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CONSTITUTIVELY ACTIVE MUTANTS OF THE HISTAMINE H_1 RECEPTOR SUGGEST A CONSERVED HYDROPHOBIC ASPARAGINE-CAGE THAT CONSTRAINS THE ACTIVATION OF CLASS A GPCRS

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RUNNING TITLE PAGE

a) Running title:

CAM H_1Rs reveal the role of residue 6.40 in GPCR activation

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d) List of nonstandard abbreviations:

CAM, constitutively-active mutant; GPCRs, G protein-coupled receptors; TM, transmembrane domain.

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ABSTRACT

The aim of this study was to create and characterize constitutively-active mutant (CAM) histamine H_1 receptors (H_1R) using random mutagenesis methodology to further investigate the activation process of the rhodopsin-like family of G protein-coupled receptors (GPCRs). This approach identified position 6.40 in TM 6 as a 'hot spot' since mutation of Ile6.40^{420} either to Glu, Gly, Ala, Arg, Lys, or Ser results in highly active CAM H_1Rs, for which almost no histamine-induced receptor activation response can be detected. The highly conserved hydrophobic amino acid at position 6.40 defines, in a computational model of the H_1R, the asparagine cage motif that restrains the side chain of Asn7.49 of the NPxxY motif towards TM 6 in the inactive state of the receptor. Mutation of the asparagine cage into Ala or Gly, removing the interfering bulky constraints, increases the constitutive activity of the receptor. The fact that the Ile6.40^{420}Arg/Lys/Glu mutant receptors are highly active CAM H_1Rs leads us to suggest that a positively charged residue, presumably the highly conserved Arg3.50 from the DRY motif, interacts in a direct or an indirect (through other side chains or/and internal water molecules) manner with the acidic Asp2.50··Asn7.49 pair for receptor activation.

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INTRODUCTION

GPCRs play a crucial role in many physiological functions (Kristiansen, 2004) and are major drug targets (Hopkins and Groom, 2002). The notion of constitutive, agonist-independent, signaling of GPCRs has fundamentally changed insights in receptor pharmacology. Receptor activity can be modulated by ligands that possess either negative (inverse agonists) or positive (agonists) intrinsic activity, whereas neutral antagonists lack intrinsic activity and only compete for GPCR binding sites (reviewed in Seifert and Wieland, 2006). Constitutive receptor activity may also be induced by mutations. Such constitutively-active mutant (CAM) GPCRs have been used to provide insight into the mechanism of receptor activation (Pardo et al., 2007; Smit et al., 2007).

It is now generally accepted that several highly conserved motifs, in the rhodopsin-like family of GPCRs, are key in the process of GPCR activation. First, a conserved hydrogen bond network linking Asp2.50 of the NLxxxD motif in transmembrane domain (TM) 2 with Trp6.48 of the CWxP motif in TM 6 maintains GPCRs in the inactive conformation (Jongejan et al., 2005; Li et al., 2004; Xu et al., 2005). This network of interactions constrains Trp6.48 in the inactive *gauche*$^+$ conformation, impeding its conformational transition toward the observed active *trans* conformation (Ruprecht et al., 2004). Second, Asn7.49 of the highly conserved NPxxY motif in TM 7 acts as an on/off switch by adopting alternative conformations in the inactive and active receptor states (Govaerts et al., 2001; Urizar et al., 2005). Asn7.49 is restrained towards TM 6 in the inactive *gauche*$^+$ conformation by molecular interactions that diverge among GPCR subfamilies (Urizar et al., 2005) or via a water molecule in rhodopsin (and possibly other receptors) (Okada et al., 2002). Upon receptor activation Asn7.49 adopts the *trans* conformation to interact with Asp2.50 in TM 2 (Urizar et al., 2005). And third, the ionic lock between Arg3.50 of the highly conserved DRY motif in TM3 with its adjacent Asp/Glu3.49 residue (Alewijnse et al., 2000; Ballesteros et al.,

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2001; Scheer et al., 1996) and an additional Asp/Glu6.30 amino acid in TM 6 (Alewijnse et al., 2000; Ballesteros et al., 2001; Scheer et al., 1996). These ionic interactions are disrupted during the process of receptor activation facilitating the movement of the cytoplasmic end of TM 6 and the conformational transition of Arg3.50 (Alewijnse et al., 2000; Ballesteros et al., 2001; Scheer et al., 1996).

The aim of this study was to create and characterize CAM histamine H_1 receptors (H_1Rs) through random mutagenesis to further investigate the activation process of the rhodopsin-like family of GPCRs. Mutant receptors were initially screened using the Receptor Selection and Amplification Technology (R-SAT) functional assay, an assay platform that has previously been successfully applied to identify for instance the G-protein-coupling domain of muscarinic receptors (Hill-Eubanks et al., 1996), as well as for the generation of CAM calcium-sensing (Jensen et al., 2000) and muscarinic (Spalding et al., 1997) receptors. Spalding *et al.* (1997) used the R-SAT procedure successfully to identify a face of TM6 of the muscarinic m5 receptor as a region to stabilize the inactive state and, therefore, as a hot spot for generating CAM GPCRs by random mutagenesis. Our initial functional R-SAT screen of randomly mutated H_1Rs resulted in the identification of several highly constitutively active mutant H_1Rs, which were further analyzed by assessing NF-κB activation in COS-7 cells as well as radioligand binding studies. In addition, rhodopsin-based molecular models of wild-type and mutant histamine H_1Rs were built to explore the mechanisms responsible for constitutive activity. We provide the first examples of CAM H_1Rs that harbor a mutation in the highly conserved hydrophobic amino acid residue 6.40 in TM 6. Furthermore, combining our observations with data from other GPCRs resulted in the identification of a putative hydrophobic cage for Asn7.49. This proposed Asn-cage is highly

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conserved in the family A GPCRs and appears to serve as an important constraint for GPCR activation.

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MATERIALS AND METHODS

Materials. Cell culture media, penicillin, and streptomycin were obtained from Life Technologies (Merelbeke, Belgium). Cyto-SF3 was obtained from Kemp laboratories (Frederick, MD.), [^{3}H]mepyramine (20 Ci/mmol) from PerkinElmer (Zaventem, Belgium). Doxepin hydrochloride, mepyramine (pyrilamine maleate), and tripelennamine hydrochloride were obtained from SigmaAldrich (Natick, MA). ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, and polyethyleneimine, were purchased from Sigma Chemical (St. Louis, MO). D-Luciferin was obtained from Duchefa Biochemie BV (Haarlem, The Netherlands), glycerol from Sigma-Aldrich Laborchemikalien (Seelze, Germany), and Triton X-100 from Fluka (Buchs, Switzerland). pNF-κB-Luc was obtained from Stratagene (La Jolla, USA), pSI from Promega (Madison, WI), the TOPO 2.1 vector from Invitrogen (Carlsbad, CA), Superfect from Qiagen (Dusseldorf, Germany), High-Fidelity Platinum Taq DNA Polymerase and High Fidelity buffer from Life Technologies (Rockville, MD), and Taq DNA polymerase from Boehringer Mannheim. Gifts of mianserin hydrochloride (Organon NV, The Netherlands), pcDEF$_3$ (Dr. J. Langer, Robert Wood Johnson Medical School, Piscataway, NJ), are greatly acknowledged.

Molecular Cloning. The human H_1R was cloned by PCR using the following oligodeoxynucleotides primers: 5'(5'-gct act aag tgg cca ctc atc acc caa gtc-3'), 3'(5'-caa cac aca ggc ctg cgg ccg cta ttt cct tg-3'). PCR conditions employed 100 ng (~125 pmol) of each primer, 250 uM dNTPs, 80 ng human genomic DNA, 2m M $MgSO_4$, 1x High Fidelity buffer and 1.75 units of High-Fidelity Platinum *Taq* DNA Polymerase. PCR reactions conditions were: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 60°C for 35 sec and 72°C for 1 min; 35 sec; followed by a final 10 min extension at 72°C. The resultant PCR product was subcloned

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into the TOPO 2.1 vector as per manufacturer's protocols, and subsequently subcloned into the mammalian expression vector pSI for R-SAT based functional studies.

Mutagenesis of the human H_1R gene and Isolation of CAM H_1Rs. Mutations were introduced into the human H_1R gene by PCR. Plasmid pSIhH_1R (Weiner et al., 2001) was used as template for all PCRs. The PCR primers were complementary to the H_1R sequence, except for the codon corresponding to the desired amino acid residue in the H_1R. Receptor genes containing mutations were constructed with a heterogeneous PCR primer that randomly introduced a combination of all four bases at the three positions of the codon that was to be mutated. Constitutively active mutant (CAM) H_1R genes were isolated by functional screening based on the ability of this mutant to activate growth of NIH 3T3 cells in the functional assay R-SAT in the absence of histamine, and the inhibition of agonist independent proliferative responses by 10 μM of the inverse H_1R agonist mepyramine (Bakker et al., 2000; Bakker et al., 2001). For each residue that was mutated in the H_1R 25 potential mutant cDNAs were tested in this way (except for I^{433}). Mutant H_1Rs exhibiting the desired phenotype, were subsequently sequenced to identify the amino acid substitution due to the mutation that was introduced via PCR.

Cell culture and transfection. COS-7 African green monkey kidney cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere in Dulbecco's modified essential media (DMEM) containing 2 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin and 5% (v/v) FCS. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described (Wieland et al., 1999; Bakker et al., 2000; Bakker et al., 2001). NIH-3T3 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 1% penicillin and streptomycin and 10% bovine calf serum and maintained at 37°C in a humidified 5%

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CO_2/95% air atmosphere. NIH-3T3 cells were transiently transfected using the Superfect transfection reagent following the manufacture's protocols. The total amount of DNA transfected was maintained constant by addition of either $pcDEF_3$ or $pcDNA_3$.

Receptor selection and amplification technology (R-SAT) assays. R-SAT assays were essentially performed as described previously (Weiner et al., 2001). Briefly, on day one NIH-3T3 cells were plated into 96 well cell culture plates at a density of 7500 cells/well. On day two cells were transfected with 25 ng/well of (mutant) H_1R DNA, with 20 ng/well of plasmid DNA encoding β-galactosidase. On day three, the media was replaced with DMEM supplemented with 1% penicillin and streptomycin, 2% Cyto-SF3 and varying drug concentrations. After five days of cell culture, media was removed and the cells were incubated in phosphate-buffered saline containing 3.5 mM *O*-nitrophenyl-β-D-galactopyranoside, and 0.5% Nonidet P-40 detergent. The 96-well plates were incubated at room temperature for up to 8h, and the resulting colorimetric reaction was measured by spectrophotometric analysis at 420 nm on an automated plate reader (Biotek Instruments Inc., Burlington, VT). Data were analysed by a non-linear, least squares curve-fitting procedure using Graphpad Prism® version 4 (GraphPad Software, Inc., San Diego, CA). All data shown are expressed as mean ± S.E.M..

Reporter-gene assays. COS-7 cells transiently co-transfected with pNFκB-Luc (125 μg/10^7 cells) and either $pcDEF_3hH_1R$ (25 μg/10^7 cells) were seeded in 96 well blackplates (Costar) in serum free culture medium and incubated with drugs. After 48 hrs, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μL/well luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100 and 2.6 μM DTT). After

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30 min luminescence was measured for 3 sec/well in a Victor^2 (Perkin Elmer). All data shown are expressed as mean ± S.E.M..

H_1R binding studies. Cells used for radioligand binding-studies were harvested 48h after transfection and homogenized in ice-cold H_1R-binding buffer (50 mM Na_2/K^+-phosphate buffer (pH 7.4)). The cell homogenates were incubated for 30 min at 25ºC in a total volume of 200 µL H_1R-binding buffer with ~1 nM [^{3}H]mepyramine. The non-specific binding was determined in the presence of 1 µM ketotifen. The incubations were stopped by rapid dilution with 3 mL ice-cold H_1R-binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 mL buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

Molecular models of wild-type and mutant H_1Rs. The previously reported 3D model of the H_1R was employed (Jongejan et al., 2005). Molecular models for the mutant H_1Rs were obtained as described in our previous manuscript (Jongejan et al., 2005). In the I6.40^{420}K, I6.40^{420}R, and I6.40^{420}S mutant H_1Rs the side chain of Asn*7.49* is modeled in the proposed active *trans* conformation and is interacting with Asp2.50^{73} (Urizar et al., 2005). The accessible surface of the O_δ atom of Asn7.49^{464}, in the inactive conformation, was obtained with the NACCESS program (Hubbart and Thornton, University College London).

Analytical methods. All data shown are expressed as mean ± S.E.M. Protein concentrations were determined according to Bradford (Bradford, 1976), using BSA as a standard. Data from radioligand binding assays and functional assays data were evaluated by a non-linear,

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least squares curve-fitting procedure using Graphpad Prism® version 4 (GraphPad Software, Inc., San Diego, CA).

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RESULTS

Generation and identification of CAM H_1Rs. A variety of amino acids in the human histamine H_1R were selected for mutagenesis (Figure 1), these include amino acids present in the highly conserved DRY motif in TM 3, the top of TM 5, and of TM 6. These regions of the hH_1R were selected based on the well documented role of both the DRY motif and TM 6 in activation of class A GPCRs (reviewed in Gether et al., 2002; Flanagan, 2005). In contrast, the top of TM 5 was chosen for its role in interaction with antihistamines (Wieland et al., 1999), which are currently known as inverse H_1R agonists (Bakker et al., 2000; Bakker et al., 2001), and was therefore postulated to be involved in H_1R inactivation. The random saturation mutagenesis was performed via degenerate PCR as described in the Materials and Methods section above. A fair number of mutant receptor cDNAs were generated by this method. Yet, the wild-type receptor is clearly preferably generated in this approach, as it is largely present in each pool of cDNAs that was generated. Undoubtedly, our approach did not result in the generation of all possible mutant H_1Rs at the selected amino acids that were included in the mutagenesis approach. Yet, the degenerate PCR approach resulted in a number of arbitrary H_1R mutants that were evaluated for their signaling properties in our quest for CAM H_1Rs.

A variety of assays have been utilized to demonstrate constitutive H_1R activity, since our initial demonstration of this phenomenon for the H_1R (Bakker et al., 2000). These assays include the measurements of inositol phosphates (Bakker et al., 2000), cell shape (Yu et al., 2006), as well as the activities of a variety of reporter genes (Bakker et al., 2001; Wu et al., 2004; Smit et al., 2002; Weiner et al., 2001). As we aimed at the generation and characterization of a large number of mutant receptors, we selected R-SAT functional assays for the initial characterization, since the R-SAT assay is extremely robust, allows high-throughput and yields a similar H_1R pharmacological profile for a wide variety of inverse

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H_1R agonists compared to the more standard NF-κB reporter-gene assay (see Bakker et al., 2007).

The initial functional screen using mutated H_1Rs, consisting of the evaluation of H_1R signaling under both basal conditions and after incubation with 10μM of the inverse H_1R agonist mepyramine, identified several residues in the H_1R that upon mutation could yield CAM H_1Rs, including some receptors with mutations in the DRY motif. However, three residues were identified in TM 6 (Ile6.40^{420}, Ala6.43^{423}, or Ile6.45^{425}) that are very prone to yield highly activated CAM H_1Rs upon mutation (Figure 1). From our experimental approach especially mutant H_1Rs with a mutation at position 6.40^{420} jump out and therefore were analyzed in more detail. All potential mutant H_1Rs harboring a mutation at this position were subsequently sequenced and characterized by performing NF-κB reporter gene assays.

Functional evaluation of CAM H_1Rs. Of the evaluated mutants the H_1Rs mutated at position 6.40^{420} exhibit the largest degree of constitutive signaling (Figures 1 and 2). Mutation of Ile6.40^{420} either to Glu, Gly, Ala, Arg, Lys, or Ser results in highly active CAM H_1Rs, for which almost no additional histamine-induced receptor activation can be detected. Thus, these receptors appear to be fully activated due to their respective mutations (Figure 2). Among the mutations we have analyzed, the I6.40^{420}F mutation constitutes a unique substitution at this position exhibiting a level of constitutive activity comparable to that of the wild-type H_1R (Figure 2 and Table 1).

Histamine and a variety of inverse H_1R agonists were subsequently assayed for their potency and intrinsic activity (α) for the mutant H_1R6.40^{420} receptors (Table 1). The ability of histamine to activate the mutant H_1R6.40^{420} receptors greatly varies depending on the mutant. Whereas the H_1R I6.40^{420}F mutant receptor is activated by histamine similarly to the wild-type receptor, for other mutant receptors, notably the H_1R I6.40^{420}R, H_1R I6.40^{420}E, H_1R

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I6.40^{420}K, and H_1R I6.40^{420}S mutant receptors, hardly any histamine-induced activation could be detected (see also Figure 3). The pharmacological profiles of the evaluated inverse H_1R agonists also vary depending on the mutation in the H_1R receptor. Whereas α for most tested inverse H_1R agonists remains constant for the mutant H_1R6.40^{420} receptors, the α values for mepyramine, d-chlorpheniramine, and mirtazepine, exhibit a mutant H_1R6.40^{420} receptor-dependent variation with a general tendency of becoming weaker partial inverse H_1R agonists for the mutant H_1R6.40^{420} receptors. The potencies of the inverse H_1R agonists obtained for the mutant H_1R6.40^{420} receptors, on the other hand, indicate that the potencies of cyproheptadine, astemizole, and loratadine are reduced to a lesser extent than that observed for the other tested inverse H_1R agonists, while in comparison those of doxepine and d-chlorpheniramine are reduced to a greater extent (Table 1). Figure 3 illustrates the differences observed in the pharmacological profiles of histamine and cyproheptadine for the various mutant H_1R6.40^{420} receptors as well as the differences observed in the basal activity of the mutant receptors.

As shown in Figure 4, a linear correlation is found between the pIC_{50} values of the inverse H_1R agonists obtained for the wild-type H_1R and for the H_1Rs mutated at position 6.40, and the slope of the correlation appears not to be influenced by the mutations. The intercept of the correlation, however, is clearly rightward shifted for the CAM H_1Rs compared to the H_1R6.40^{420}F receptor which exhibits a constitutive activity comparable to that of the wild-type H_1R, suggesting higher concentrations of inverse H_1R agonist are required to silence the constitutive activity of the identified highly active CAM H_1Rs.

Radioligand binding studies of Ile6.40^{420} mutants. We evaluated the binding characteristics of [^{3}H]mepyramine to wild-type and mutant H_1Rs upon expression in COS-7 cells. The lower potency of mepyramine observed for the mutant receptors in the functional studies suggests

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saturation binding assays not to be feasible for characterization of all mutant $H_1R6.40^{420}$ receptors as high amounts of radioligand would be required. We therefore performed homologous displacement studies to determine the pK_b value of [^{3}H]mepyramine for the mutant $H_1R6.40^{420}$ receptors as well as to estimate their respective expression levels (B_{max} values) upon heterologous expression (Table 2).

Analysis of the binding data indicates the expression levels of the mutant $H_1R6.40^{420}$ receptors to be considerable lower than that of the wild-type H_1R. Especially the mutant $H_1RI6.40^{420}E$ and $H_1RI6.40^{420}K$ receptors, and to a lesser extent $H_1RI6.40^{420}S$ and $H_1RI6.40^{420}A$, have expression levels that are about 10 and 25 percent of the expression level achieved for the wild-type H_1R, respectively. The mutant $H_1RI6.40^{420}F$, $H_1RI6.40^{420}R$ and $H_1RI6.40^{420}G$ receptors reach expression levels of about 40 to 50 percent of that observed for the wild-type H_1R. Except for the mutant $H_1R6.40^{420}S$ and $H_1R6.40^{420}G$ receptors, where [^{3}H]mepyramine binds about three fold less potent than to the wild-type H_1R, the obtained pK_b values obtained for [^{3}H]mepyramine binding to the mutant $H_1R6.40^{420}$ receptors are rather similar to the values for the wild type H_1R. We subsequently determined the affinities of histamine for the mutant $H_1R6.40^{420}$ receptors. Whereas both $H_1R6.40^{420}F$ and $H_1R6.40^{420}S$ receptors exhibit affinities for histamine equal to the affinity of histamine for the wild-type H_1R, the other mutant $H_1R6.40^{420}$ receptors exhibit substantially higher affinity for histamine than the wild-type H_1R (see Table 2). These data indicate that for some, but not all ($H_1R6.40^{420}S$), CAM H_1Rs the affinity towards the endogenous agonist is increased.

Molecular modeling of Ile6.40^{420} mutant receptors. Ile6.40^{420} is located between Arg3.50^{125} of the DRY motif in TM 3 and Asn7.49^{464} of the NPxxY motif in TM 7 (Figure 5). Consequently, the observed effects of the different Ile6.40^{420} mutations on the constitutive H_1R activity probably can be explained by modification of any of these key motifs. Insertion

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of a negatively charged side chain at this locus in the I6.40^{420}E mutation facilitates the interaction with Arg3.50^{125} (Figure 6A). This is in agreement with previous suggestions that Arg3.50^{125} performs a conformational change, during the process of receptor activation, from being engaged in the ionic lock with the contiguous Asp3.49^{124} and Glu6.30^{410} in the inactive state (Ballesteros et al., 2001) to point towards the protein core (Ballesteros et al., 1998). Based on our modeling and mutational data we propose that Arg3.50^{125} elicits the conformational change from the inactive χ_1:*trans*, χ_2:*gauche*$^-$, χ_3:*gauche*$^+$, χ_4:*gauche*$^-$ (Figure 5A) to the active χ_1:*gauche*$^+$, χ_2:*trans*, χ_3:*trans*, χ_4:*trans* conformations (Figures 5B and 6A). In contrast, addition of the positively charged side chain of either Arg or Lys in the I6.40^{420}R or I6.40^{420}K mutant receptors modify Asn7.49^{464} of the NPxxY motif. We have recently proposed that Asn7.49^{464} changes its conformation from pointing towards TM 6 in the inactive *gauche*$^+$ conformation, to interact with Asp2.50^{73} in the active *trans* conformation (Govaerts et al., 2001; Urizar et al., 2005). The formation of the Asp2.50^{73}··Asn7.49^{464} pair conveys acidic properties to the Asn7.49^{464} side chain (Urizar et al., 2005). Thus, either Arg or Lys in the I6.40^{420}R or I6.40^{420}K mutant receptors, respectively, interacts with the acidic O_δ atom of Asn7.49^{464} (Figures 6B and 6C). Similarly, Ser6.40^{420} in the I6.40^{420}S mutant receptor stabilizes this active conformation of Asn7.49^{464} by forming a hydrogen bond interaction between both side chains (not shown). During the preparation of this manuscript Proneth *et al.* suggested the potential involvement of a rearrangement of hydrogen bonding networks between 6.40 in TM6 and the DRY and NPxxY motifs, as the explanation for the observed constitutive activity of the hMC4R L6.40^{250}Q mutant (Proneth et al., 2006). Their findings corroborate and strengthen our findings on the proposed role of residue 6.40 in GPCR activation.

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Notably, mutation of Ile6.40^{420} to either Ala or Gly also causes a significant increase in the constitutive activity of the resultant mutant H_1Rs, which is comparable to the magnitude of constitutive activity of the mutant H_1R I6.40^{420}R receptor. Thus, the side chain of Ile6.40^{420} plays an important role in maintaining an inactive state of the receptor. We hypothesize that the bulky and β-branched Ile6.40^{420} buries the O_δ atom of Asn7.49^{464} from the intracellular counterpart (i.e. a positive charge, see discussion). The accessible surface (see Materials and Methods) of the O_δ atom, in the inactive conformation of Asn7.49^{464} (Figure 5A), is 0.5 Å^2 in the wild-type receptor and increases to 6.5 Å^2 or 8.5 Å^2 in the mutation of Ile6.40^{420} to Ala or Gly, respectively. Thus, removal of the Ile6.40 side-chain through mutation into Ala or Gly renders the O_δ atom accessible, hence facilitating the conformational transition of Asn7.49^{464} towards Asp2.50^{73}.

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DISCUSSION

Activation of GPCRs is thought to involve disruption of intramolecular interactions that stabilize their inactive conformations. Such disruptions are induced by agonists but may also be induced upon mutation of the receptor. Disruption of these stabilizing interactions has a large energetical cost that must be compensated by the formation of new stabilizing interactions in the resulting active state of the receptor. In the present work, based on our pharmacological data for CAM H_1Rs that were obtained through a random mutagenesis approach, complemented with a molecular modeling approach, we propose stabilizing interactions acting both at the inactive as well as the active states of the H_1R.

On the basis of both our observations and previous work by others, we suggest Asp2.50 is involved in maintaining Trp6.48 pointing towards TM 7 in the inactive receptor state (Figure 5A) through a conserved hydrogen bond network (Pardo et al., 2007; Smit et al., 2007). This network of interactions impedes the reported conformational transition of Trp6.48 from pointing towards TM7, in inactive rhodopsin, to pointing towards TM5, in metarhodopsin I (Ruprecht et al., 2004). Binding of agonists to the extracellular domain of the receptor modifies the conformation of Trp6.48 towards TM5 through a specific hydrogen bond interaction (López-Rodríguez et al., 2005). The rotamer toggle switch of Trp6.48 occurs in a concerted manner with the side chain at position 3.36 (Figure 5B) (Urizar et al., 2005; Jongejan et al., 2005). These conformational transitions of Ser3.36 and Trp6.48 have two effects in the structure of the helical bundle. First, it disrupts the conserved hydrogen bond network between Trp6.48 and Asp2.50, triggering the conformational transition of Asn7.49 towards Asp2.50 (Figure 5B) (Urizar et al., 2005; Jongejan et al., 2005). Second, it decreases the proline-kink of TM 6 leading to movement of the cytoplasmic end of TM 6 away from

TM 3, disrupting the ionic interaction between Arg3.50 with the nearby negatively charged side chains at positions 3.49 and 6.30 (Shi et al., 2002).

The mutant H_1Rs that we generated in this study have been largely characterized upon transient expression in COS-7 cells. We observed differences in expression levels between the wild-type and mutant receptors that could be due, at least in part, to an increased instability of CAM receptors, alike our previous findings for CAM H_2Rs having mutations in the DRY motif (Alewijnse et al., 2000). However, the instability of the receptor protein or its expression does not appear to be solely correlated with the level of its constitutive activity. The $I6.40^{420}F$ mutant shows a level of constitutive activity comparable to the wild-type receptor while having a reduced expression level. Therefore, also yet unidentified mechanisms contribute to the overall lower expression levels of these mutant receptors.

In this manuscript we have shown that the physico-chemical properties of the amino acid side chain at position 6.40 in TM 6 are key in the process of receptor activation since it is located midway between the NPxxY motif and the ionic lock (Figure 5). Statistical analysis shows that GPCRs do not contain either positive (Arg, Lys) or negative (Asp, Glu) side chains at this locus (Mirzadegan et al., 2003). I6.40R or I6.40K mutant receptors are highly active CAM H_1Rs with almost no additional histamine-induced activation (Figure 3). Thus, Arg6.40 or Lys6.40 are not observed in the rhodopsin family of GPCRs because a positively charged residue at this position would induce constitutive receptor activation by triggering the conformational change of Asn7.49 towards Asp2.50 (Figures 6B and 6C). Remarkably, insertion of a negatively charged residue (the I6.40E mutant receptor) at this 6.40 position also induces histamine H_1R constitutive activity (Figure 3). In contrast to I6.40R or I6.40K, the I6.40E mutant receptor disrupts the ionic lock between the cytoplasmic ends of TM 3 and

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6 by triggering the conformational change of Arg3.50 towards the protein core (Figure 6A). Notably, Arg6.40 or Lys6.40 in the I6.40R or I6.40K mutant receptors create an intracellular positive field, which is similar to the positive field created by Arg3.50 in the I6.40E mutation because of their similar position (compare Figure 6A with 6B and 6C). Thus, the fact that insertion of either a negatively or positively charged side chain at the 6.40 position enhances constitutive H_1R activity, suggests that the creation of this positive electrostatics between TMs 3, 6, and 7 is an important determinant for receptor activation. These findings let us to propose that the highly conserved Arg3.50 of the (D/E)RY motif at the bottom of TM 3 performs a conformational change from being engaged in the ionic lock with the contiguous Asp3.49 and Glu6.30 in the inactive state (Figure 5A) to interact with the Asp2.50··Asn7.49 pair in the active state of wild-type H_1R (Figure 5B). Because the distance between these two motifs, as observed in the crystal structure of rhodopsin, is large, we suggest either a direct interaction if rigid-body movements of the TM helices occur or an indirect interaction through other side chains or/and internal water molecules. This proposal is in agreement with previous findings: *i*) Addition of the N7.49A mutation, to the highly constitutively active E3.49A or E3.49Q mutant receptors, which releases Arg3.50 from the ionic lock, dramatically lowers the constitutive activity of the double mutants to levels of wild-type for the thyrotropin receptor (Claeysen et al., 2002). Thus, the release of the Arg3.50 side chain by mutation of Glu3.49 is only stable in the presence of Asn7.49. *ii*) Similarly, the E3.49Q mutation in rhodopsin favours the formation of metharhopsin II, whereas the double E3.49Q/N7.49A mutation decreases the relative activation rate (Fritze et al., 2003). These data led the authors to propose a clear interplay between Asn7.49 of the NPxxY motif and the D(E)RY motif. *iii)* Finally, the D2.50N point mutation in the M_3 muscarinic receptor abolishes agonist-induced receptor/G-protein coupling in yeast; whereas the D2.50N/R3.50M and D2.50N/R3.50W double mutant receptors showed E_{max} values similar to the wild-type

receptor (Li et al., 2005). The authors proposed a conformational link between Asp2.50 and Arg3.50, which is critical for receptor activation.

Interestingly, the 6.40 position appears to be a highly conserved hydrophobic residue in the rhodopsin-like family of GPCRs (L, 14%; V, 42%; I, 28%; M, 5%) (Mirzadegan et al., 2003). Removal of this hydrophobic and bulky side chain (mutation to Ala or Gly) induces constitutive activity comparable in magnitude to addition of a positively (mutation to Arg or Lys) or a negatively (mutation to Glu) charged side chain at this locus (Figures 1 and 2). The mechanistic role of Ile6.40 probably is to restrain Asn7.49 in the inactive *gauche*+ conformation. Thus, alike to the arginine cage (Ballesteros et al., 1998), we would like to propose that Asn7.49 is also located in a cage that restrains its conformation towards TM 6 in the inactive state. The asparagine cage is formed, in addition to this hydrophobic side chain at position 6.40, by the hydrophobic Leu2.46 of the NLxxxD motif in TM 2 of the H_1R (Figure 5). Removal (mutation to Ala or Gly) of the bulky and β- or γ-branched hydrophobic side chain at positions 2.46 in rhodopsin (Madabushi et al., 2004) or the thyrotropin receptor (Urizar et al., 2005); or 6.40 in rhodopsin (Han et al., 1996), the serotonin $5HT_{2A}$ receptor (Shapiro et al., 2002), and the H_1R (Figures 1 and 2) induces constitutive activity. These considerations make us propose that the suggested constraining action of the Asn-cage in the H_1R might be more widespread in the family of class A GPCRs.

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FOOTNOTES

Unnumbered footnotes

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LEGENDS FOR FIGURES

Figure 1. Functional R-SAT screen for the identification of CAM H_1Rs. Selected amino acids in either the DRY motif (1), TM 5 (2), or in TM 6 (3) of the human histamine H_1R were mutated by PCR and subsequently screened for their constitutive activity. NIH-3T3 cells were transiently transfected with cDNAs encoding a potential mutant H_1R and assayed for constitutive H_1R activity. Data in the graph is plotted as the basal signal/observed signal in the presence of 10 μM of the inverse H_1R agonist mepyramine (Bakker et al., 2000; Bakker et al., 2001). The dotted lines indicate the minimal and maximal effect obtained for the wild-type H_1R under these assay conditions. Of the amino acids selected for mutagenesis, only a limited number of amino acid residues appear to be ´hot spots´ for the creation of CAM H_1Rs: Ile6.40^{420}, Ala6.43^{423}, and Ile6.45^{425}, while also the random mutation of F6.54^{434} and F6.55^{435} may yield CAM H_1Rs. All these residues are located in TM 6.

Figure 2. Effects of the expression of the various mutant H_1Rs that are mutated at Ile6.40^{420} in COS-7 cells on the basal activation of NF-κB. The basal activation of NF-κB by the wild-type H_1R that is observed under the same experimental conditions is given as a comparision. Data shown is the average of five independent experiments, each performed in triplicate, and is expressed as the percentage of wild-type H_1R-mediated basal NF-κB activation (WT on Y-axis represents 100%).

Figure 3. Effects of histaminergic ligands on mutant H_1R-mediated activation of NF-κB. Modulation of NF-κB activation by the various isolated mutant H_1Rs that are mutated at Ile6.40^{420} by the H_1R agonist histamine (●) and the inhibition of constitutive NF-κB activation by the inverse H_1R agonist cyproheptadine (○). Representative concentration

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response curves are shown. The maximum effect observed for histamine was set to 100% stimulation.

Figure 4. Correlation graphs of the potencies of various histaminergic ligands for the wild-type H_1R *versus* the Ile6.40^{420} mutant H_1Rs. The potencies of various inverse H_1R agonists (●) to mediate the inhibition of constitutive wild-type or mutant H_1R-induced activation of NF-κB are plotted, as well as the potency of histamine to induce wild-type and mutant H_1R-mediated NF-κB activation (○); see also Table 1. The dotted lines represent the 95% confidence bands of the best-fit line; the H_1R agonist histamine was not included in the fitting of the inverse H_1R agonist data.

Figure 5. (A) Computational model of the histamine H_1R in the inactive state showing the local environment of Ile6.40^{420}. The hydrogen bond network linking Asp2.50^{73} and Trp6.48^{428}; the water-mediated inter-helical interaction between Asn7.49^{464} and the backbone carbonyl at position 6.40; and the ionic interaction between Arg3.50^{125} and Asp3.49^{124} and Glu6.30^{410} are shown. (B) Schematic representation of the conformational changes of the Ser3.36^{111}/Trp6.48^{428} concerted rotamer toggle switch; the conformational transition of Asn7.49^{464} towards Asp2.50^{73}; and the conformational change of Arg3.50^{125} towards the protein core, during the process of histamine H_1R activation. This modelling exercise only aims at exploring these localized rotamer changes, which correspond to early stages of the activation process when side chain relocations have not yet been translated into major conformational changes of TM segments (Ruprecht et al., 2004), probably through a structural reorganization of the highly conserved proline-induced distortions. Structural water molecules 1a and 1c that mediate inter-helical interactions are also shown (Pardo et al., 2007; Smit et al., 2007). Only polar hydrogens are depicted for clarity. The color code for the α-

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carbon ribbons are goldenrod (TM 2), dark red (TM 3) orange (TM 6), and blue (TM 7).

Figure 6. Computational model of (*A*) I6.40^{420}E, (*B*) I6.40^{420}R, and (*C*) I6.40^{420}K mutant receptors. (A) Glu6.40^{420} in the I6.40^{420}E mutant receptor triggers the conformational change of Arg3.50^{125} from being engaged in the ionic lock with the contiguous Asp3.49^{124} and Glu6.30^{410} in the inactive state to point towards the protein core. It is not possible to determine the conformation of Asn7.49^{464} in this mutant receptor, thus, it has been modeled arbitrarily as in the inactive state. (B, C) Arg6.40^{420} and Lys6.40^{420} in the I6.40^{420}R or I6.40^{420}K mutant receptors trigger the conformational change of Asn7.49^{464} towards Asp2.50^{73}. It is not possible to determine the conformation of Arg3.50^{125} in these mutant receptors, thus, it has been modeled arbitrarily engaged in the ionic lock as in the inactive state. Only polar hydrogens are depicted for clarity. The color code for the α-carbon ribbons are goldenrod (TM 2), dark red (TM 3), orange (TM 6), and blue (TM 7).

Table 1. Pharmacological characterization of the mutant H_1Rs obtained at the $I6.40^{420}$ position by NF-κB reporter gene assays. Assayed are the agonist histamine yielding a positive intrinsic activity (α) set to 1.0, and a variety of inverse H_1R agonists. For each mutant H_1R the intrinsic activities of the inverse H_1R agonists are related to the inverse H_1R agonist yielding the greatest inhibition of mutant H_1R-mediated basal NF-κB activation which was set to -1.0 by definition. Data are presented as the mean of the indicated number of separate experiments (n), each of which was performed in triplicate, the S.E.M. values for the pEC_{50} values are ≤ 0.1, unless indicated otherwise.

	H_1R WT			H_1R $I6.40^{420}$K			H_1R $I6.40^{420}$F			H_1R $I6.40^{420}$S			H_1R $I6.40^{420}$A			H_1R $I6.40^{420}$R			H_1R $I6.40^{420}$G			H_1R $I6.40^{420}$E		
	pEC_{50}	α	n	pEC_{50}	α	n	pEC_{50}	α	n	pEC_{50}	α	n	pEC_{50}	α	n	pEC_{50}	α	n	pEC_{50}	α	n	pEC_{50}	α	n
Histamine	6.8	1.0	89	6.6[e]	1.0	2	6.6	1.0	8	NE			6.7[b]	1.0	5	6.1[c]	1.0	7	7.1[b]	1.0	5	7.4[a]	1.0	4
Mepyramine	7.9	-0.9	89	6.2[a]	-0.6	6	8.4[a]	-0.6	4	6.5[b]	-0.6	4	6.5[a]	-0.6	7	6.5[b]	-0.6	5	6.7[a]	-0.7	3	5.3[c]	-0.8	3
Cyproheptadine	8.5	-0.9	12	7.1	-1.0	6	8.6[a]	-0.9	4	8.1[a]	-1.0	7	7.4	-1.0	7	7.0	-1.0	7	7.5	-1.0	7	7.6	-1.0	7
Ketotifen	9.2	-0.9	10	7.8[a]	-0.8	5	9.4	-0.8	3	8.3	-0.9	3	7.8[a]	-0.9	6	7.7[a]	-0.9	3	8.2[a]	-0.9	3	8.1	-0.9	3
Doxepin	9.3	-0.9	12	7.2[a]	-0.7	3	8.7	-0.9	3	7.6[a]	-0.9	3	7.2[d]	-0.8	3	6.9[a]	-0.7	3	7.5	-0.9	3	7.3[b]	-0.8	3
Mianserin	8.8	-0.9	9	6.9	-0.9	5	8.3[a]	-0.9	3	7.8	-0.9	5	7.5[a]	-1.0	5	6.9	-0.9	5	7.7	-1.0	5	7.6[a]	-1.0	5
Tripelennamine	7.4	-0.8	11	5.7[a]	-0.5	7	7.2[a]	-0.8	3	5.9	-0.8	4	5.3[a]	-0.7	7	5.4	-0.8	3	5.8[a]	-0.8	4	ND		
d-chlorpheniramine	7.8	-0.9	7	5.5[b]	-0.7	5	8.0[a]	-0.6	3	6.0[a]	-0.7	3	6.1[a]	-0.6	3	6.0[a]	-0.7	3	6.3[a]	-0.7	3	ND		
Mirtazepine	9.2[a]	-0.9	3	7.6[c]	-0.6	3	9.1	-0.7	3	8.2[c]	-0.8	3	8.3[b]	-0.7	3	7.8	-0.6	3	7.6[a]	-0.6	3	ND		
Triprolidine	8.3	-0.7	11	6.1	-0.8	3	8.6[a]	-0.6	3	6.7[a]	-0.9	3	6.8[b]	-0.6	3	6.4	-0.8	3	6.7	-0.8	3	ND		
Levoclabastine	8.0	-1.0	14	6.3	-0.8	3	7.4[a]	-0.9	4	6.8[b]	-0.9	3	6.4[b]	-0.8	3	6.3	-0.8	3	6.5	-0.9	3	6.3	-1.0	3
Astemizole	8.3[a]	-1.0	7	7.3	-1.0	3	7.9[b]	-1.0	3	7.5	-1.0	3	7.5[a]	-0.9	4	7.5	-1.0	3	7.5	-1.0	3	8.1	-0.5	3
loratadine[f]	6.4[a]	-1.0	10	5.4	-0.9	5	5.4	-1.3	3	5.4	-1.0	3	5.4	-0.9	6	5.3	-1.1	3	5.2	-1.0	3	5.2	-1.2	3

[a] SEM ≤ 0.2; [b] SEM ≤ 0.3; [c] SEM ≤ 0.4; [d] SEM ≤ 0.5; [e] SEM ≤ 0.6; [f] Loratadine was not taken as a reference full inverse agonist with an intrinsic activity (α) of -1, due to its previously reported inhibition of non-H_1R mediated NF-κB activation in this assay (Bakker et al., 2001).
ND not determined
NE could not be determined

Table 2. Expression levels of the various Ile6.40^{420} mutant H_1Rs upon transfection of COS-7 cells (B_{max} values), their affinity for mepyramine as determined by homologous [^{3}H]mepyramine displacement studies (pK_b values) and their subsequently determined affinities (pK_i values) for histamine. Data are presented as means ± S.E.M. of at least three separate experiments, each performed in triplicate.

Receptor	B_{max} (pmol/mg protein)	pK_b [^{3}H]mepyramine	pK_i histamine
H_1R WT	44 ± 11	8.0 ± 0.1	6.0 ± 0.4
H_1R I6.40^{420}K	5 ± 1	8.2 ± 0.1	7.4 ± 0.1
H_1R I6.40^{420}F	23 ± 1	8.1 ± 0.1	5.9 ± 0.3
H_1R I6.40^{420}S	10 ± 2	7.5 ± 0.1	6.0 ± 0.1
H_1R I6.40^{420}A	11 ± 1	8.2 ± 0.1	6.9 ± 0.2
H_1R I6.40^{420}R	18 ± 1	7.7 ± 0.1	7.4 ± 0.1
H_1R I6.40^{420}G	18 ± 5	7.6 ± 0.1	7.2 ± 0.1
H_1R I6.40^{420}E	4 ± 1	7.9 ± 0.1	6.7 ± 0.1

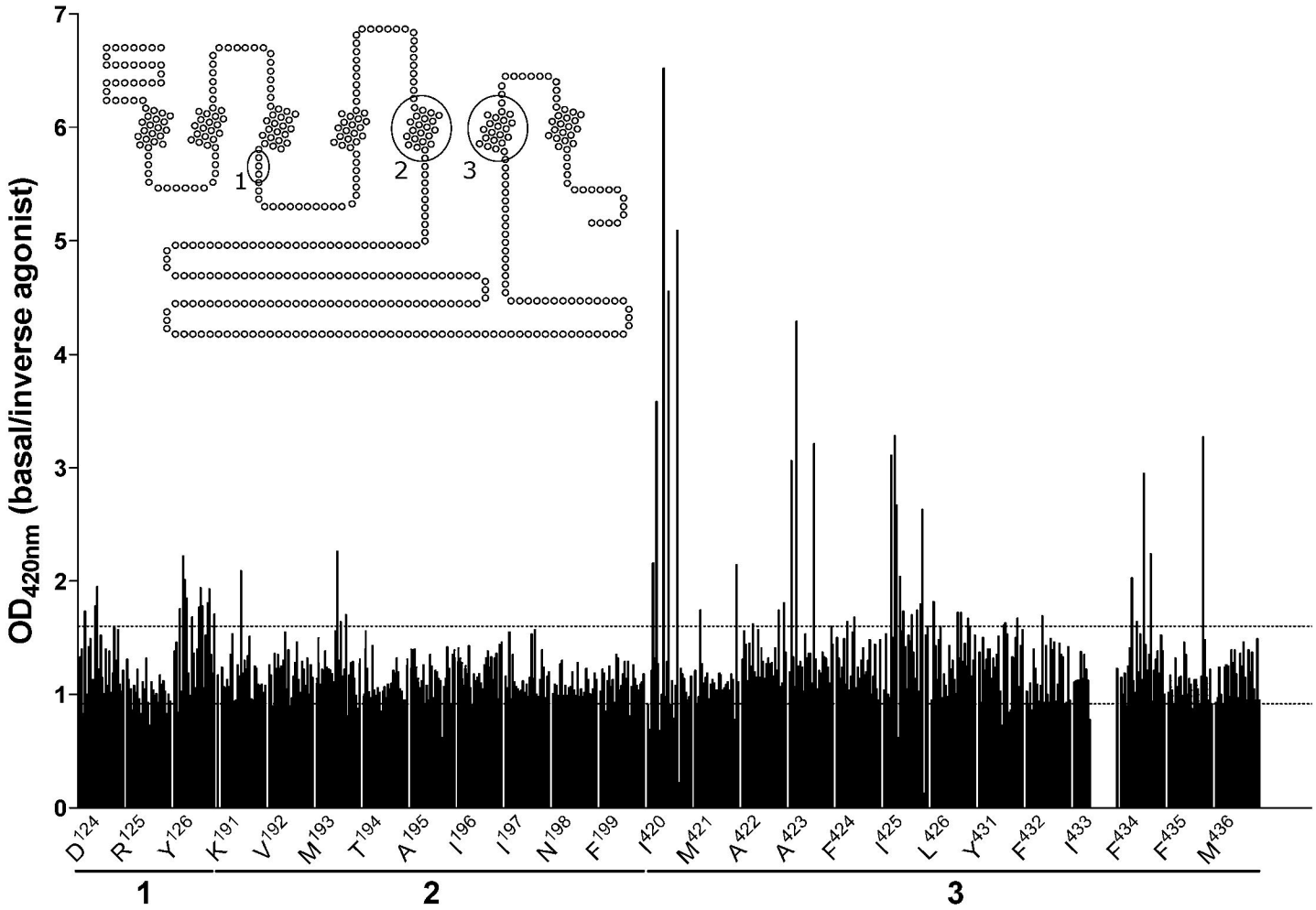

Figure 1

Molecular Pharmacology Fast Forward. Published on October 24, 2007 as DOI: 10.1124/mol.107.038547
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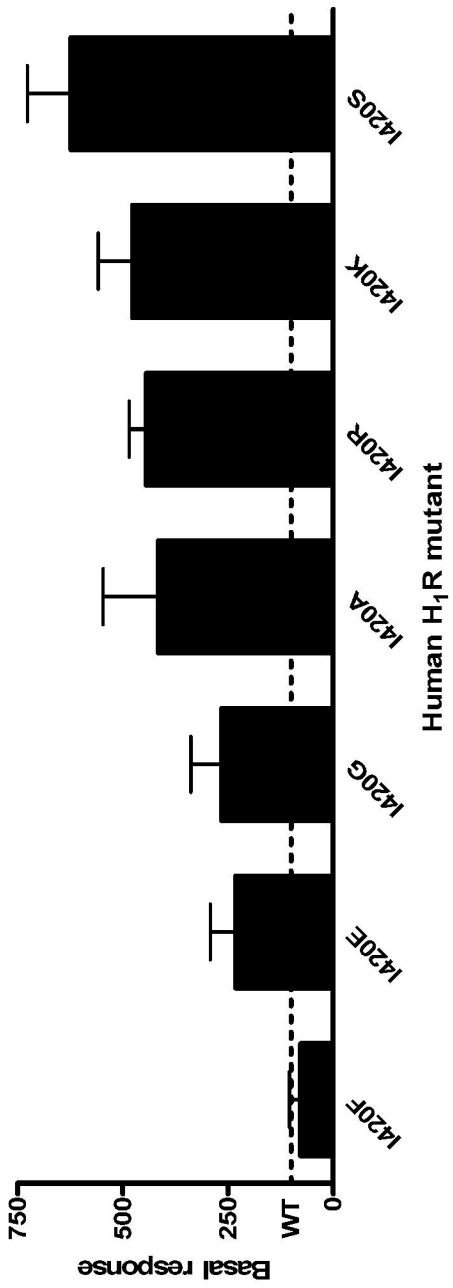

Figure 2

Molecular Pharmacology Fast Forward. Published on October 24, 2007 as DOI: 10.1124/mol.107.038547
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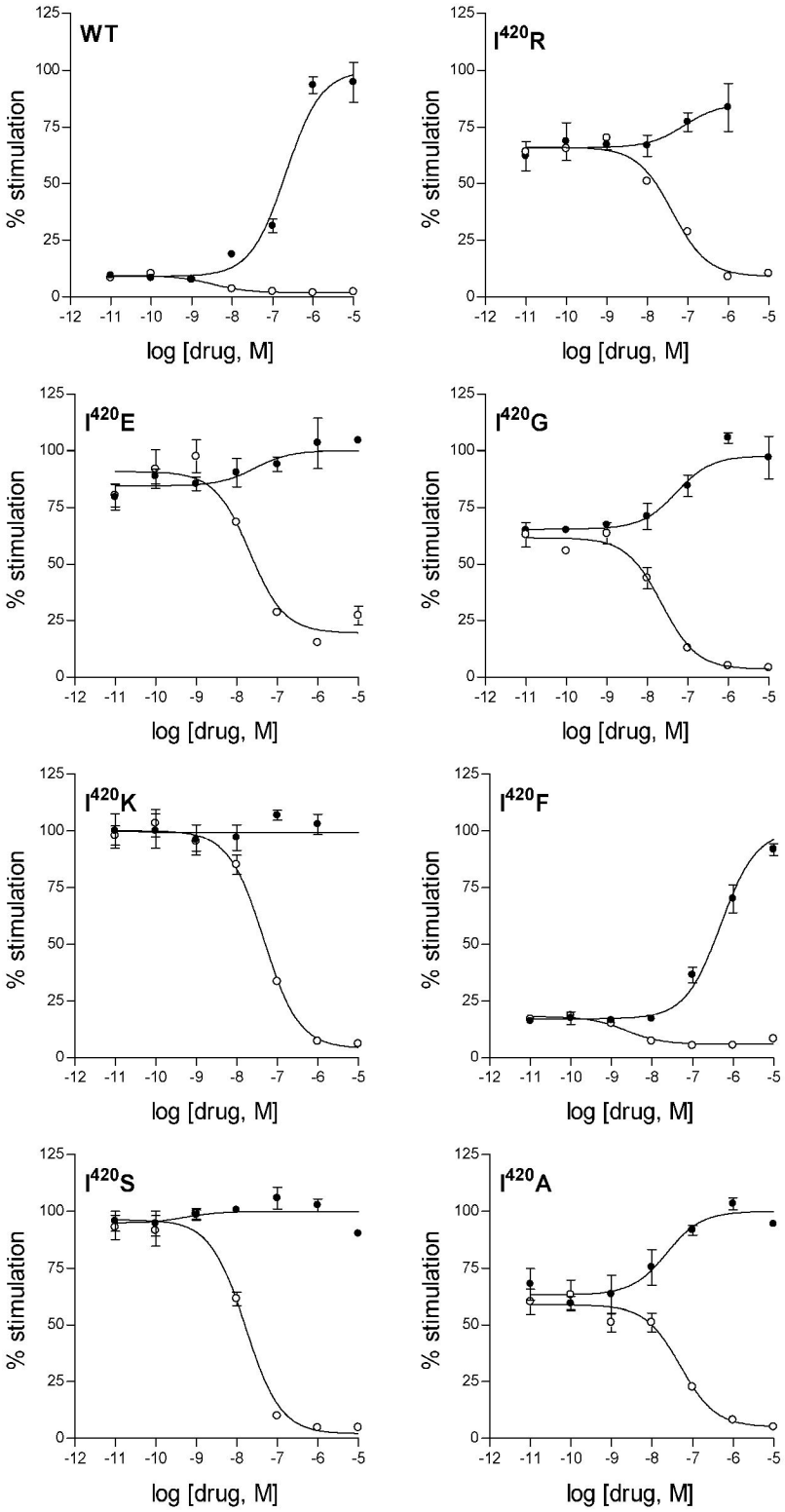

Figure 3

Molecular Pharmacology Fast Forward. Published on October 24, 2007 as DOI: 10.1124/mol.107.038547
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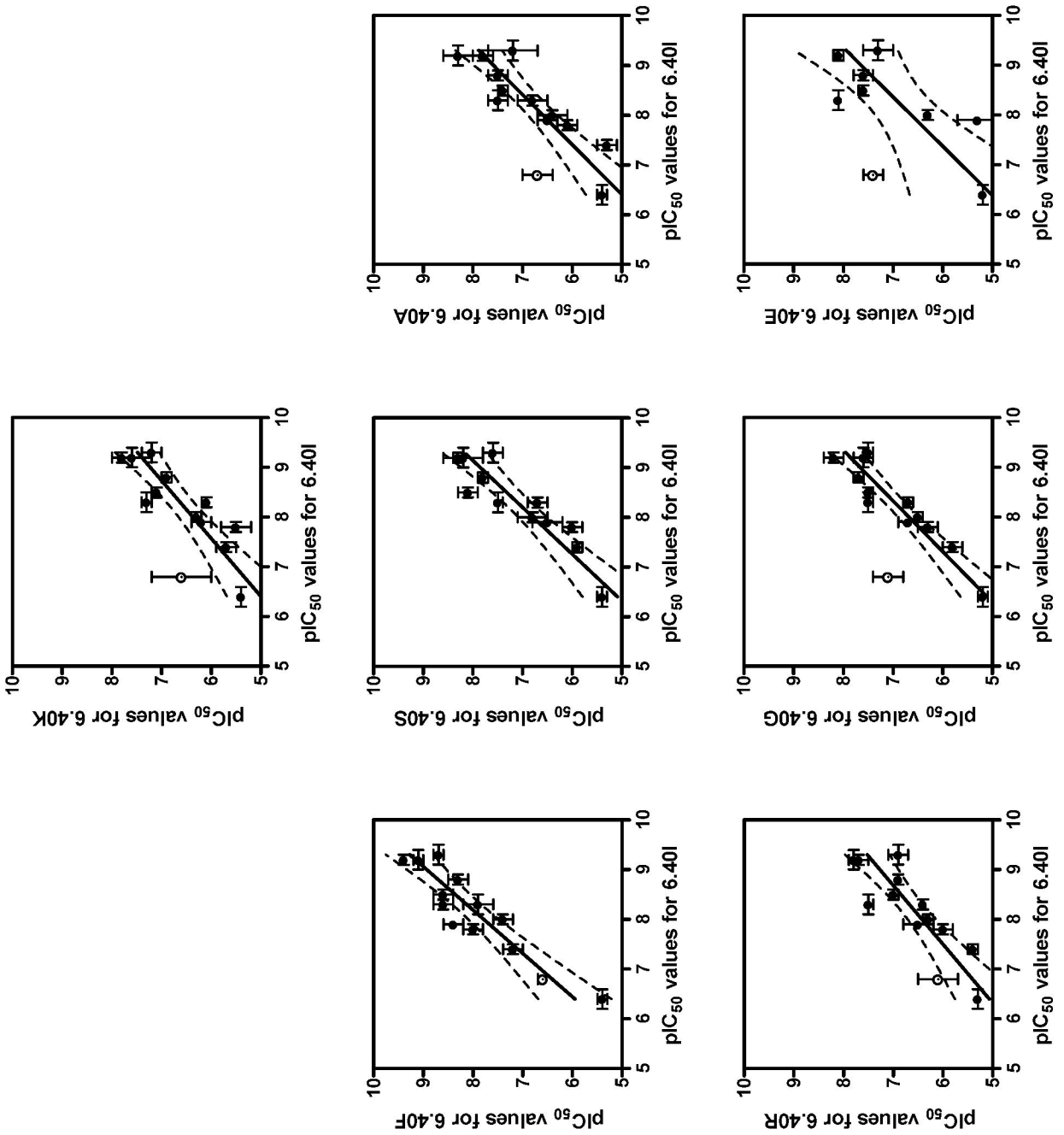

Figure 4

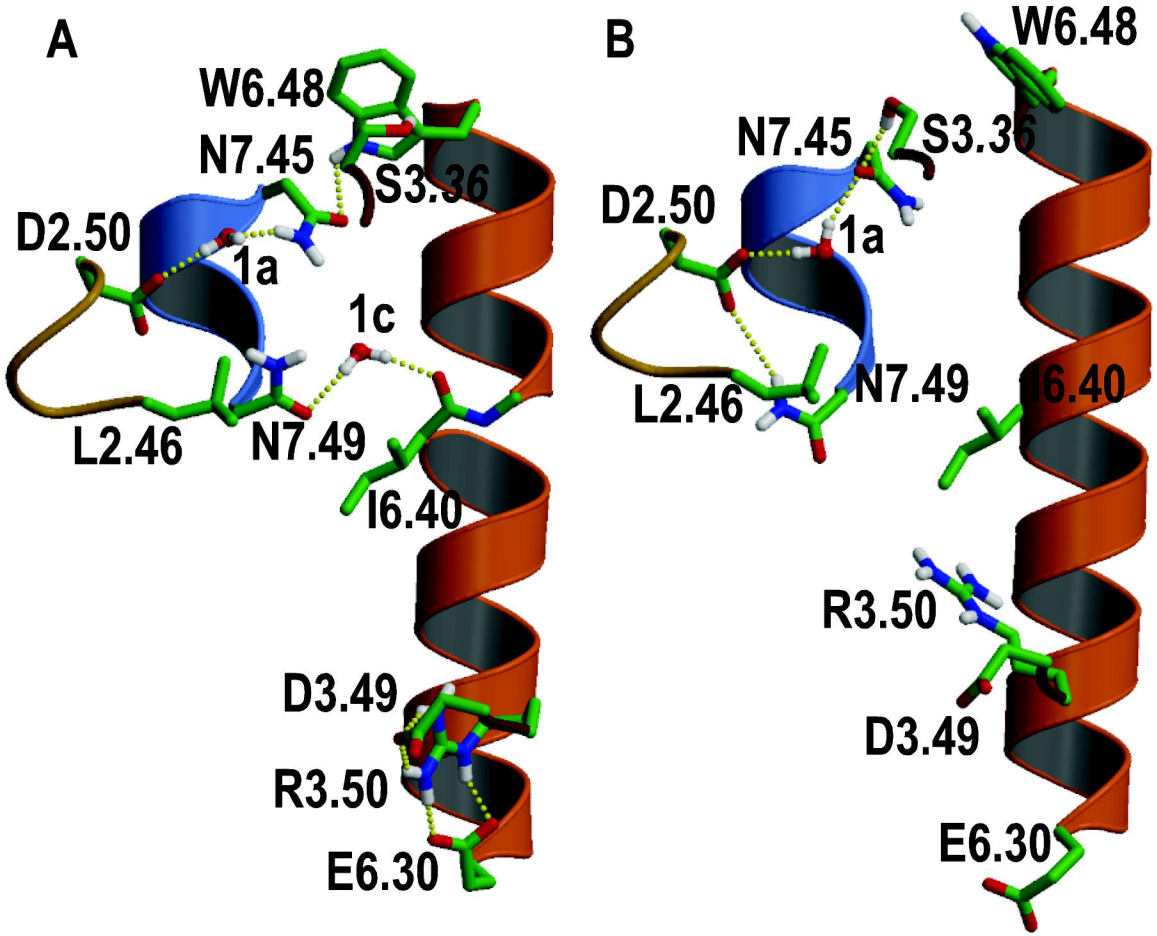

Figure 5

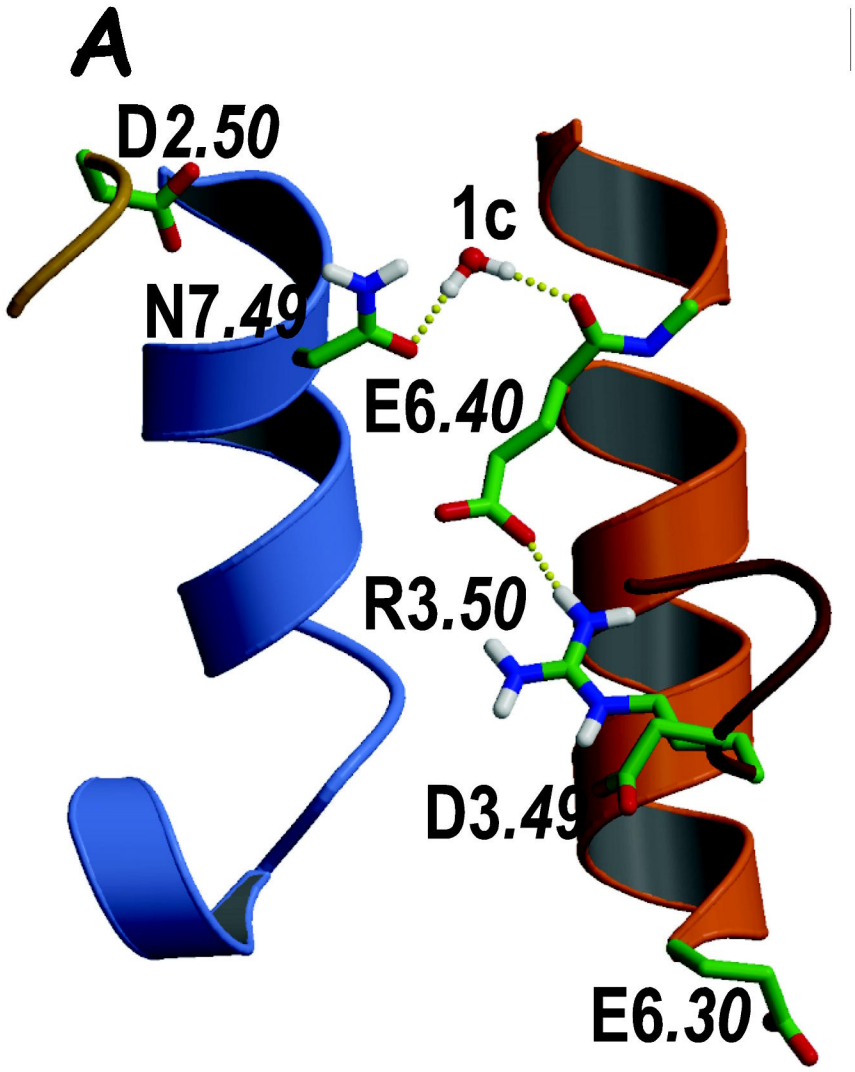

Figure 6A

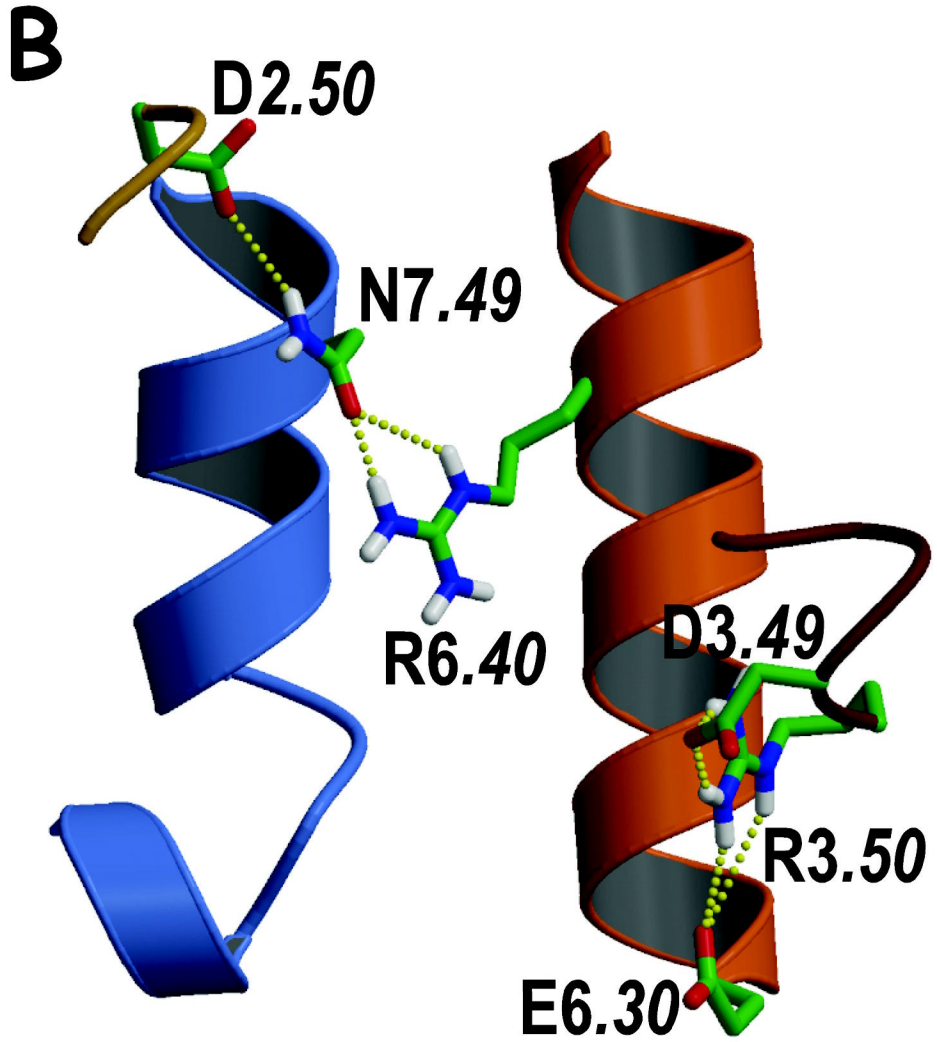

Figure 6B

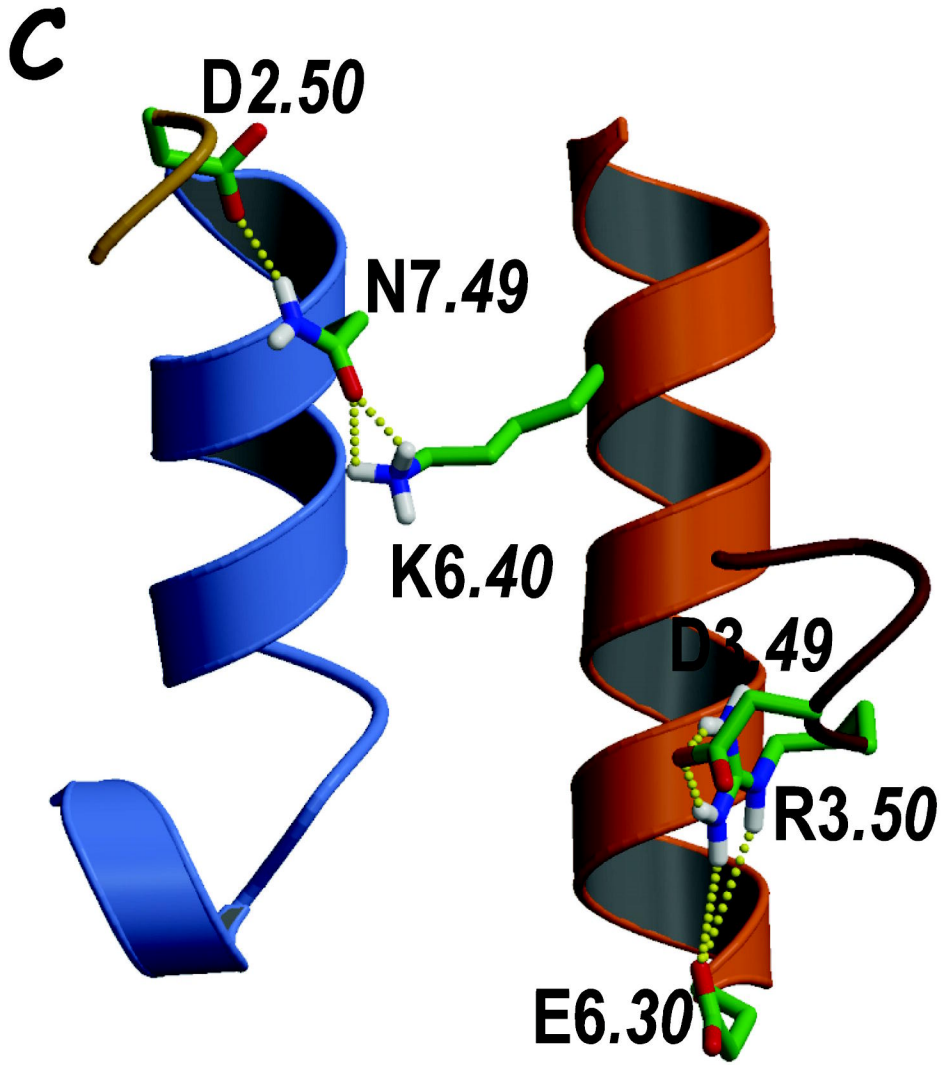

Figure 6C