [³H]EMPA and [³H]almorexant. To investigate 7TMD pocket of OX₁ and OX₂ receptors, three radioligand antagonists; [³H]SB-674042, [³H]EMPA and [³H]almorexant were selected for current study (**Fig. 1A**). [³H]SB-674042 is the first radioligand antagonist selective for hOX₁ to be described (Langmead et al., 2004). In the filtration binding assay, [³H]SB-674042 has displayed a high affinity binding to hOX₁ with a K_d value of 0.74 nM. SB-674042 has also showed a 275-fold selectivity in functional assay (FLIPR) for hOX₁ over hOX₂ (Malherbe et al., 2009b). Recently, the biochemical characterization of an OX₂ antagonist, EMPA, with 900-fold selectivity in binding for OX₂ over OX₁ has been reported (Malherbe et al., 2009a). [³H]EMPA is a high affinity radioligand that binds to HEK293-hOX₂ membrane with K_d value of 1.1 nM. [³H]almorexant bound with high affinity to a single saturable site on recombinant hOX₁ and hOX₂ with K_d values of 1.3 nM and 0.17 nM, at 37°C, respectively (Malherbe et al., 2009b). Furthermore, SB-674042 and EMPA were able to displace the [³H]almorexant binding from hOX₁ and hOX₂ membranes with K_i values of 1.9 nM and 1.2 nM, respectively. Therefore, it is concluded that almorexant should share a common binding pocket in the transmembrane region of the orexin receptors or at least overlapping with those of SB-674042 on hOX₁ and EMPA on hOX₂.

Alignment of 7TM domains of the OX receptors towards hβ2AR and selection of the orexin receptor mutations. The orexin receptors are highly conserved across mammalian species. Human OX₁ and OX₂ display 64% overall sequence identity (Sakurai et al., 1998), an even higher degree of identity of 84% is found when comparing 7TMD regions (**Fig. 2**). To elucidate the binding modes of almorexant, EMPA and SB-674042, an alignment of the seven transmembrane helices of the hOX₁ and hOX₂ towards the transmembrane helices of human β₂-adrenergic receptor (PDB ref code 2RH1) (Cherezov et al., 2007; Rosenbaum et al., 2007)

was made (**Fig. 3**). In **Fig. 3**, an alignment of 7TM of the hOX₁ and hOX₂ towards the 7TM of bovine rhodopsin (PDB ref code 1F88) (Palczewski et al., 2000) and human A_{2A} adenosine receptor (PDB ref code 3EML) (Jaakola et al., 2008) were also shown for comparison. Since at early stage of current study, OX₂ was our target, the first site directed mutagenesis campaign has focused on the elucidation of the binding mode of the selective hOX₂ EMPA (Malherbe et al., 2009a) and the dual acting molecule almorexant (Malherbe et al., 2009b) against the human orexin 2 receptor. The partial inverse agonist of human β₂-adrenergic receptor, carazolol, was employed as a template for the locations of EMPA and almorexant. Amino acids, which were found 6.0 Å away from carazolol in the X-ray crystal structure of β₂-adrenergic receptor (Cherezov et al., 2007; Rosenbaum et al., 2007), were generally considered as possible candidates to affect bindings of EMPA and almorexant. **Fig. 3** shows a set of 17 mutations covering the whole binding site (TM2, -3, -5, -6, -7 and ECL2b) that was suggested from the manual docking of almorexant in the hOX₂ 3D homology model. Seventeen residues of OX₂ were mutated to alanine and OX₂ Y317^{6,48} was additionally mutated to Phenylalanine to discriminate between its aromatic and hydrogen bond donor capabilities. For OX₁, we selected a smaller list of residues that had been proven to be important for OX₂. Ten residues of OX₁ were mutated to alanine and OX₁ A127^{3,33} was changed to threonine to mimic the OX₂ residue (**Fig. 3**). Overall, twenty-nine point-mutations, eighteen in hOX₂ and eleven in hOX₁ were accordingly introduced in the 7TMD region by site-directed mutagenesis.

Effect of mutations on hOX₁- or hOX₂-mediated of orexin-A-evoked [Ca²⁺]_i response. In HEK293 cells transiently expressing hOX₁ or hOX₂, orexin-A (0.0001-10 μM) elicited a concentration-dependent increase in intracellular free calcium [Ca²⁺]_i as monitored using the Ca²⁺-sensitive dye Flou-4 and a Fluorometric Imaging Plate Reader (FLIPR-96). As seen in **Table 1** and **Table 2**, orexin-A activates hOX₂ and hOX₁ with similar potency.

The EC_{50} , n_H and relative E_{max} values, calculated from concentration-response curves of orexin-A in the cells expressing WT and mutated hOX₂ receptors, are given in **Table 1**. The mutations T111A^{2.61}, D211A^{45.51}, W214A^{5.54}, Y223A^{5.38}, F227A^{5.42}, F346A^{7.35} and H350A^{7.39} caused a large decrease in the potency of orexin-A (by 243.5-, 416.1-, 62.4-, 183.9-, 240.3-, 54.5- and 49.5-fold) respectively, without affecting their efficacy, in comparison to the WT. The mutations Y232A^{5.47} and Y317A^{6.48} resulted in a moderate reduction of both potency (by 28.4- and 17.7-fold) and efficacy (relative E_{max} of 44.9% and 49.6%) of orexin-A, respectively. The mutation Q134A^{3.32} caused a moderate decrease in potency of orexin-A (by 22.3-fold) without having effect on its efficacy.

The effect of mutations on potency (EC_{50}) and efficacy (relative E_{max}) of orexin-A in the HEK293 cells transiently expressing WT and mutated hOX₁ receptors, are given in **Table 2**. The mutations D203A^{45.51}, W206A^{45.54}, Y215A^{5.38}, F219A^{5.42}, Y224A^{5.47}, Y311A^{6.48} and H344A^{7.39} caused large decreases in the potency of orexin-A (by 408.2-, 417.8-, 407.8-, 139.6-, 84.4-, 163.9- and 241.1-fold), in comparison to the WT. Except for the mutation of W206A^{45.54}, which caused a moderate decrease of orexin-A's efficacy (relative E_{max} of 45.0%), other mutations had no major effect on efficacy of orexin-A. The mutation V130A^{3.36} affected moderately the potency of orexin-A (30.6-fold), without having any effect on its efficacy.

Effect of mutations on binding affinity and function potency of [³H]EMPA and [³H]almorexant at OX₂ receptor. To characterize the binding pockets of EMPA and almorexant, 18 point-mutations located in the TM2, -3, -5, -6, and -7 and ECL2b regions of hOX₂, were selected based on proposed docking mode of almorexant (**Fig. 3**). With the exception of the Y232A^{5.47} and Y317A^{6.48}, which produced a reduction in orexin-A-stimulated fluorescence responses in the FLIPR experiment, the 16 mutations had no effect on or partially affected the orexin-A-induced FLIPR signal. Saturation binding analyses were

performed on membranes isolated from the HEK293 transiently transfected with the WT and mutated hOX₂ receptors using 0.01 to 12 nM concentrations of [³H]EMPA or 0.03 to 15 nM concentrations of [³H]almorexant. The dissociation constants (K_d) and the maximum binding sites (B_{max}) derived from the saturation isotherms are given in **Table 3**.

The mutations T135A^{3.33}, W214A^{45.54}, Y223A^{5.38}, F227A^{5.42}, Y232A^{5.47}, Y317A^{6.48}, I320A^{6.51}, H350A^{7.39} and Y354A^{7.43}, abolished [³H]EMPA binding to undetectable levels (K_d>30 nM can not be detected because of high non-specific binding, NSB>SB). The binding affinity of [³H]EMPA was decreased by 7.4-, 14.9-, 8.7- and 3.9-fold by mutations T111A^{2.61}, V138A^{3.36}, D211A^{45.51} and Y317F^{6.48}, and were statistically significant (P = 0.03, 0.0001, 0.003, 0.002), respectively (**Table 3**). In functional FLIPR assay (**Fig. 4, A, C and E, and Table 3**), in cells expressing the mutants T135A^{3.33}, W214A^{45.54}, Y223A^{5.38}, F227A^{5.42}, Y232A^{5.47}, Y317A^{6.48}, I320A^{6.51}, H350A^{7.39} and Y354A^{7.43}, that did not bind [³H]EMPA, EMPA was not able to efficiently inhibit orexin-A-evoked [Ca²⁺]_i response and thus resulted in the large increases in K_b values (2972.7-, >10,000-, 339.0-, 420.0-, 37.1-, 54.5-, 415.5-, 279.1- and 366.4-fold, respectively). The mutations D211A^{45.51} and Y317F^{6.48}, which caused the decreases in the EMPA's binding affinity, resulted similarly in decreases of functional potency by 16.8 and 9.5-fold. In general, a good agreement was observed between the effect of mutations on binding affinity and functional potency of EMPA, except for the mutants T111A^{2.61} and V138A^{3.36} in which the effect on functional potency of EMPA was greater than that on binding affinity (51.1- and 90.9-fold increases in K_b values versus 7.4- and 14.9-fold increases in K_d values, respectively).

As seen in **Table 3**, the mutations W214A^{45.54}, Y223A^{5.38}, F227A^{5.42}, Y317A^{6.48} and H350A^{7.39} abolished [³H]almorexant binding to undetectable levels (K_d>20 nM can not be detected because of high non-specific binding, NSB>SB). The binding affinity of [³H]almorexant was decreased by 10.7- and 10.0-fold by mutations Q134A^{3.32} and Y232A^{5.47},

which were statistically significant ($P = 0.04$ and 0.006), respectively. Interestingly, the mutation F346A^{7.35} caused 3.25-fold increase in affinity of [³H]almorexant with high statistical significant ($P = 0.0002$). In functional FLIPR assay (**Fig. 4, B, D and F, and Table 3**), in cells expressing the mutants W214A^{45.54}, Y223A^{5.38}, F227A^{5.42}, Y317A^{6.48} and H350A^{7.39} that did not bind [³H]almorexant, almorexant was not able to efficiently inhibit orexin-A-evoked [Ca^{2+}]_i response and thus resulted in the large increases in K_b values (>10,000-, 10,000-, 200.4-, 25.0- and 94.2-fold, respectively). The mutation Y232A^{5.47}, which caused a decrease in the almorexant's binding affinity by 10.0-fold, resulted similarly in a decrease of functional potency by 7.5-fold. Therefore, with the exception of mutation Q134A^{3.32}, which led to a decrease in binding affinity (10.7-fold) and had no effect on functional potency, a good correlation between binding and functional potency of almorexant was observed at OX₂.

Comparison of mutation effects on binding and functional potencies of SB-674042 and almorexant at OX₁. To determine the residues forming the binding pockets of SB-674042 and almorexant at hOX₁ receptor, 11 point-mutations proposed from docking of almorexant onto OX₁ TM cavity (**Fig. 3**). Saturation binding analyses were performed on membranes isolated from the HEK293 cells transfected with the WT and mutated OX₁ receptors using 0.03 to 15 nM concentrations of [³H]SB-674042 and 0.02-20 nM of [³H]almorexant. The dissociation constants (K_d) and the maximum binding sites (B_{max}) derived from the saturation isotherms are given in **Table 4**. The functional potencies of SB-674042 and almorexant were not measured at D203^{45.51}, W206A^{45.54} and Y215A^{5.38} due to high EC₈₀ values (>600 nM) of orexin-A.

As seen in the **Table 4**, the mutations W206A^{45.54}, Y215A^{5.38} and F219A^{5.42} abolished [³H]SB-674042 binding to undetectable levels ($K_d > 100$ nM can not be detected because of high non-specific binding, NSB>SB). The mutants Q126A^{3.32}, A127T^{3.33} and H344A^{7.39}

caused dramatic decreases in the binding affinity of [³H]SB-674042 by 50.9-, 20.2- and 22.7-fold, with high statistical significance (P = 0.0005, 0.003 and 0.0005), respectively. The mutations Y311A^{6.48} and Y348A^{7.43} led to decreases in the binding by 10.8- and 9.3-fold, and were statistically significance (P = 0.01 and 0.0003), respectively. In the functional FLIPR assay (**Fig. 5, A and C, and Table 4**), in cells expressing the mutant F219A^{5.42}, that did not bind [³H]SB-674042, SB-674042 was not able to efficiently inhibit orexin-A-evoked [Ca²⁺]_i response and thus resulted in the large increase in K_b value by 311.7-fold. The mutants Q126A^{3.32}, A127T^{3.33} and H344A^{7.39} that caused dramatic decreases in the binding affinity of [³H]SB-674042, similarly led to large decreases in functional potency by 40.3-, 22.8- and 20.8-fold, respectively. However, the mutations Y311A^{6.48} and Y348A^{7.43}, which led to decreases in the binding affinity of SB-674042, had no effect on its functional potency.

As seen in **Table 4**, the mutations Q126A^{3.32}, A127T^{3.33}, D203A^{45.51}, W206A^{45.54}, Y215A^{5.38}, F219A^{5.42}, Y224A^{5.47}, Y311A^{6.48}, H344A^{7.39} and Y348A^{7.43} abolished [³H]almorexant binding to undetectable levels (K_d>20 nM can not be detected because of high non-specific binding, NSB>SB). In the functional FLIPR studies (**Fig. 5, B and D, and Table 4**), in cells expressing the mutants Q126A^{3.32}, A127T^{3.33}, F219A^{5.42}, Y311A^{6.48}, H344A^{7.39} and Y348A^{7.43} that did not bind [³H]almorexant, almorexant was not able to efficiently inhibit orexin-A-evoked [Ca²⁺]_i response and thus resulted in the large increases in K_b values (>10,000-, >10,000-, 191.9-, >10,000-, 61.3- and 32.7-fold, respectively). However, the mutation Y224A^{5.47}, which caused loss of almorexant's binding affinity, had a marginal effect on its functional potency.

Docking of SB-674042, almorexant and EMPA onto the OX₁-7TMD and OX₂-7TMD binding cavities. Our mutational data indicate that the complexes of antagonists with long-lasting pseudo-irreversible mode of antagonism such as almorexant-OX₂ and SB-674042-OX₁ needed fewer contact sites with the respective receptor, while the complexes of

competitive, reversible antagonists such as EMPA–OX₂ and almorexant–OX₁ were more affected by amino acid replacements, their interactions involving more molecular determinants (**Tables 3 and 4**). To visualize the mutation data, a 3D-model of the hOX₁–7TMD and hOX₂–7TMD using the atomic coordinates of hβ₂AR (PDB code, 2RH1) were constructed and three small molecule antagonists docked onto the transmembrane cavity. **Fig. 6A and B** show the docking of almorexant and EMPA to hOX₂. **Fig. 6C** shows both compounds superposed in the hOX₂ binding site. According to the mutational results, both antagonists share important interactions with residues on TM5, -6 and -7. This fits nicely with our predicted docking poses: Y223^{5,38}, F227^{5,42} and Y232^{5,47} form a subpocket filled by both antagonists equally well. Also, both almorexant and EMPA can nicely interact with one of their aromatic rings with H350^{7,39} to form an aromatic π - π interaction. Both antagonists are thus docked onto the same region in the homology model. However, due to their singular chemical structures they show different effects with further surrounding residues. Most importantly, only the OX₂ selective ligand EMPA was affected by the T135A^{3,33} mutation. This might be explained by the fact that EMPA is located closer to this residue than almorexant and can form a hydrogen bond with the pyridyl group. Besides, only EMPA was affected by the V138A^{3,36}, Y317F^{6,48} and I320^{6,51} mutations. As **Fig. 6B/C** shows, these four residues are located closely to each other deep in the binding cavity and are forming a subpocket filled with the para-methoxy substituted pyridyl group of EMPA. Almorexant, on the other side, does not reach as deep into this part of the pocket and is thus not influenced by these three mutations. The effect of F346A^{7,35} on only almorexant can be explained by the suboptimal geometry of the edge-to-face aromatic interaction of the phenyl substituent of almorexant with F346^{7,35}: The aromatic hydrogens of the phenyl substituent of almorexant point onto the aromatic hydrogens of F346^{7,35} rather than onto the aromatic face. This is an unfavorable situation. Consequently, removal of the aromatic ring of F346^{7,35} results in a

statistically significant gain of binding affinity. EMPA is not affected by this mutation since it does not reach close enough to this sidechain in order to form a direct interaction. Furthermore, the effect of Q134A^{3.32} on almorexant can be explained by a hydrogen bond between Q134^{3.32} and the amide group in this antagonist. EMPA cannot form such an interaction with these residues. It should be noted that the importance of the W214^{45.54} and Y354^{7.43} can not be explained from our docking model. W214^{5.54} is located on the extracellular loop E2 and is, according to our model, far away from the antagonist binding side. Nevertheless, its location at the entrance of the channel leading to the binding cavity might affect the kinetic characteristics of the antagonists by influencing their entry into the binding site.

Fig. 7A shows the docking of almorexant to hOX₁, which is essentially the same than its docking to the hOX₂ homology model. The only difference between OX₂ and OX₁ in the closer environment of almorexant is T135A^{3.33}. Interestingly, while the T135A^{3.33} mutation in the OX₂ receptor did not affect almorexant binding, A127T^{3.33} in OX₁ affected almorexant. This cannot be explained from the docking model. However, the introduction of the threonine residue and thus an OH group with its capabilities to form hydrogen bond might result in a change of the local environment that is different from the OX₂ receptor due to additional differences between OX₂ and OX₁ further away from almorexant. Thus, it might be an indirect effect on almorexant binding and functionality. Finally, **Fig. 7B** shows a possible docking mode of SB-674042 to hOX₁. It is essentially located in the same region as almorexant. Since it does not come so close to the extracellular loop E2 than almorexant, it is however not affected by the D203A^{45.51} mutation.

Discussion

Here, we have determined the likely binding pockets of OX antagonists, almorexant, EMPA, and SB-674042 using h β 2AR-based modeling of OX-7TMDs and site-directed mutagenesis. Based on the proposed docking mode of almorexant onto the OX₁- and OX₂-7TMD binding cavities, twenty-nine point-mutations (eighteen in OX₂ and eleven in OX₁) located in the TM2, -3, -5, -6, -7, and ECL2b regions were selected as candidates for mutational studies. These mutated hOX₁ and hOX₂ made also possible to probe the orexin-A-binding pockets of OX₁ and OX₂ on the basis of orexin-A-evoked [Ca²⁺]_i response in the HEK293 cells transiently expressing the mutated receptors.

Binding Site of orexin-A in OX₁ and OX₂. The conserved residues OX₁ D203^{45.51} (D211^{45.51} in OX₂), W206^{45.54} (W214^{45.54} in OX₂), Y215^{5.38} (Y223^{5.38} in OX₂), F219^{5.42} (F227^{5.42} in OX₂), Y224^{5.47} (Y232^{5.47} in OX₂), Y311^{6.48} (Y317^{6.48} in OX₂), and H344^{7.39} (H350^{6.39} in OX₂) were found to be contributing to both orexin-A-binding sites at OX₁ and OX₂, but these residues had yet a more prominent effect on orexin-A's potency at OX₁ than that at OX₂. Orexin-A behaved differently on two residues at TM3 helix positions 3.32 and 3.36 between OX₁ and OX₂. The mutation of OX₂ Q134^{3.32} to alanine caused a 22-fold drop in orexin-A potency at OX₂, whereas the mutation of corresponding residue in OX₁, Q126A^{3.32}, had no effect on orexin-A's potency. Conversely, the mutation OX₁ V130A^{3.36} led to a decrease of 31-fold in orexin-A's potency at OX₁, without having any effect on the corresponding residue V138^{3.36} in OX₂. Therefore, the residues OX₁ V130^{3.36} and OX₂ Q134^{3.32} might contribute to the selectivity of orexin-A–OX₁ and –OX₂ binding pockets, respectively. For orexin-A, the conformation and residues required for a high activity at the OX₁ and OX₂ receptors have been extensively characterized. These studies demonstrated that alanine replacement of the same amino acids of orexin-A produced a more prominent reduction in the potency for the OX₁ than that for the OX₂ even though the determinants

required from orexin-A for activation of the receptor was similar between OX₁ and OX₂ (Ammoun et al., 2003; Lang et al., 2004). Interestingly, the reported observations that OX₁ was in general more sensitive to amino acid replacements in orexin-A than OX₂ are in good agreement with our mutational studies of OX₁ and OX₂.

Binding Site of OX antagonists in OX₁ and OX₂. Among eighteen point-mutations that are located in TM2, -3, -5, -6, -7 and ECL2b of hOX₂, we observed that the W214A^{45.54}, Y223A^{5.38}, F227A^{5.42}, Y317A^{6.48} and H350A^{7.39} mutations resulted in complete loss of the [³H]EMPA and [³H]almorexant binding affinities and also blocked the inhibition by both antagonists of orexin-A-evoked [Ca²⁺]_i response. However, the conversion of Y317^{6.48} to a phenylalanine had no significant effect on almorexant's binding, but a small significant effect on EMPA's binding affinity. This may indicate that the forces involved in the interaction between Y317^{6.48} and almorexant might be via hydrophobic C-H/π interactions. The mutation Y232A^{5.47} led to the complete loss of [³H]EMPA binding affinity and a 37-fold decrease in EMPA's potency, had yet only a moderate effect on [³H]almorexant binding affinity and potency. Notably, three mutations T135A^{3.33}, I320A^{6.51}, Y354A^{7.43} behaved differently between interacting modes of two antagonists in OX₂, having a detrimental effect on the affinity and potency of EMPA, while almorexant's affinity and potency remained unaffected. Two mutations, T111A^{2.61} and D211A^{45.51}, also affected only moderately the binding of EMPA, but not that of almorexant. Of interest is the conserved residue OX₂ V138^{3.36} (V130^{3.36} in OX₁): mutation of this residue affected the binding affinity and functional potency of EMPA (by 15- and 91-fold decrease, respectively), but had no effect on almorexant's binding in OX₁ and OX₂, or SB-674042's binding in OX₁. The V138^{3.36} could be contributing specifically to binding selectivity of EMPA for OX₂. Therefore, it is concluded that the residues T135^{3.33} and V138^{3.36} represent the minimal structural motif responsible for the high selectivity of OX₂ for EMPA. Indeed, in our model, only EMPA's

para-methoxy substituted pyridyl group can reach deep in the subpocket formed by residues T135^{3.33}, V138^{3.36} and Y317^{6.48} and additionally, its pyridyl ring can form a H-bond with the hydroxyl group of T135^{3.33} located in close proximity (**Fig. 6B**).

Among the eleven point-mutations that are located in TM3, -5, -6, -7 and ECL2b of hOX₁, the mutations Q126A^{3.32}, A127T^{3.33}, W206A^{45.54}, Y215A^{5.38}, F219A^{5.42} and H344A^{7.39} abolished the binding affinity and functional potency of both almorexant and SB-674042, yet with a more dramatic effect on almorexant than on SB-674042. The conserved residues D203^{45.51}, Y224^{5.47}, Y311^{6.48} and Y348^{7.43} of OX₁ had completely different behavior between two antagonists: their mutations had detrimental effects on almorexant's binding and function, but had no or small effects on SB-674042. The residue at position 3.33 is of special interest, because it is a non-conservative residue between OX₁ (A127^{3.33}) and OX₂ (T135^{3.33}). Conversion of A127^{3.33} to threonine affected dramatically SB-674042 and almorexants' binding affinities and functions at OX₁; conversely, conversion of OX₂ T135^{3.33} to alanine was detrimental only on EMPA's binding affinity and potency. Hence, the position 3.33 of orexin receptors was identified as a critical position that must be involved in subtype selectivity and also in differentiating two different antagonists for the same receptor, as observed for EMPA and almorexant at OX₂.

Comparison of the ligand-binding site in OX₁ and OX₂ to other class A GPCRs.

In Table 5, we have summarized our mutational studies of OX antagonists and compared the critical residues contributing to the ligand-binding sites of OX₁ and OX₂ with those of rhodopsin (Palczewski et al., 2000; Teller et al., 2001), hβ₂AR (Cherezov et al., 2007; Rosenbaum et al., 2007) and hA_{2A} (Jaakola et al., 2008). The ligand-binding pockets of OX₁ and OX₂ are very similar, and both offer numerous hydrophobic interactions, predominantly aromatic. Besides, the local conformation of positions 3.32, 3.33 and 3.36 in TM3 and 45.51 in ECL2b might provide the structural basis for pharmacologic selectivity between OX₁ and

OX₂. It is interesting to note that among seven critical residues that are shared between orexin-A–OX₁ and –OX₂ binding sites, five residues D^{45.51}, W^{45.54}, Y^{5.38}, F^{5.42} and H^{7.39} are located on the ECL2b and in the top of TM domains at the interface to the main binding crevice, thereby suggesting superficial OX receptor interactions of orexin-A. Similar observation has been reported for other large peptides, such as chemokine receptor interaction with chemokine (Schwarz and Wells, 2002). High resolution β₂AR X-ray structure has revealed that the main ligand-binding pocket is a funnel with a partial lid, and that ECL-2b region (highly variable region between the receptors) is an important 7TM structural element forming the edge of this protein lid. In hβ₂AR, the formation of a salt bridge between D192^{45.51} on ECL2b and K305^{7.32} on ECL3 at the extracellular end of TM7 is the major structural feature that blocks access to the ligand binding site. Furthermore, an interaction between F193^{45.53} on ECL2b and carazolol was also observed in hβ₂AR X-ray structure (Ahuja and Smith, 2009; Cherezov et al., 2007; Rosenbaum et al., 2007). In analogy, OX₁ D203^{45.51} (D211^{45.51} in OX₂) on ECL2b was found to be crucial for orexin-A–OX₁ and –OX₂, EMPA–OX₂ and almorexant–OX₁ binding sites. The OX₁ W206^{45.54} (W214^{45.54} in OX₂), another residue located on ECL2b was also important for OX ligand-binding pocket. Hence, OX receptors might operate with a similar mechanism of activation as β₂AR.

We observed that six OX residues, W^{45.54}, Y^{5.38}, F^{5.42}, Y^{5.47}, Y^{6.48} and H^{7.39}, are important contributors to the orexin-A–OX₁ and –OX₂, EMPA–OX₂ and almorexant–OX₁ and –OX₂ binding pockets. We speculate that these aromatic residues are involved in a tight network of interhelical aromatic/hydrophobic interactions, which maintains the OX receptors in a constrained/inactive conformation. Consequently, the OX antagonist via intramolecular interactions could further reinforce this network, thereby hindering the structural rearrangements necessary for activation. Of note are the important helix positions 5.47 and 6.48 (Y^{5.47} and Y^{6.48} in OX); as a recent report investigating ghrelin receptor, β₂AR and NK₁

has demonstrated that two residues F^{5.47} in TM5 and W^{6.48} (key residue of the rotamer toggle switch) in TM6 are located in close proximity at the bottom of the main ligand-binding pocket and an aromatic interaction between two residues could stabilize the active conformation of W^{6.48} (Holst et al., 2009). Interestingly, two critical residues, F^{5.42} and H^{7.39} (in the top of TM5 and TM7), are positioned on opposite extremities of the ligand-binding pocket. In hβ₂AR, the corresponding positions (S203^{5.42} and N312^{7.39}) are involved in H-bond interactions with carazolol (Cherezov et al., 2007; Rosenbaum et al., 2007). Moreover, the position 7.39 in chemokine receptors (E^{7.39}, a conserved residue among CCRs) provides an important anchor-point for interaction with nonpeptide agonists and antagonists (Jensen et al., 2007; Rosenkilde and Schwartz, 2006). The position 7.39 was also found to be the critical residue in the binding pocket of other peptide receptors including hNK₂ (F293^{7.39}), hNK₃ (F342^{7.39}), hV_{1a} (A334^{7.39}) and hV_{1b} (M324^{7.39}) (Derick et al., 2004; Huang et al., 1995; Malherbe et al., 2008). In conclusion, we have demonstrated for the first time the important molecular determinants of ligand-binding site of OX₁ and OX₂.

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Legends for Figures

Fig. 1: (A) Chemical structures of the selective OX₁, OX₂ and dual OX₁/OX₂ antagonists. T, tritium. (B) The amino acid sequences of orexin-A and -B. Orexin-A have a pyroglutamate (2-pyrrolidone-5-carboxylic acid) at the first N-terminal residue site, which is indicated by U. The C-terminal of both orexins (-NH₂) are amidated. Two intramolecular disulfide bonds in orexin-A formed between C6 and C12, and between C7 and C14 are shown as lines. The residues, identical in both orexins, are highlighted in gray.

Fig. 2: Amino acid sequence alignment of the TM1-Helix 8 region of the hOX₁ with the hOX₂ using pairwise global alignment (Needleman-Wunsch algorithm). The numbers on the right refer to the amino acids of hOX₁ and hOX₂ receptors. Vertical lines indicate identical residues and colons indicate conservative replacement of residues (chemically homologous residues) between hOX₁ and OX₂. The transmembrane (TM) domains are boxed. The conserved residue in each TM which is assigned to 50 according to Ballesteros Weinstein numbering scheme is shown by black arrows. The critical residues (indicating by Ballesteros Weinstein numbering scheme of the amino acids) in the binding pocket of orexin receptor antagonists (almorexant, EMPA, SB-674042) are shown by red boldface type and red short arrows. Three conserved motifs in TM3 (DRY), TM6 (CWxP) and TM7 (NPxxY) that is believed to function as a micro-switches (Nygaard et al., 2009), are shown in blue boldface type and underlined. ECL, extracellular loop; ICL, intracellular loop.

Fig. 3: A, Alignment of the amino acids forming the binding site of OX₁ and OX₂ relative to bovine rhodopsin (P02699), human β_2 -adrenergic (P07750) and human A_{2A} (P29274) receptors. Ballesteros Weinstein numbering scheme of the amino acids (indicated above the TMs in the bottom row) are given to facilitate the comparison with other GPCR's (see

Materials and Methods). The numbers above the OX1R_HUMAN and OX2R_HUMAN receptors give the sequence number of the positions of the mutations carried out in this study. The residue at helix position 3.33, which is different between OX₁ and OX₂ binding pockets are boxed and yellow high-lighted.

Fig. 4: Effects of orexin antagonists on orexin-A-evoked [Ca²⁺]_i in WT and mutated hOX₂ receptors. Concentration-dependent inhibition of OX-A (EC₈₀ value) stimulated increases in [Ca²⁺]_i by EMPA (panels A, C and E) and almorexant (panels B, D and F) as assayed using the Ca²⁺-sensitive dye, Flou-4 and a Fluorometric Imaging Plate Reader in HEK293 transiently transfected with the hOX₂ WT and mutated receptors. Responses are normalized to the first control response. Each curve represents the mean of six dose-response measurements (each performed in duplicate) from three independent transfections.

Fig. 5: Effects of orexin antagonists on orexin-A-evoked [Ca²⁺]_i in WT and mutated hOX₁ receptors. Concentration-dependent inhibition of orexin-A (EC₈₀ value) stimulated increases in [Ca²⁺]_i by SB-674042 (panels A and C) and almorexant (panels B and D) as assayed using the Ca²⁺-sensitive dye, Flou-4 and a Fluorometric Imaging Plate Reader in HEK293 transiently transfected with the hOX₁ WT and mutated receptors. Responses are normalized to the first control response. Each curve represents the mean of 6 dose-response measurements (each performed in duplicate) from three independent transfections.

Fig. 6: (A) and (B): Predicted docking poses of almorexant (A) and EMPA (B) in the OX₂ binding site. Shown are the residues that were found to be important according to our mutational studies. Ligand carbon atoms are shown in magenta, protein carbon atoms are shown in green. Nitrogen atoms are shown in blue, oxygen in red, sulfur atoms in yellow and

fluorine atoms in green. The possible hydrogen bond interactions between Q134^{3.32} with almorexant in A and T135^{3.33} with EMPA in B are visualized by red dotted lines. (C): Predicted docking poses of both almorexant (cyan) and EMPA (magenta) in the OX₂ binding site. Shown in grey are protein carbon atoms of residues that were important for almorexant and EMPA according to our mutational studies. Shown in cyan are protein carbon atoms of residues that were important for only almorexant according to our mutational studies. Shown in magenta are protein carbon atoms of residues that were important for only EMPA according to our mutational studies. Nitrogen atoms are shown in blue, oxygen in red, sulfur atoms in yellow and fluorine atoms in green.

Fig. 7: Predicted docking poses of almorexant (A) and SB674042 (B) in the OX₁ binding site. Shown are the residues that were found to be important according to our mutational studies. Ligand carbon atoms are shown in magenta, protein carbon atoms are shown in green. Nitrogen atoms are shown in blue, oxygen atoms in red and fluorine atoms in green.

Tables

Table 1. The effect of mutations on OX₂-mediated of orexin-A-induced [Ca²⁺]_i response. EC₅₀, Hill slope (n_H), and relative efficacy (E_{max}) values for the orexin-A-induced [Ca²⁺]_i response in the HEK293 cells transiently transfected with the WT and mutated hOX₂ receptors. The data is mean ± S.E. of eight concentration-response measurements (each performed in duplicate) from four independent transfections. The mutations that affected the potency of orexin-A in comparison to WT are shown in boldface type.

hOX ₂	7TM position	orexin-A			
		EC ₅₀ nM	EC ₅₀ (mut)/ EC ₅₀ (WT)	Hill	E _{max} relative
WT		0.6 ± 0.1		0.5 ± 0.0	100
T111A	2.61	151.0 ± 22.2	243.5	0.7 ± 0.0	114.4 ± 13.0
Q134A	3.32	13.8 ± 2.1	22.3	1.4 ± 0.1	62.3 ± 1.1
T135A	3.33	0.5 ± 0.1	0.8	0.6 ± 0.1	109.3 ± 11.2
V138A	3.36	2.8 ± 0.4	4.4	0.7 ± 0.0	92.1 ± 10.0
S139A	3.37	1.5 ± 0.3	2.5	0.6 ± 0.0	86.1 ± 8.6
D211A	45.51	258.0 ± 57.2	416.1	0.8 ± 0.1	126.4 ± 16.4
W214A	45.54	38.7 ± 12.2	62.4	0.7 ± 0.1	71.3 ± 7.4
Y223A	5.38	114.0 ± 14.1	183.9	1.0 ± 0.1	61.2 ± 1.2
F227A	5.42	149.0 ± 34.8	240.3	1.1 ± 0.1	64.3 ± 6.3
F228A	5.43	1.8 ± 0.7	3.0	0.7 ± 0.1	76.2 ± 2.1
Y232A	5.47	17.6 ± 3.1	28.4	1.2 ± 0.1	44.9 ± 4.5
Y317A	6.48	11.0 ± 6.1	17.7	1.0 ± 0.1	49.6 ± 6.7
Y317F	6.48	0.8 ± 0.3	1.3	0.6 ± 0.1	72.3 ± 9.7
I320A	6.51	0.6 ± 0.2	0.9	0.6 ± 0.0	97.0 ± 5.4
F346A	7.35	33.8 ± 8.8	54.5	1.1 ± 0.2	60.7 ± 13.2
H350A	7.39	30.7 ± 5.2	49.5	1.1 ± 0.2	63.0 ± 10.7
V353A	7.42	1.2 ± 0.5	1.9	0.6 ± 0.1	84.8 ± 14.7
Y354A	7.43	2.3 ± 0.9	3.7	0.7 ± 0.1	66.1 ± 12.0

Table 2. The effect of mutations on OX₁-mediated of orexin-A-induced [Ca²⁺]_i response. EC₅₀, Hill slope (n_H), and relative efficacy (E_{max}) values for the orexin-A-induced [Ca²⁺]_i response in the HEK293 cells transiently transfected with the WT and mutated hOX₁ receptors. The data is mean ± S.E. of eight concentration-response measurements (each performed in duplicate) from four independent transfections. The mutations that affected the potency and relative efficacy of orexin-A in comparison to WT are shown in boldface type.

hOX ₁	7TM position	orexin-A			
		EC ₅₀ nM	EC ₅₀ (mut)/ EC ₅₀ (WT)	Hill	E _{max} relative
WT		0.9 ± 0.2		0.7 ± 0.1	100
Q126A	3.32	2.2 ± 0.2	2.4	0.7 ± 0.1	59.1 ± 10.1
A127T	3.33	1.6 ± 0.4	1.8	0.8 ± 0.0	97.9 ± 32.9
V130A	3.36	27.5 ± 2.5	30.6	0.7 ± 0.1	98.9 ± 31.4
D203A	45.51	367.4 ± 75.0	408.2	0.8 ± 0.1	155.2 ± 53.1
W206A	45.54	376.0 ± 102.0	417.8	0.8 ± 0.2	45.0 ± 8.0
Y215A	5.38	367.0 ± 95.0	407.8	0.6 ± 0.1	65.0 ± 9.0
F219A	5.42	125.6 ± 26.5	139.6	0.8 ± 0.1	90.1 ± 48.6
Y224A	5.47	76.0 ± 41.3	84.4	0.6 ± 0.1	83.7 ± 40.6
Y311A	6.48	147.5 ± 67.1	163.9	0.9 ± 0.1	53.4 ± 12.9
H344A	7.39	217.0 ± 63.9	241.1	0.9 ± 0.1	64.5 ± 7.3
Y348A	7.43	7.8 ± 3.0	8.7	0.8 ± 0.0	62.4 ± 18.9

Table 3. Comparison of mutation effects on binding and functional potencies of antagonists at OX₂.

[³H]EMPA and [³H]almorexant binding properties at human wild type and mutated hOX₂ receptors. Saturation binding isotherms of [³H]EMPA and [³H]almorexant were performed on membrane preparations from HEK293 cells transiently transfected with the WT and mutated hOX₂ as described under “Materials and Methods”. The K_d and B_{max} values are mean ± S.E., calculated at least from 3-5 independent experiments (each performed in triplicate). Statistical significance was determined using the two-tailed t-test: *P<0.05, **P<0.01, ***P<0.001. Effects of mutations on inhibition of orexin-A-induced [Ca²⁺]_i response by EMPA and almorexant. K_b and Hill coefficient (n_H) values for the inhibition by EMPA and almorexant of orexin-A (EC₈₀ value)-evoked [Ca²⁺]_i response in the HEK293 cells transiently transfected with the hOX₂ WT and mutated receptors. Data are means ± S.E. of the six dose-response measurements (each performed in duplicate) from three independent transfections. The mutations that affected the binding and functional potencies of EMPA and almorexant in comparison to the WT are shown in boldface type. N.D.B., no detectable binding due to high nonspecific binding.

hOX ₂	Position in the 7TMD	³ H]EMPA binding			EMPA (FLIPR)			³ H]almorexant binding			almorexant (FLIPR)		
		K _d nM	K _d (mut)/ K _d (WT)	B _{max} pmol/mg protein	K _b nM	K _b (mut)/ K _b (WT)	n _H	K _d nM	K _d (mut)/ K _d (WT)	B _{max} pmol/mg protein	K _b nM	K _b (mut)/ K _b (WT)	n _H
WT		1.1 ± 0.0		24.8 ± 0.2	1.1 ± 0.1		0.6 ± 0.0	1.2 ± 0.0		23.1 ± 0.6	2.4 ± 0.1		1.8 ± 0.1
T111A	2.61	8.1 ± 1.9	7.4*	20.8 ± 3.4	56.2 ± 8.5	51.1	0.6 ± 0.1	0.8 ± 0.2	0.7	18.3 ± 2.9	3.4 ± 0.4	1.4	1.1 ± 0.1
Q134A	3.32	1.9 ± 0.3	1.7	4.1 ± 0.2	2.5 ± 0.3	2.3	0.9 ± 0.0	12.8 ± 5.5	10.7*	18.0 ± 6.1	4.4 ± 0.5	1.8	1.7 ± 0.3
T135A	3.33	N.D.B.			3270.0 ± 97.4	2972.7	0.6 ± 0.1	1.0 ± 0.2	0.8	11.9 ± 1.3	7.2 ± 0.1	3.0	2.6 ± 0.2
V138A	3.36	16.4 ± 0.8	14.9***	18.1 ± 1.4	100.0 ± 12.6	90.9	0.6 ± 0.0	1.6 ± 0.1	1.3	18.4 ± 1.8	3.1 ± 0.8	1.3	2.0 ± 0.1
S139A	3.37	1.8 ± 0.1	1.6	4.0 ± 0.3	2.2 ± 0.4	2.0	0.8 ± 0.0	1.8 ± 1.0	1.5	4.5 ± 1.2	3.6 ± 0.6	1.5	2.1 ± 0.1
D211A	45.51	9.6 ± 1.2	8.7**	11.6 ± 1.0	18.5 ± 2.0	16.8	0.7 ± 0.0	0.7 ± 0.2	0.6	9.4 ± 0.9	6.3 ± 0.6	2.6	1.7 ± 0.1
W214A	45.54	N.D.B.			> 10,000			N.D.B.			> 10,000		
Y223A	5.38	N.D.B.			372.9 ± 56.0	339.0	3.1 ± 0.9	N.D.B.			> 10,000		
F227A	5.42	N.D.B.			462.0 ± 13.9	420.0	1.4 ± 0.0	N.D.B.			481.0 ± 42.1	200.4	0.4 ± 0.1
F228A	5.43	3.3 ± 0.9	3.0	10.1 ± 0.8	3.1 ± 0.6	2.8	0.5 ± 0.1	1.1 ± 0.2	0.9	10.0 ± 1.1	2.6 ± 0.3	1.1	1.6 ± 0.2
Y232A	5.47	N.D.B.			40.8 ± 6.5	37.1	1.0 ± 0.1	11.6 ± 1.9	10.0**	14.6 ± 2.1	18.1 ± 1.6	7.5	1.3 ± 0.2
Y317A	6.48	N.D.B.			60.0 ± 7.1	54.5	1.0 ± 0.1	N.D.B.			59.9 ± 5.1	25.0	1.2 ± 0.3
Y317F	6.48	6.2 ± 0.9	3.9**	12.6 ± 2.5	10.4 ± 0.7	9.5	1.0 ± 0.1	1.0 ± 0.0	0.8	5.2 ± 0.1	4.0 ± 0.6	1.7	1.8 ± 0.2
I320A	6.51	N.D.B.			457.0 ± 20.9	415.5	0.7 ± 0.2	0.8 ± 0.2	0.7	11.2 ± 1.3	3.1 ± 0.8	1.3	1.0 ± 0.2
F346A	7.35	1.6 ± 0.1	1.4	11.8 ± 0.3	1.5 ± 0.2	1.4	1.0 ± 0.0	0.4 ± 0.1	0.3***	10.6 ± 0.4	1.9 ± 0.3	0.8	1.5 ± 0.4
H350A	7.39	N.D.B.			307.0 ± 35.1	279.1	1.0 ± 0.2	N.D.B.			226.0 ± 63.2	94.2	0.5 ± 0.1
V353A	7.42	3.1 ± 0.8	2.8	20.8 ± 2.8	4.4 ± 0.2	4.0	0.7 ± 0.1	3.0 ± 1.1	2.5	20.7 ± 3.6	6.2 ± 0.7	2.6	1.1 ± 0.1
Y354A	7.43	N.D.B.			403.0 ± 44.8	366.4	0.6 ± 0.1	2.6 ± 0.8	2.2	20.1 ± 2.4	4.7 ± 0.3	2.0	0.9 ± 0.0

Table 4. Comparison of mutation effects on binding and functional potencies of antagonists at OX₁.

[³H]SB-674042 and [³H]almorexant binding properties at human wild type and mutated hOX₁ receptors. Saturation binding isotherms of [³H]SB-674042 and [³H]almorexant were performed on membrane preparations from HEK293 cells transiently transfected with the WT and mutated hOX₁ as described under “Materials and Methods”. The K_d and B_{max} values are mean ± S.E., calculated at least from 3-5 independent experiments (each performed in triplicate). Statistical significance was determined using the two-tailed t-test: *P<0.05, **P<0.01, ***P<0.001.

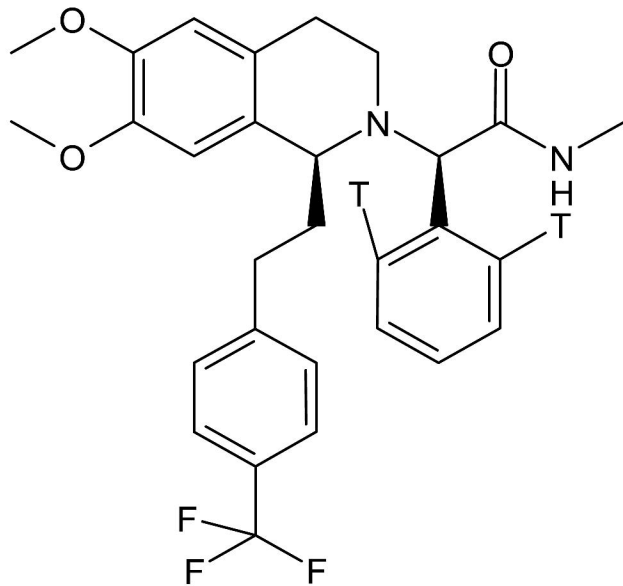
Effects of mutations on inhibition of orexin-A-induced [Ca²⁺]_i response by SB-674042 and almorexant. K_b and Hill coefficient (n_H) values for the inhibition by SB-674042 and almorexant of orexin-A (EC₈₀)-evoked [Ca²⁺]_i response in the HEK293 cells transiently transfected with the hOX₁ WT and mutated receptors. Data are means ± S.E. of the 6 dose-response measurements (each performed in duplicate) from three independent transfections. The mutations that affected the binding and functional potencies of [³H]SB-674042 and [³H]almorexant in comparison to the WT are shown in boldface type. N.D.B., no detectable binding due to high nonspecific binding. N. M., not measured.

hOX ₁	Position in the 7TMD	³ H]SB-674042 binding			SB-674042 (FLIPR)			³ H]almorexant binding			almorexant (FLIPR)		
		K _d nM	K _d (mut)/ K _d (WT)	B _{max} pmol/mg protein	K _b nM	K _b (mut)/ K _b (WT)	n _H	K _d nM	K _d (mut)/ K _d (WT)	B _{max} pmol/mg protein	K _b nM	K _b (mut)/ K _b (WT)	n _H
WT		1.2 ± 0.0		14 ± 0.4	0.6 ± 0.1		0.5 ± 0.1	2.3 ± 0.3		22.9 ± 2.2	4.8 ± 0.7		0.5 ± 0.0
Q126A	3.32	61.1 ± 6.0	50.9***	10.0 ± 3.8	24.2 ± 8.8	40.3	0.9 ± 0.1	N.D.B.			>10,000		
A127T	3.33	24.2 ± 7.0	20.2**	15.2 ± 3.0	13.7 ± 1.6	22.8	0.5 ± 0.1	N.D.B.			>10,000		
V130A	3.36	2.5 ± 1.2	2.1	17.6 ± 0.5	0.6 ± 0.3	1.0	0.7 ± 0.0	3.4 ± 1.0	1.5	13.4 ± 2.3	13.1 ± 1.9	2.7	1.1 ± 0.1
D203A	45.51	0.8 ± 0.1	0.7	9.0 ± 0.2	N.M.			N.D.B.			N.M.		
W206A	45.54	N.D.B.			N.M.			N.D.B.			N.M.		
Y215A	5.38	N.D.B.			N.M.			N.D.B.			N.M.		
F219A	5.42	N.D.B.			187.0 ± 77.1	311.7	0.5 ± 0.1	N.D.B.			921.0 ± 346.5	191.9	0.9 ± 0.3
Y224A	5.47	2.2 ± 1.0	1.8	2.2 ± 0.2	0.3 ± 0.0	0.5	0.6 ± 0.0	N.D.B.			22.8 ± 3.7	4.8	0.9 ± 0.1
Y311A	6.48	12.9 ± 3.4	10.8**	2.7 ± 0.3	0.7 ± 0.1	1.2	0.6 ± 0.0	N.D.B.			>10,000		
H344A	7.39	27.2 ± 4.0	22.7***	1.6 ± 0.8	12.5 ± 1.1	20.8	0.8 ± 0.0	N.D.B.			294.0 ± 97.1	61.3	0.3 ± 0.1
Y348A	7.43	11.2 ± 1.0	9.3***	3.2 ± 0.1	4.1 ± 0.6	6.8	0.6 ± 0.1	N.D.B.			157.0 ± 18.8	32.7	0.8 ± 0.1

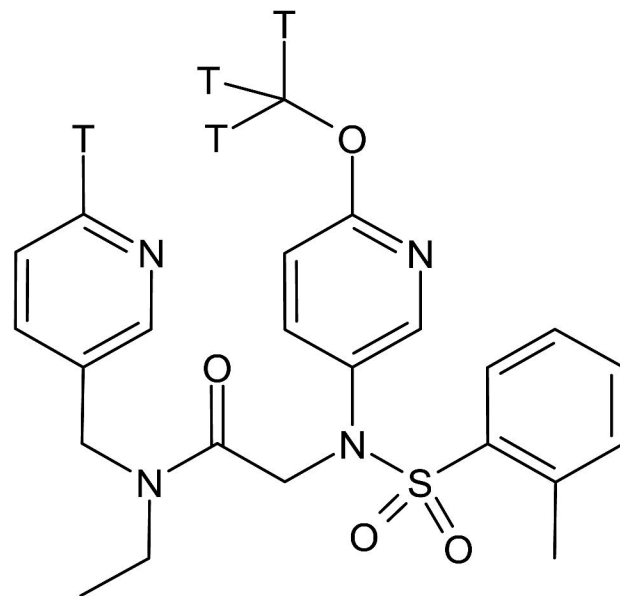
Table 5. Comparison of ligand-binding pocket of orexin receptor antagonists with those of bovine rhodopsin, h β_2 AR and hA $_{2A}$.

The residues located at a distance of 4.5 Å from 11-*cis*-retinal (inverse agonist) in the 3D structure of rhodopsin (2.8 Å, PDB ref code 1F88) (Palczewski et al., 2000; Teller et al., 2001), within 4 Å of the carazolol (partial inverse agonist) in the 2.4 Å resolution crystal structure of human β_2 -adrenergic receptor (PDB, 2RH1) (Cherezov et al., 2007; Rosenbaum et al., 2007), and within 5 Å of the ZM241385 (subtype-selective antagonist) in the 3D structure of human A $_{2A}$ adenosine receptor (2.6 Å, code 3EML) (Jaakola et al., 2008) are shown for comparison with the critical residues in the binding pocket of orexin receptor antagonists. The generic numbering system proposed by Ballesteros and Weinstein (Ballesteros and Weinstein, 1995) was used to compare residues in the 7TMD of the different GPCR's. WT, the same potency as wild type; (↓), decrease in both binding affinity and potency in comparison with WT; S.E., small but statistically significant decrease in binding affinity; N.A., not available (the mutation was not performed); (↑), increase in binding affinity.

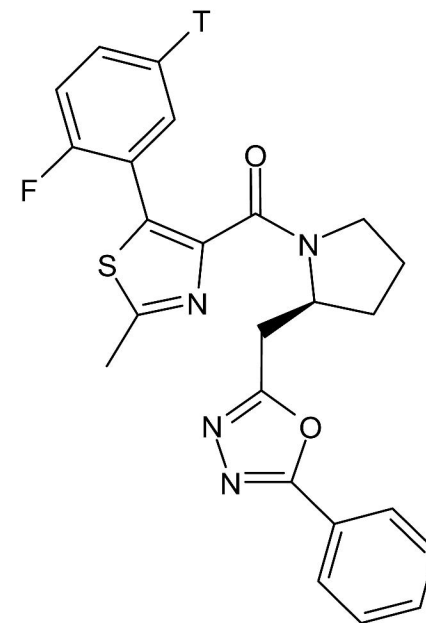
Position in the 7TMD	Opsd_bovin	h β_2 AR	hA $_{2A}$	hOX $_1$		hOX $_2$	
	11- <i>cis</i> -retinal	carazolol	ZM241385	SB-674042	almorexant	almorexant	EMPA
2.61				N.A.	N.A.	WT	T111A (↓)
3.32	A117	D113		Q126A (↓)	Q126A (↓)	S. E.	WT
3.33	T118	V114	L85	A127T (↓)	A127T (↓)	WT	T135A (↓)
3.36	G121	V117		WT	WT	WT	V138A (↓)
ECL2b (45.51)		D192		WT	D203A (↓)	WT	D211A (↓)
ECL2b (45.54)	Y191			W206A (↓)	W206A (↓)	W214A (↓)	W214A (↓)
5.38	F203	Y199	M177	Y215A (↓)	Y215A (↓)	Y223A (↓)	Y223A (↓)
5.42	M207	S203	N181	F219A (↓)	F219A (↓)	F227A (↓)	F227A (↓)
5.47	F212	F208		WT	Y224A (↓)	Y232A (↓)	Y232A (↓)
6.48	W265	W286	W246	S.E.	Y311A (↓)	Y317A (↓)	Y317A (↓)
6.51	Y268	F289	L249	N.A.	N.A.	WT	I320A (↓)
7.35	M288	Y308	M270	N.A.	N.A.	F346A (↑)	WT
7.39	A292	N312	I274	H344A (↓)	H344A (↓)	H350A (↓)	H350A (↓)
7.43	K296	Y316		S.E.	Y348A (↓)	WT	Y354A (↓)

A

[³H]almorexant
(dual OX₁/OX₂)



[³H]EMPA
(OX₂-sel)



[³H]SB-674042
(OX₁-sel)

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B

Orexin-A



Orexin-B



Fig. 1

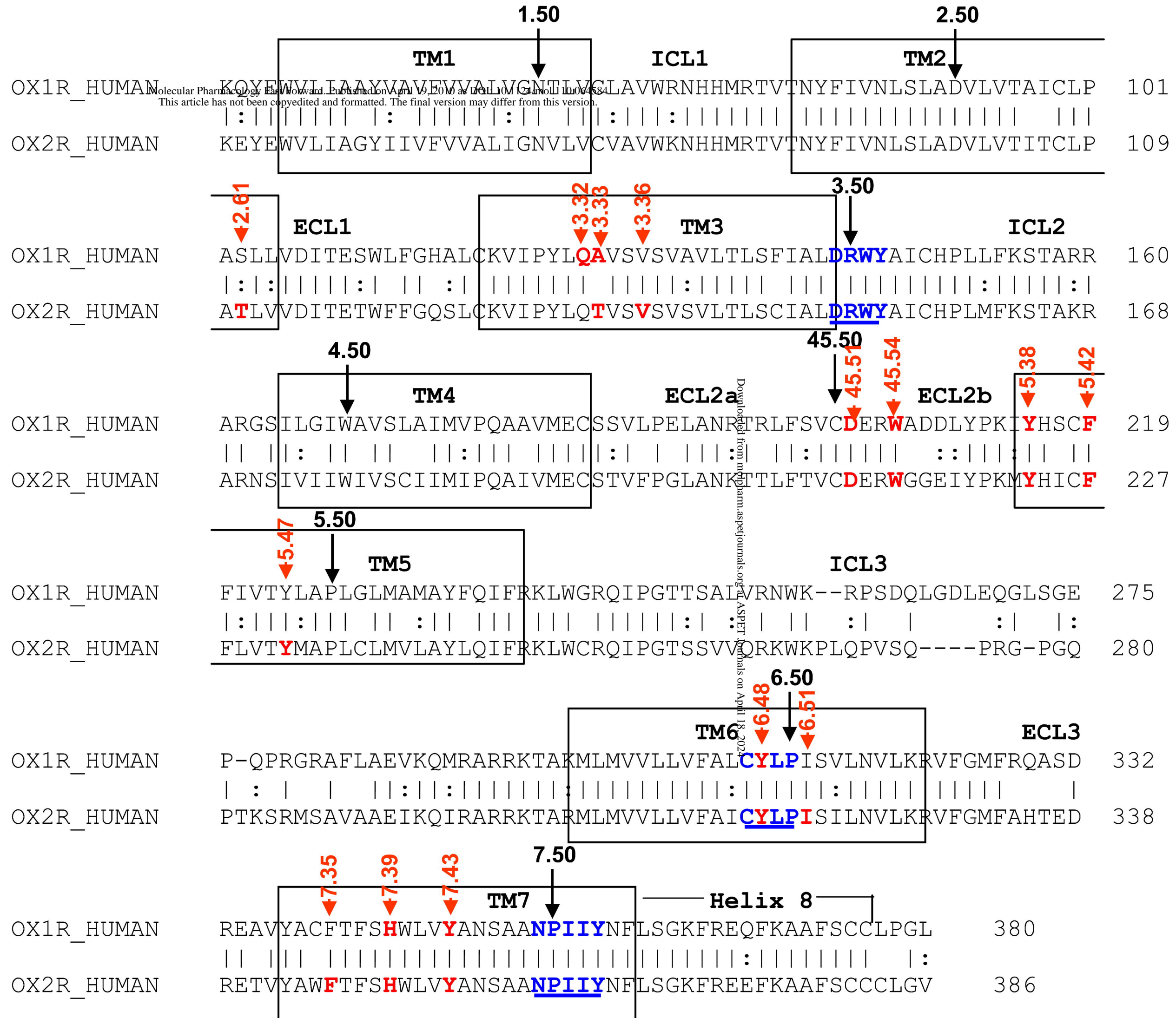


Fig. 2

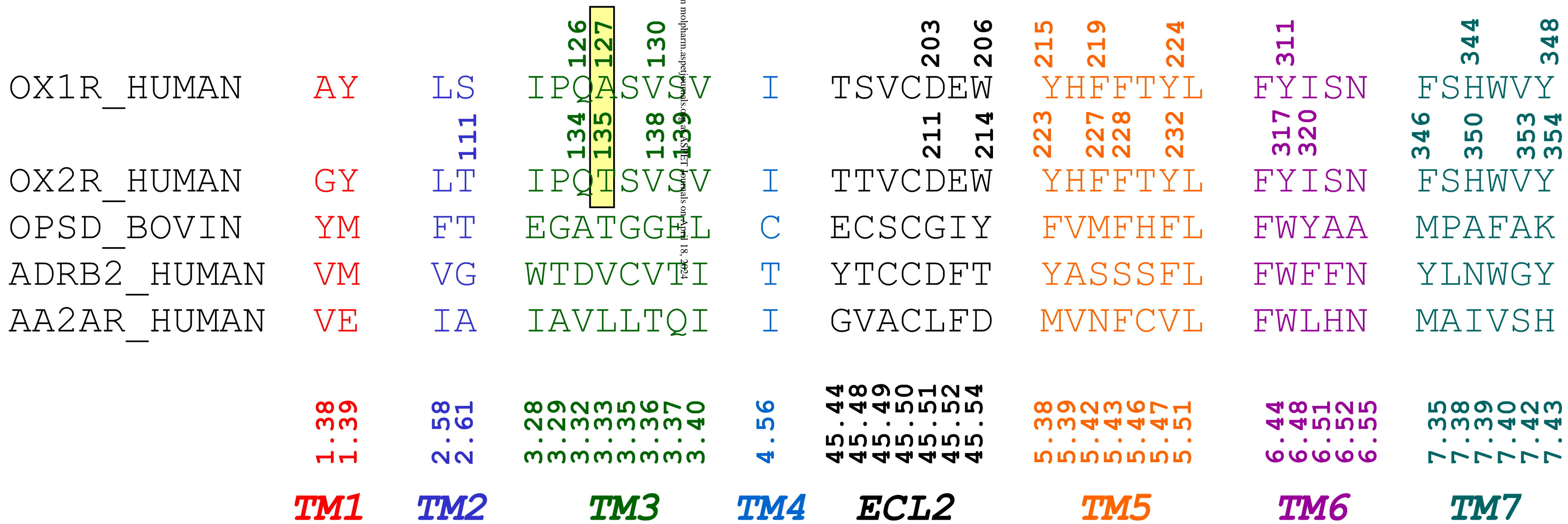


Fig. 3

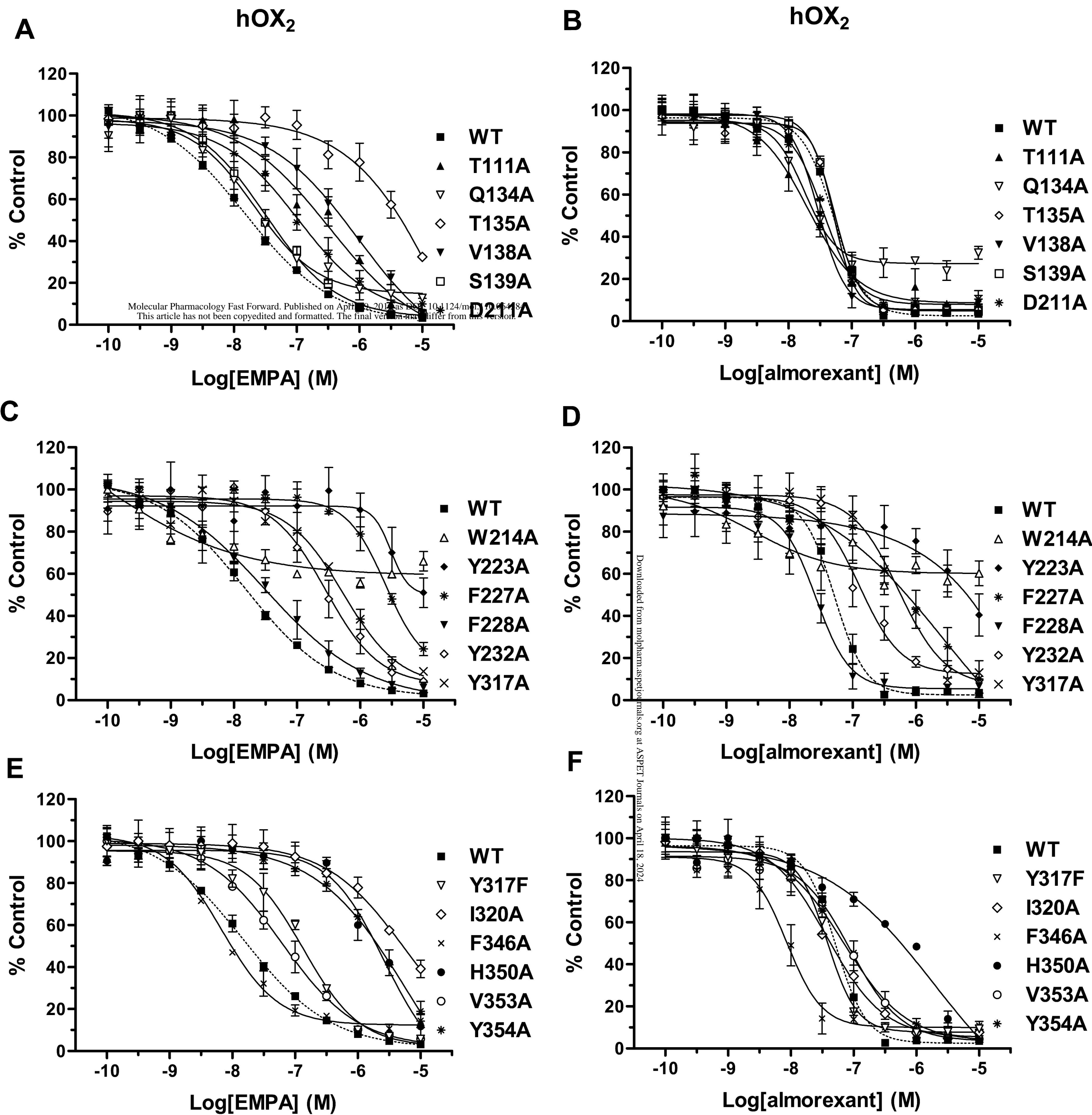


Fig. 4

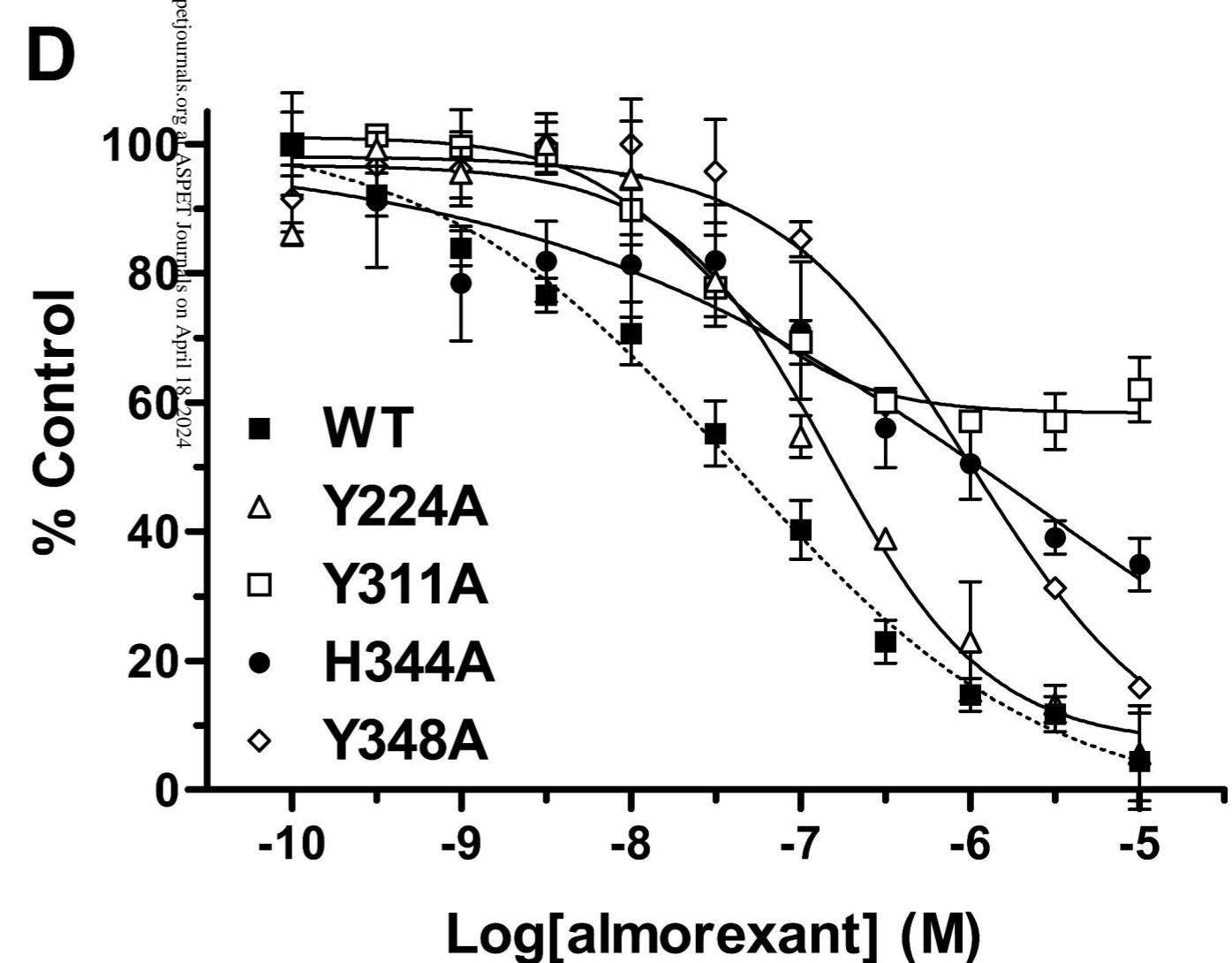
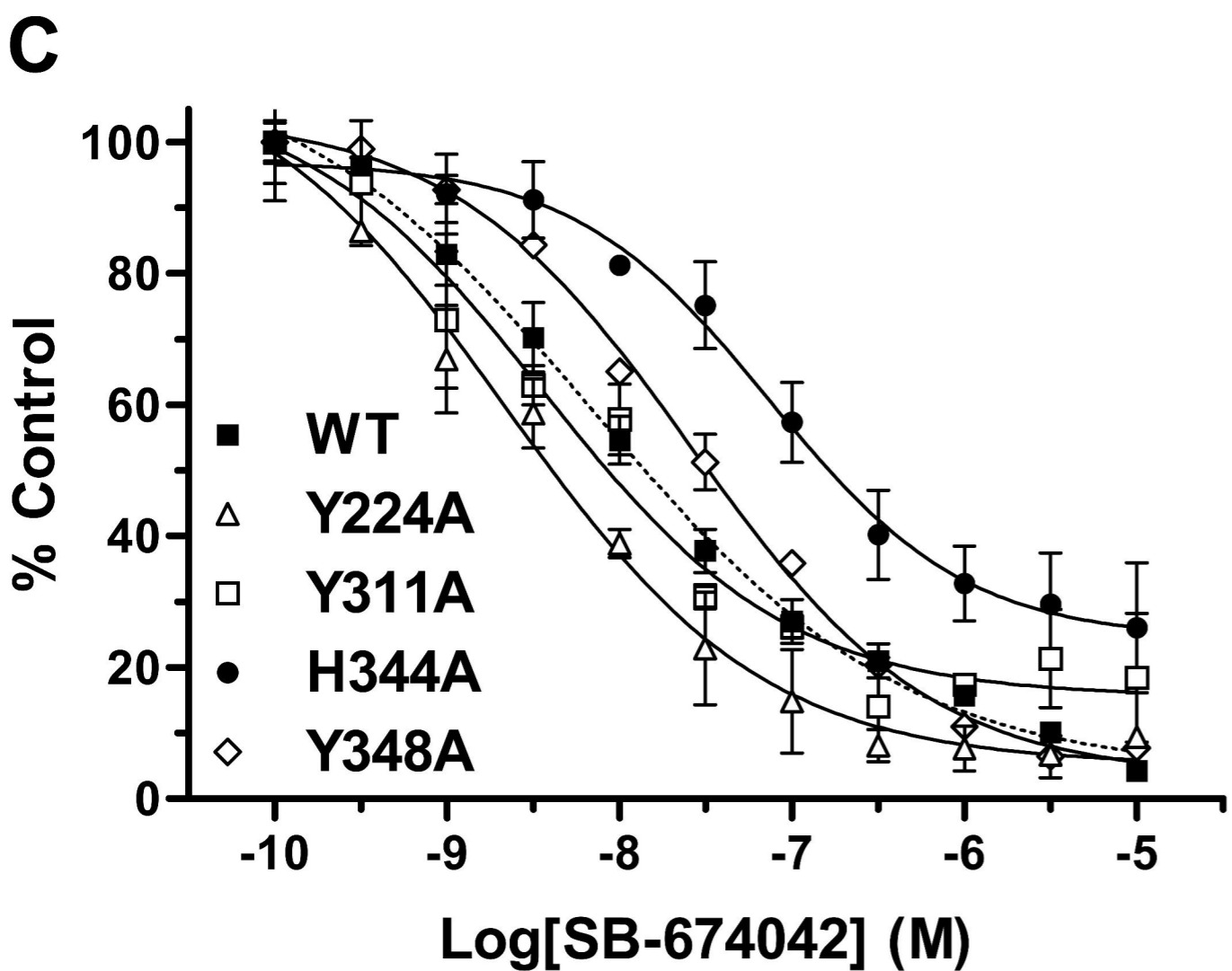
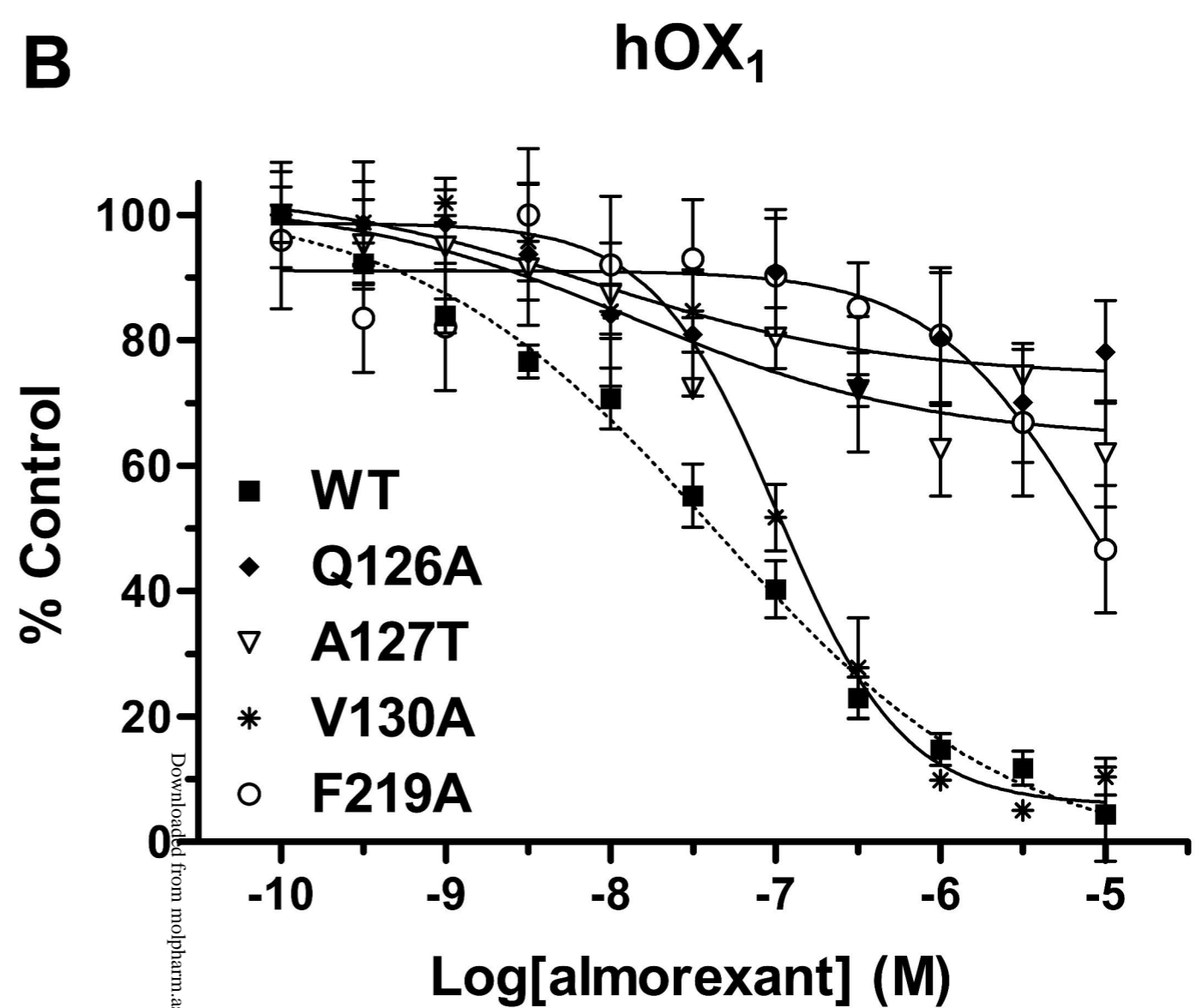
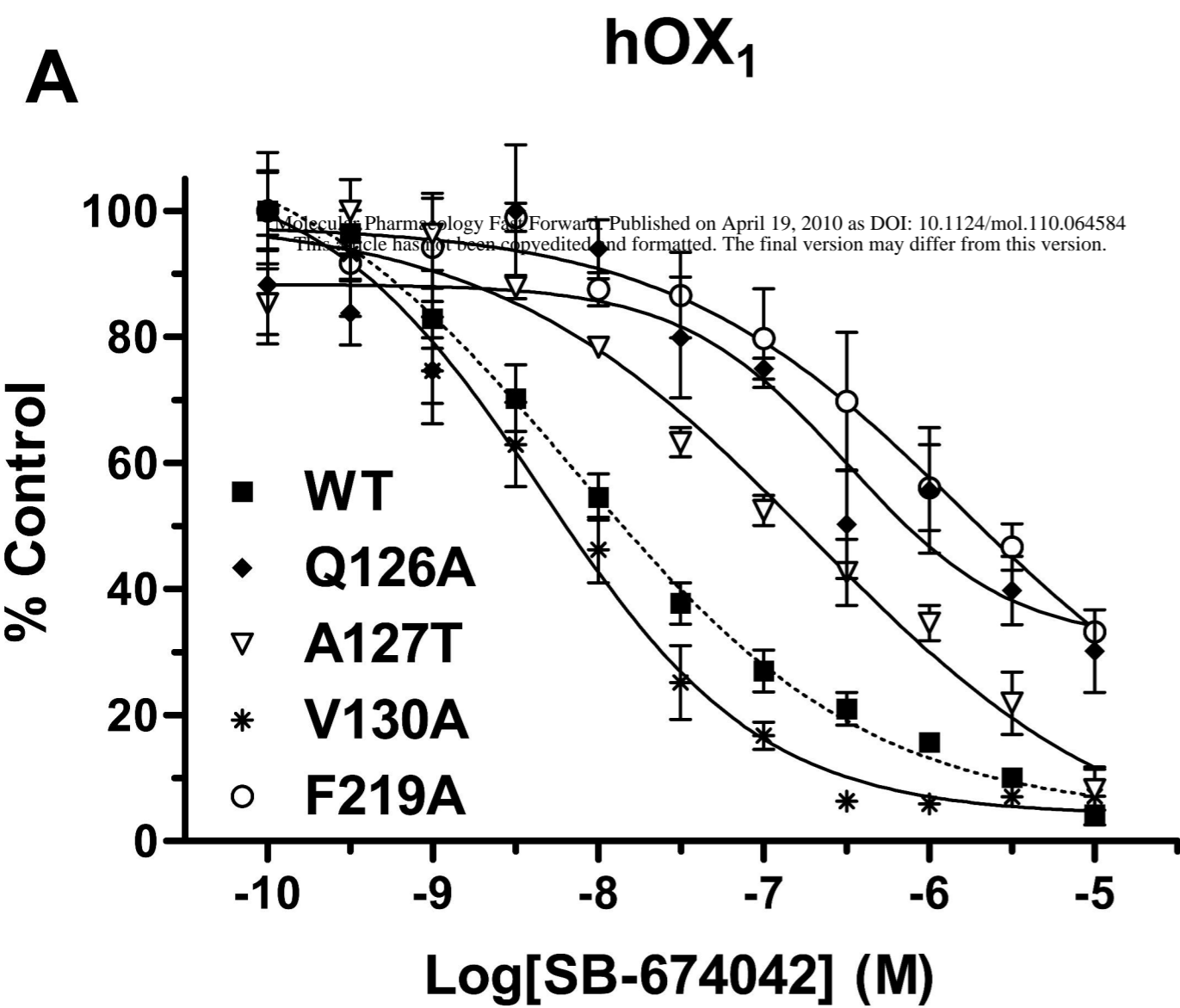


Fig. 5

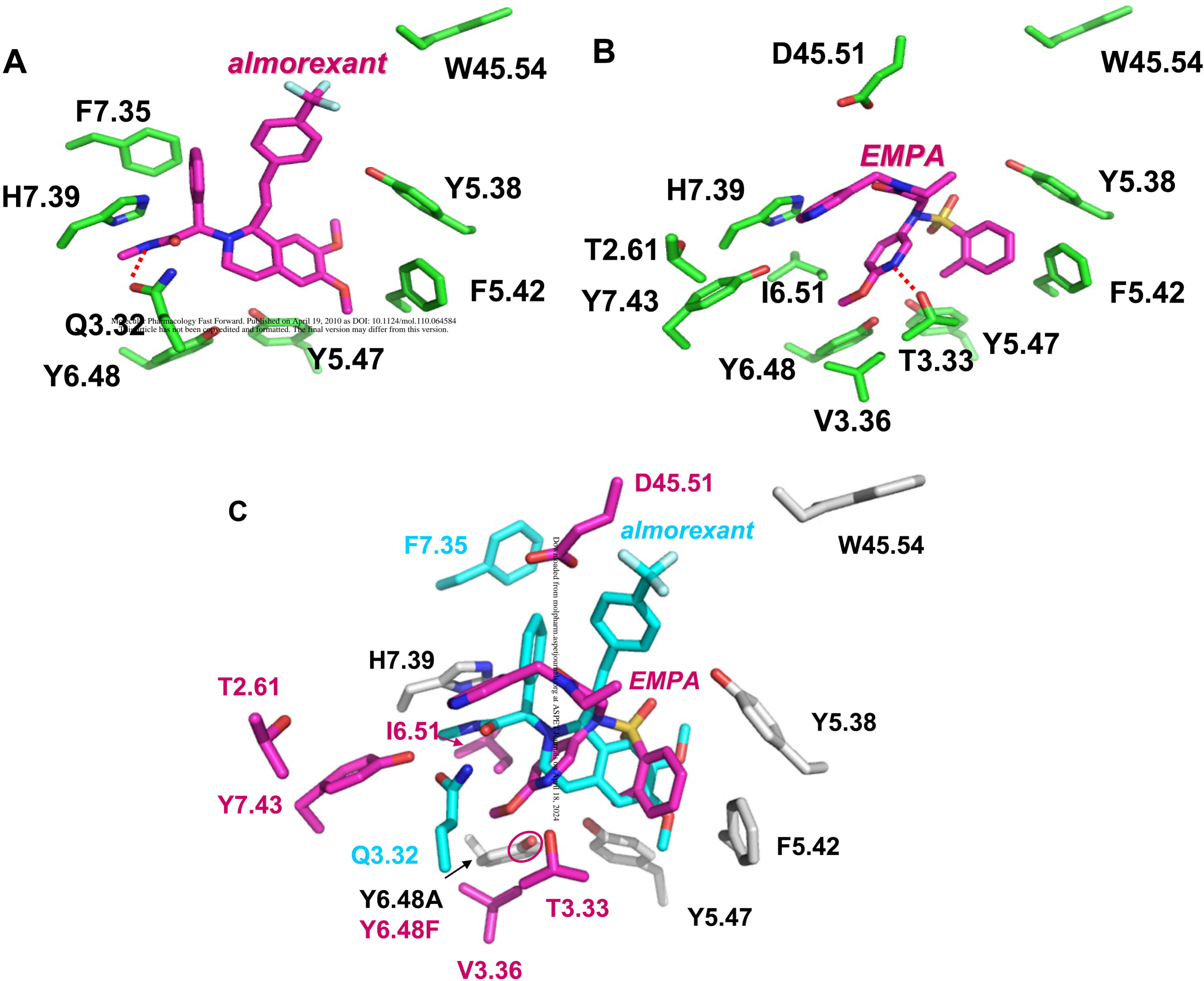


Fig. 6

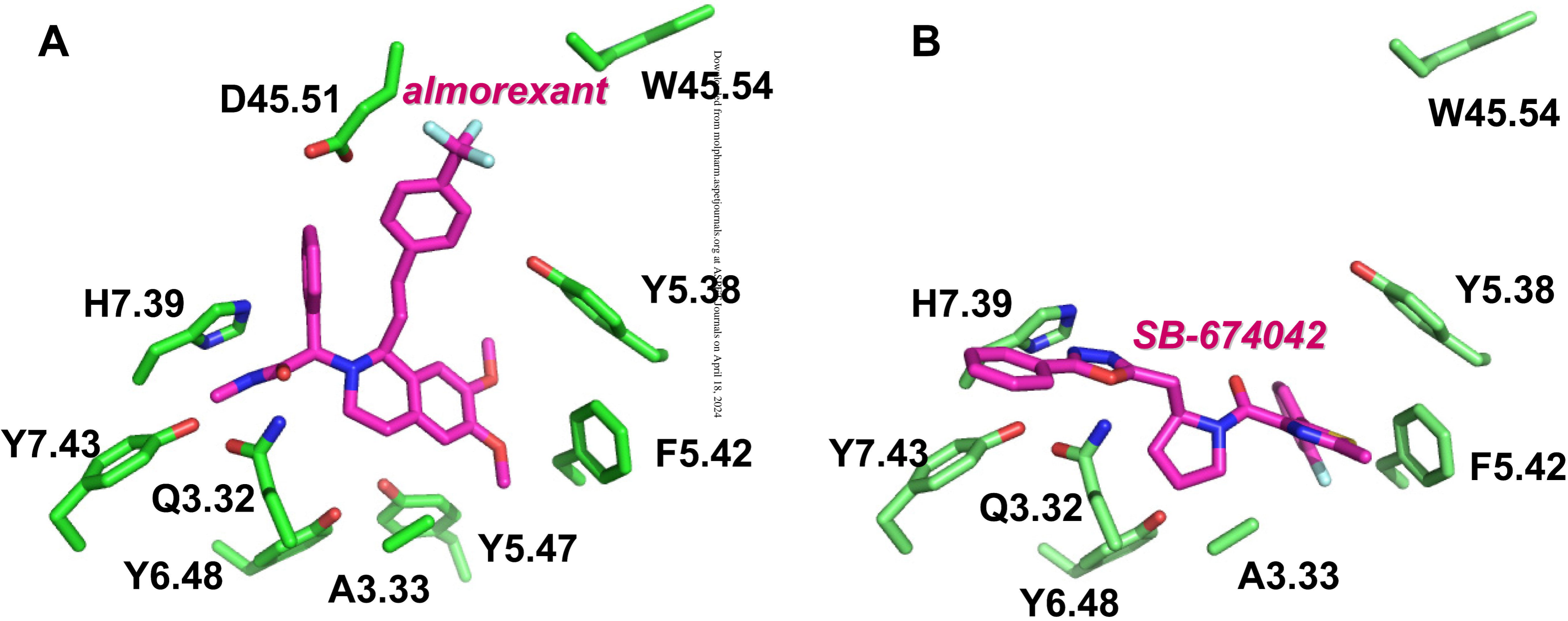


Fig. 7