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Single amino acid residue determinants of non-peptide antagonist binding to the corticotropin-releasing factor$_1$ (CRF$_1$) receptor

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ABSTRACT

The molecular interactions between non-peptide antagonists and the corticotropin-releasing factor type 1 (CRF$_1$) receptor are poorly understood. A CRF$_1$ receptor mutation has been identified that reduces binding affinity of the non-peptide antagonist NBI 27914 (M276I in transmembrane domain 5). We have investigated the mechanism of the mutation's effect using a combination of peptide and non-peptide ligands and receptor mutations. The M276I mutation reduced binding affinity of standard non-peptide antagonists 5–75-fold while having no effect on peptide ligand binding. We hypothesized that the side chain of isoleucine, β-branched and so rotationally constrained when within an α-helix, introduces a barrier to non-peptide antagonist binding. In agreement with this hypothesis, mutation of M276 to the rotationally constrained valine produced similar reductions of affinity as M276I mutation, whereas mutation to leucine (with an unbranched β-carbon) minimally affected non-peptide antagonist affinity. Mutation to alanine did not appreciably affect non-peptide antagonist affinity, implying the methionine side chain does not contribute directly to binding. Three observations suggested M276I/V mutations interfere with binding of the heterocyclic core of the compounds: (1) all compounds affected by M276I/V mutations possess a planar heterocyclic core. (2) None of the M276 mutations affected binding of an acylic compound. (3) The mutations differentially affected affinity of two compounds that differ only by core methylation. These findings imply that non-peptide antagonists, and specifically the heterocyclic core of such molecules, bind in the vicinity of M276 of the CRF$_1$ receptor. M276 mutations did not affect peptide ligand binding and this residue is distant from determinants of peptide binding (predominantly in the extracellular regions), providing molecular evidence for non-overlapping (allosteric) binding sites for peptide and non-peptide ligands within the CRF$_1$ receptor.

1. Introduction

Corticotropin-releasing factor (CRF) is the principal physiological regulator of an organism's response to stress. Responses to stress involve activation of a number of pathways via the CRF$_1$ receptor including hypothalamic activation of pituitary adrenocortocotropin, activation of autonomic responses via lower brainstem neuronal projections, and extrahypothala-

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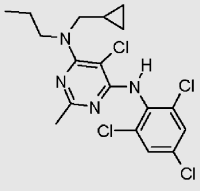
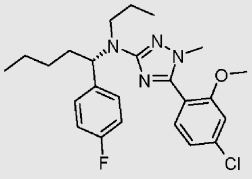
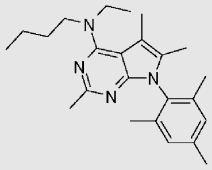
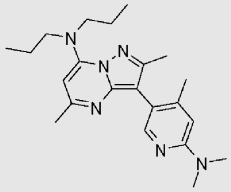
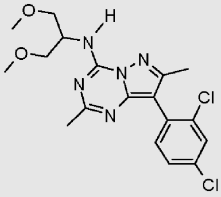
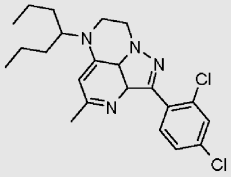
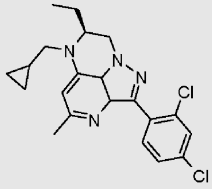
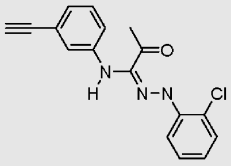
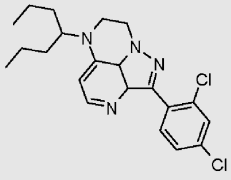
mic activation of a number of cerebral cortical and limbic nuclei regulating behavior [1–4]. Owing to its well-described physiological role in stress responses, the CRF system has been proposed as a potential therapeutic target for anxiety and depression. A pathophysiological role of the CRF system in these disorders has been implied by several clinical and a limited number of post-mortem studies [5,6]. For example, major depressive disorder has been associated with elevated activation of the HPA axis [7], and elevated CRF levels have been observed post mortem in brains from depressed patients [8]. Taken together, data from clinical human studies and from a large array of preclinical animal model experiments have been used to rationalize central CRF_1 receptors as a potential therapeutic target for anxiety, depression and stress disorders (for review, see [9–11]).

The potential clinical utility of CRF_1 receptor antagonism has stimulated considerable research efforts in developing CRF_1 receptor antagonists. Numerous orally bioavailable non-peptide ligands have been developed that bind with high affinity to the CRF_1 receptor and that penetrate the blood–brain barrier (for extensive review, see [12–15]). Representative examples that have been widely used in preclinical animal studies include CP-154,526 [16], antalarmin [17], DMP696 [18], DMP904 [19], SR125543A [20] and NBI 30775 (also known as R121919) [21]. The latter compound decreased depression and anxiety scores in a small open-label Phase IIA clinical trial of major depressive disorder patients [5]. Almost all CRF_1 receptor antagonists conform to a common topology (Table 1) [12–15]. These molecules contain a central, planar hetrocyclic core (mono-, bi- or tricylic) that bears a potential hydrogen bond acceptor nitrogen atom. A lower aryl or heteroaryl ring is separated from the core nitrogen by a one- or two-atom spacer. An ortho-substituent on the lower ring is required for high affinity binding, likely to maintain this ring orthogonal to the plane of the core. This orthogonal constraint might also be maintained by a small alkyl (typically methyl) or alkoxy substituent on the core, adjacent to the nitrogen atom. Lipophillic groups, typically alkyl or alkoxy, are attached at the top of the core and are required for high affinity binding (Table 1 and for review see [12–15]).

Table 1 – Chemical structure of CRF_1 receptor antagonists used in this study

Nomenclature	Chemical structure	Reference
'Standard' antagonists		
NBI 27914		3b of [34]
aryltriazole-(S)-71		(S)-71 of ref. [35]
antalarmin		[17,36]
NBI 30775		26 h of ref. [21]
DMP696		11i of ref. [18]
NBI 34041		12t of ref. [37]
NBI 35965		12a of ref. [37]
'Non-standard' antagonists		
arylamidrazone-51		51 of ref. [38]
desmethyl-NBI 34041		[37]

Compound syntheses are described in Section 2. All compounds except arylamidrazone-51 and desmethyl-NBI 34041 conform to the 'standard' CRF_1 receptor antagonist topology, possessing an ortho-substituted lower aromatic group, a cyclic core and aliphatic top side chains [12–15]. The cyclic core bears a potential hydrogen bond acceptor nitrogen atom separated from the lower aromatic group by a one- or two-atom spacer. The core is methylated at the position adjacent to the nitrogen on the other side of the spacer. The 'non-standard' antagonists are arylamidrazone-51, a representative acyclic antagonist, and desmethyl-NBI 34041, the analogue of NBI 34041 that lacks the core methyl substituent.

Beyond the detailed structure–activity relationship (SAR) analysis of non-peptide ligands elucidated over the past 15 years, little is currently known of the molecular mechanisms involved in non-peptide ligand binding. The CRF_1 receptor [22–24] is a Family B G-protein-coupled receptor (GPCR) [25,26]. Typical of this receptor family, the CRF_1 receptor comprises a structured N-terminal extracellular domain bearing three disulphide bonds [27–29], connected to a juxtamembrane domain of seven membrane-spanning α-helices and intervening loops (Fig. 1). The juxtamembrane domain expressed in isolation binds non-peptide ligands with the same affinity as the full-length receptor, suggesting most if not all ligand binding determinants are within this domain. Mutation of two residues within the transmembrane region reduces affinity of non-peptide ligand [30] (H199V in transmembrane domain (TM) 3 and M276I in TM5; Fig. 1). These mutations represent exchange with the corresponding residue of the closely related CRF_2 receptor [31,32], which binds CRF_1 receptor antagonists with much lower affinity [12,13,33]. The molecular mechanisms by which these mutations affect binding have not been established, for example, whether they represent direct binding determinants or indirect modulators of receptor conformation. Consequently, the localization of non-peptide ligand binding within the transmembrane region has not been determined. In this study we examined the molecular mechanism of the M276I mutation's effect using an array of non-peptide ligands and additional mutations. The findings suggest the mutation introduces a rotational constraint within the side chain at this position that interferes with non-peptide ligand binding, implying the ligands bind in the vicinity of M276 of the CRF_1 receptor.

2. Materials and methods

2.1. Materials

Sauvagine and astressin were synthesized by solid phase methodology on a Beckman Coulter 990 peptide synthesizer (Fullerton, CA) using t-Boc-protected amino acids. The assembled peptide was de-protected with hydrogen fluoride and purified by preparative HPLC. The purity of the final product was assessed by analytical HPLC and mass spectrometric analysis using an ion-spray source. The peptides were dissolved in 10 mM acetic acid/0.1% bovine serum albumin (BSA) at a concentration of 1 mM and stored at −80 °C. Non-

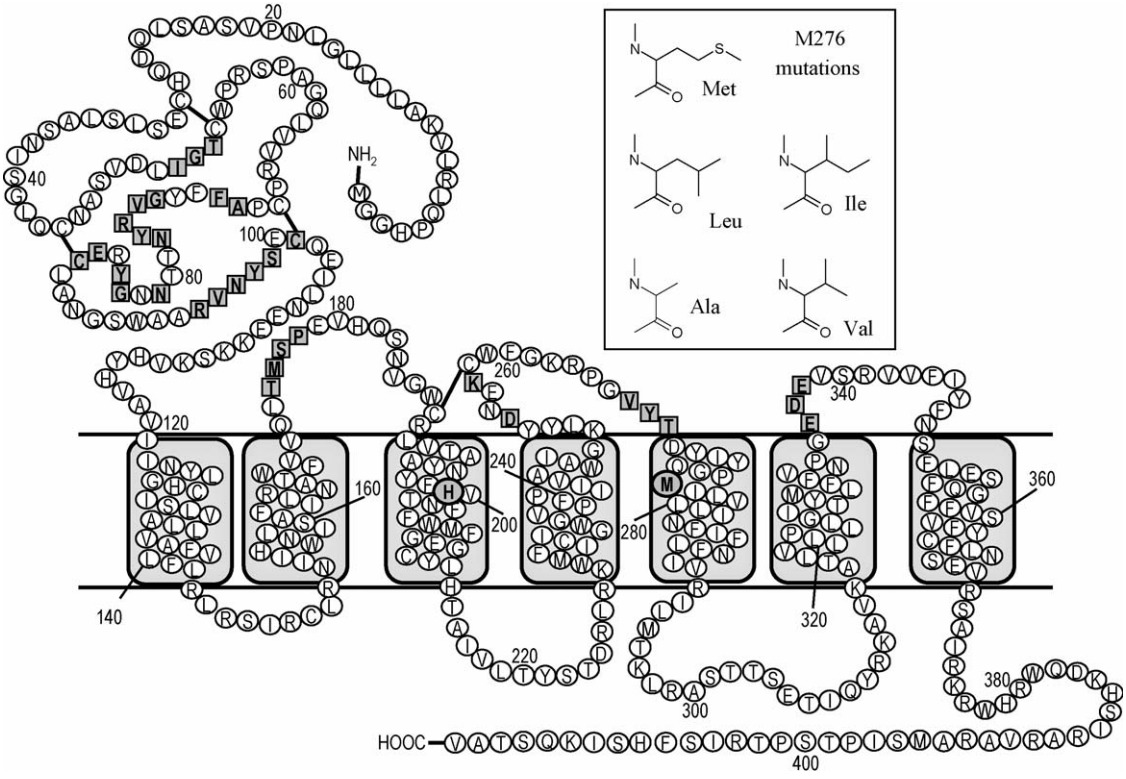


Fig. 1 – Topographical representation of the human CRF_1 receptor. The arrangement of disulphide bonds in the N-terminal extracellular region has been identified [27,28] as indicated. A further disulphide bond between extracellular loops 1 and 2 is assumed based on the complete conservation of the two cysteine residues in Class B GPCR's [25]. Amino acids determinants of peptide ligand interaction are indicated by shaded squares: ref. [29] for I51, G52, T53, G74, V75, R76, Y77, N78, G83, Y84, G86, C87, R95–S99 and C101; ref. [53] for A70 and F71; refs. [54,55] for R76, N81 and Gly83; ref. [56] for T175, M176, S177, P178, V266, Y267 and T268; ref. [30] for Asp 254; ref. [57] for E336, D337 and E338; ref. [58] for K257. Two CRF_1 receptor mutations have been identified that affect non-peptide ligand interaction—H199V and M276I (enlarged shaded circles, ref. [30]). *Inset*: chemical structure of amino acids at residue 276 tested in this study. The β-branched side chains of isoleucine and valine are rotationally constrained within an α-helix, whereas the unbranched methionine and γ-branched leucine are less constrained [43,44].

peptide ligands were synthesized as previously described (NBI 27914—compound 3b of ref. [34]; aryltriazole-(*S*)-71—compound (*S*)-71 of ref. [35]; antalarmin [17,36]; NBI 30775 (also known as R121919)—compound 26 h of ref. [21]; DMP696—compound 11i of ref. [18]; NBI 34041—compound 12t of ref. [37]; NBI 35965—compound 12a of ref. [37]; arylamidrazone-51—compound 51 of ref. [38]; desmethyl-NBI 34041 [37]. Non-peptide ligands were dissolved in 100% DMSO at a concentration of 6 mM and stored at −20 °C. [^{3}H]NBI 35965 was prepared by first brominating NBI 35965 at the 6-position of the pyrazolopyridine core, which required one equivalent of bromine in methanol at ambient temperature over 2 h. The product was purified using silica gel chromatography. Brominated NBI 35965 was tritiated by hydrogenation (American Radiolabeled Chemicals Inc., St. Louis, MO), and the tritiated product was purified by preparative HPLC and stored in ethanol at −20 °C. [^{125}I]Tyr^0-sauvagine was obtained from PerkinElmer Life Sciences (Boston, MA) (specific activity of 2200 Ci/mmol). [^{125}I]Tyr^0-astressin was prepared using the chloramine T method and purified by HPLC (specific activity 2200 Ci/mol). G418 (geneticin), Dulbecco's phosphate-buffered saline (DPBS) and cell culture supplies were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone (Logan, UT) and horse serum was from PerkinElmer Life Sciences. Hygromycin was from EMD Biosciences, San Diego, CA.

2.2. Construction of receptor expression vectors, expression in CHO cells and preparation of cell membranes

The construction of an N-terminally FLAG-tagged wild-type CRF_1 receptor has been described previously [39]. The N-terminal Flag CRF_1 receptor construct was cloned into pcDNA5/FRT/V5-His-TOPO expression vector (Invitrogen, Calsbad, CA). The N-terminal sequence was DYKDDDDA (Flag epitope) followed by the CRF_1 sequence minus the 23 amino acid signal peptide [27,28]. The presence of the N-terminal Flag epitope did not affect the peptide ligand binding affinity [39]. This construct was used as the template for mutagenesis using the QuikChange XL Site-Directed mutagenesis kit (Stratagene, La Jolla, CA). The sequence of the entire receptor was verified by DNA sequencing. CRF_1 receptor constructs were co-transfected in CHO Flp-In cells (Invitrogen) with the vector pOG44. Stable cell lines were created by selection using 1 mg/ml hygromycin in complete medium (Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/ml penicillin and 50 μg/ml streptomycin). The resulting stable cell lines were grown in the medium above supplemented with 0.5 mg/ml hygromycin. P2 cell membranes were prepared using nitrogen cavitation and differential centrifugation as described previously [40]. The protein concentration in the membrane pellet was determined using the Coomassie method (Pierce, Rockford, IL), using BSA as the standard.

2.3. Radioligand binding assays

Binding assays for wild-type and mutant CRF_1 receptors in CHO cell membranes were set up in low-binding 96-well plates (#3605, Corning, Palo Alto, CA) in assay buffer (Dulbecco's phosphate-buffered saline (DPBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 138 mM NaCl) supplemented with 10 mM $MgCl_2$, 2 mM ethylene glycol-bis[β-aminoethyl]-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 7.4 with NaOH). The following were added sequentially to the plates: 50 μl radioligand, 50 μl buffer or unlabeled ligand and 100 μl cell membrane suspension. In experiments where [^{3}H]NBI 35965 was used as the radioligand, GTPγS (10 μM final concentration) was included to maintain the form of the receptor uncoupled from G-protein [41]. In these experiments the radioligand volume was reduced to 25 μl and GTPγS was added in a volume of 25 μl. In radioligand displacement experiments 12 concentrations of unlabeled ligand were competed against a single concentration of radioligand. The radioligand concentrations used were approximately 50 pM for [^{125}I]sauvagine and 3 nM for [^{3}H]NBI 35965. In radioligand saturation experiments 8 concentrations of radioligand (for [^{125}I]astressin) or 12 concentrations (for [^{125}I]sauvagine and [^{3}H]NBI 35965) were tested in the absence and presence of unlabeled ligand, with each condition tested in duplicate. The unlabeled ligand used in these saturation experiments was the unlabeled analogue of the respective radioligand (1 μM astressin, 3 μM sauvagine or 10 μM NBI 35965). No specific binding of the three radioligands was observed in non-transfected CHO cell membranes. The amount of cell membrane protein added per assay was 0.6–2.0 μg/well for [^{125}I]sauvagine binding assays, 0.1–0.13 μg/well for [^{125}I]astressin binding assays and 2 μg/well for [^{3}H]NBI 35965 binding assays. All assays were incubated for 2 h at room temperature. Bound and free radioligand were then separated by rapid filtration, using UniFilter GF/B filters (Packard, Meriden, CT) on a UniFilter-96 vacuum manifold (Packard). GF/B filters were pre-treated for 20–40 min with 0.1% polyethylenimine in DPBS. The filter was washed three times with 0.4 ml/well 0.01% Triton X-100 in DPBS (for peptide radioligands) or DPBS (for [^{3}H]NBI 35965), then dried under electric fans for 40 min–1 h. Following addition of scintillation fluid (40 μl per filter disc, Microscint 20, Packard), the plate was sealed and scintillation counts measured in a Packard Topcount NXT. Cpm resulting from emission of Auger electrons from ^{125}I were converted to dpm, using a predetermined counting efficiency of 35%. In radioligand displacement assays total radioligand bound was less than 20% of the total amount of radioligand added to the assay well. The total amount of radioligand added to the assay well was measured using a Packard Cobra II gamma counter for [^{125}I]-labeled peptides (78% efficiency), and using a Packard 1600TR liquid scintillation counter for [^{3}H]NBI 35965 (47% efficiency).

2.4. Data analysis

Radioligand saturation was analyzed using a previously described method [40] that takes into account depletion of free radioligand by receptor-specific and non-specific binding of radioligand, providing an accurate measurement of K_d. This analysis was performed using Prism 3.0 (GraphPad Software, San Diego, CA). Initial analysis of competition binding assays using a four-parameter logistic equation (Prism 3.0) indicated a slope factor of 0.9–1.1. Consequently data were analyzed using a single-affinity state competition equation using XL*fit* (ID Business Solutions Ltd., Emeryville, CA) to provide fitted

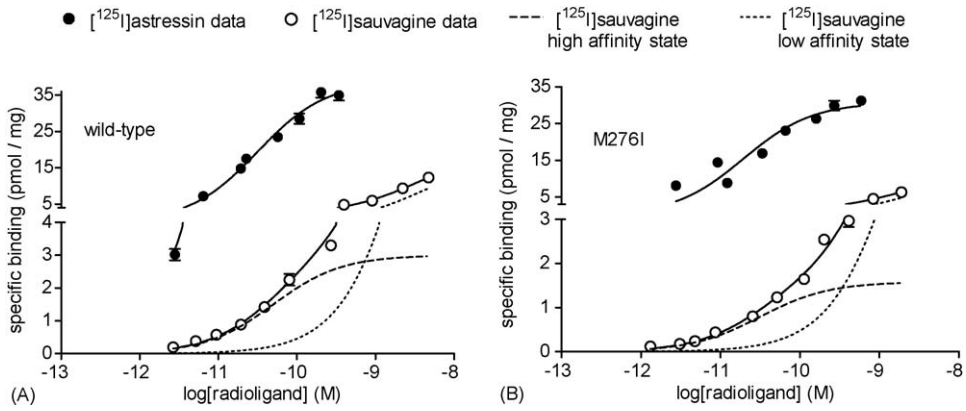


Fig. 2 – Peptide radioligand saturation of wild-type and M276I mutant CRF_1 receptors. [^{125}I]sauvagine and [^{125}I]astressin saturation was measured as described in Section 2 for CRF_1 receptors in CHO cell membranes: wild-type (A), M276I (B); and M276V, M276L and M276A (graphical data not shown). [^{125}I]astressin saturation of all receptors was best described by a single-affinity state saturation equation, whereas [^{125}I]sauvagine saturation was best described by a two-affinity state fit (solid lines). For [^{125}I]sauvagine data, the curves for saturation of each of the two states are indicated by the dashed lines. In subsequent [^{125}I]sauvagine displacement assays a low concentration of [^{125}I]sauvagine was used (approximately 50 pM) in order that the high affinity state was the predominant state labeled in these assays. Data points are mean ± S.E.M. of duplicate measurements. The data are from single experiments representative of five and four independent experiments for wild-type and M276I CRF_1 receptors, respectively.

values of K_i. Statistical analysis of differences between values was performed by analysis of variance (ANOVA) using Prism 3.0.

3. Results

3.1. *Effect of M276 mutations on peptide ligand interaction with the CRF_1 receptor*

M276I mutation in TM5 of the CRF_1 receptor has been shown previously to reduce affinity of a non-peptide antagonist [30]. The aim of this study was to investigate the mechanism of the mutation's effect using an array of non-peptide ligands and additional M276 mutations. We first characterized the peptide ligand binding properties of four mutant receptors (M276I, M276V, M276L, M276A; Fig. 1) compared to the wild-type CRF_1 receptor. All the receptors were stably expressed in CHO cells and binding was measured in membranes prepared from these cells.

All receptors were able to bind the labeled peptide agonist [^{125}I]sauvagine and the labeled peptide antagonist [^{125}I]astressin with high affinity (Fig. 2; Table 2). Binding of either radioligand was not detected in membranes from non-

Table 2 – Radioligand saturation data the CRF_1 receptor in CHO cell membranes

Radioligand	Receptor	State 1		State 2		B_{max} states 1 + 2 (pmol/mg)
		pK_d (K_d, pM)	B_{max} (pmol/mg)	pK_d (K_d, pM)	B_{max} (pmol/mg)	
[^{125}I]sauvagine	WT	10.71 ± 0.03 (19)	1.7 ± 0.3	9.17 ± 0.08 (680)	8.7 ± 1.5	10 ± 2
[^{125}I]sauvagine	M276I	10.73 ± 0.12 (19)	1.2 ± 0.4	8.97 ± 0.16 (1000)	8.6 ± 2.7	10 ± 3
[^{125}I]sauvagine	M276V	10.80 ± 0.09 (16)	2.5 ± 0.4	9.28 ± 0.07 (520)	13 ± 2	16 ± 2
[^{125}I]sauvagine	M276L	10.76 ± 0.13 (17)	1.6 ± 0.2	9.12 ± 0.08 (760)	9.7 ± 0.2	11 ± 1
[^{125}I]sauvagine	M276A	10.79 ± 0.26 (16)	1.0 ± 0.5	8.79 ± 0.15 (1600)	13 ± 2	14 ± 2
[^{125}I]astressin	WT	10.34 ± 0.17 (45)	24 ± 5			24 ± 5
[^{125}I]astressin	M276I	10.60 ± 0.11 (25)	33 ± 3			33 ± 3
[^{125}I]astressin	M276V	10.39 ± 0.44 (41)	30 ± 5			30 ± 5
[^{125}I]astressin	M276L	10.52 ± 0.22 (30)	23 ± 3			23 ± 3
[^{125}I]astressin	M276A	10.52 ± 0.01 (31)	28 ± 4			28 ± 4
[^{3}H]NBI 35965	WT	9.32 ± 0.09 (480)	26 ± 4			26 ± 4

Radioligand saturation of the receptors was measured as described in Section 2. Specific binding data were fit to single- and two-affinity state equations and the best fit determined using a partial *F*-test. For [^{125}I]sauvagine saturation the two-affinity state model provided a better fit than the single-affinity state model in all cases ($p < 0.05$), whereas for [^{125}I]astressin and [^{3}H]NBI 35965 saturation the two-affinity state model did not improve the goodness of fit ($p > 0.05$). Data are mean ± S.E.M from four to five experiments, except for [^{125}I]astressin saturation of M276I, M276V, M276L and M276A receptors (mean ± range, $n = 2$).

transfected CHO cells (data not shown). In radioligand saturation experiments, [^{125}I]sauvagine binding was best-described by a two-affinity state binding model for all mutant receptors and the wild-type receptor (Fig. 2 for wild-type and M276I receptors; Table 2 for all receptors). For each mutant receptor, neither the affinity (K_d) nor the binding site capacity (B_{max}) of each state was significantly different from the corresponding values for the WT receptor (single factor ANOVA, $p > 0.05$ in all cases, Table 2). In addition, the K_d and B_{max} for [^{125}I]astressin binding was similar for all receptors (Table 2). The [^{125}I]astressin B_{max} was greater than the sum of the B_{max} values for the two-affinity states for [^{125}I]sauvagine (Table 2), consistent with previous characterization of the CRF_1 receptor in L cell membranes and rat cerebellum homogenates [42]. We next tested the ability of peptide ligands to displace [^{125}I]sauvagine binding to the receptors. None of the M276

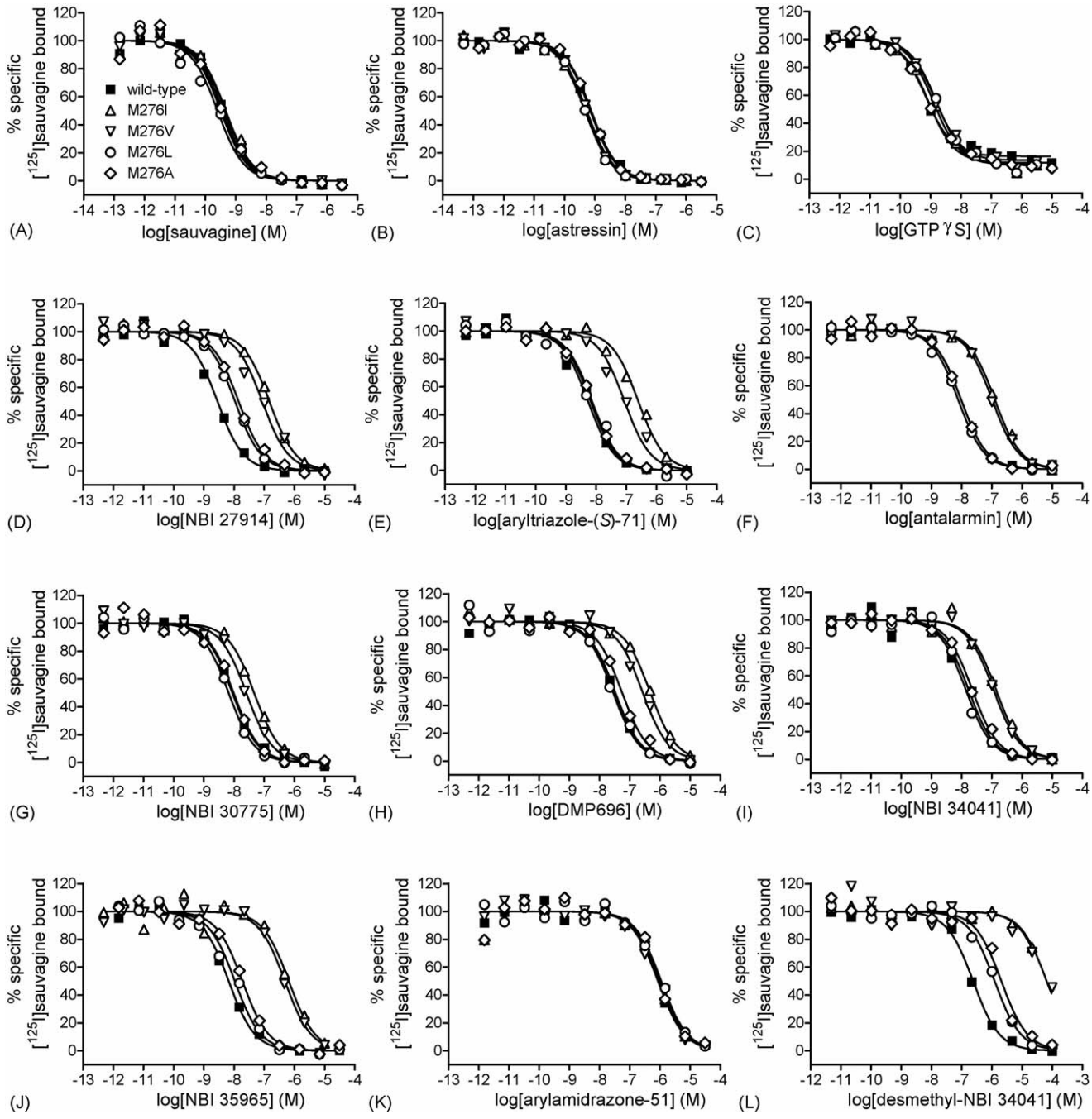


Fig. 3 – Displacement of [^{125}I]sauvagine binding to wild-type and M276 mutant CRF_1 receptors by unlabeled ligands. Inhibition of [^{125}I]sauvagine binding to wild-type, M276I, M276V, M276L and M276A receptors was measured as described in Section 2, for sauvagine (A), astressin (B), GTPγS (C), NBI 27914 (D), aryltriazole-(*S*)-71 (E), antalarmin (F), NBI 30775 (G), DMP696 (H), NBI 34041 (I), NBI 35965 (J), arylamidrazone-51 (K) and desmethyl-NBI 34041 (L). Curves are fits to a single-affinity state inhibition equation. Data points are single determinations. Data are from single experiments representative of 3–17 independent experiments.

Table 3 – Ligand affinity for wild-type and M276 mutant CRF_1 receptors

	Wild-type	M276I		M276V		M276L		M276A	
	pK_i (K_i, nM)	pK_i (K_i, nM)	$K_i/K_{i(wild\text{-}type)}$	pK_i (K_i, nM)	$K_i/K_{i(wild\text{-}type)}$	pK_i (K_i, nM)	$K_i/K_{i(wild\text{-}type)}$	pK_i (K_i, nM)	$K_i/K_{i(wild\text{-}type)}$
Peptides and GTPγS									
sauvagine	9.70 ± 0.11 (0.20)	9.70 ± 0.14 (0.20)	1.0	9.86 ± 0.13 (0.14)	0.69	9.98 ± 0.16 (0.11)	0.53	9.88 ± 0.13 (0.13)	0.67
astressin	9.66 ± 0.10 (0.22)	9.73 ± 0.05 (0.19)	0.86	9.55 ± 0.13 (0.28)	1.3	9.64 ± 0.17 (0.23)	1.0	9.83 ± 0.09 (0.15)	0.69
GTPγS	9.52 ± 0.13 (0.30)	9.44 ± 0.23 (0.37)	1.2	9.43 ± 0.07 (0.37)	1.2	9.41 ± 0.07 (0.39)	1.3	9.79 ± 0.09 (0.16)	0.54
'Standard' antagonists									
NBI 27914	9.10 ± 0.05 (0.80)	7.55 ± 0.11[a] (28)	**35**	7.31 ± 0.15[a] (49)	**61**	8.49 ± 0.11[a] (3.2)	**4.1**	8.56 ± 0.07[a] (2.8)	**3.5**
aryltriazole-(S)-71	8.83 ± 0.08 (1.5)	7.14 ± 0.14[a] (72)	**49**	7.39 ± 0.09[a] (41)	**28**	8.58 ± 0.04 (2.6)	1.8	8.65 ± 0.02 (2.3)	1.5
antalarmin	8.59 ± 0.14 (2.5)	7.28 ± 0.08[a] (53)	**21**	7.55 ± 0.02[a] (28)	**11**	8.59 ± 0.03 (2.6)	1.0	8.55 ± 0.02 (2.9)	1.1
NBI 30775	8.67 ± 0.05 (2.1)	7.97 ± 0.04[a] (11)	**5.0**	8.05 ± 0.06[a] (8.8)	**4.2**	8.66 ± 0.05 (2.2)	1.0	8.41 ± 0.07 (3.9)	1.8
DMP696	8.03 ± 0.08 (9.3)	6.78 ± 0. 11[a] (160)	**18**	7.16 ± 0.07[a] (69)	**7.4**	8.08 ± 0.01 (8.3)	0.90	7.87 ± 0.05 (13)	1.5
NBI 34041	8.36 ± 0.12 (4.4)	7.10 ± 0.16[a] (80)	**18**	7.50 ± 0.02[a] (31)	**7.2**	8.39 ± 0.06 (4.0)	0.92	8.23 ± 0.03 (5.9)	1.3
NBI 35965	8.69 ± 0.03 (2.1)	6.81 ± 0.05[a] (150)	**75**	6.97 ± 0.08[a] (110)	**52**	8.34 ± 0.11 (4.6)	2.2	8.22 ± 0.06[a] (6.0)	3.0
'Non-standard' antagonists									
arylamidrazone-51	6.82 ± 0.05 (150)	6.83 ± 0.09 (150)	0.99	6.68 ± 0.09 (210)	1.4	6.59 ± 0.15 (260)	1.7	6.65 ± 0.11 (220)	1.5
desmethyl-NBI 34041	7.15 ± 0.08 (71)	4.85 ± 0.04[a] (14000)	**200**	4.86 ± 0.02[a] (14000)	**200**	6.60 ± 0.03[a] (250)	**3.5**	6.41 ± 0.04[a] (390)	**5.5**

Ligand affinity for inhibition of [^{125}I]sauvagine binding to the receptors was measured as described in Section 2. 'Standard' and 'non-standard' antagonist structures are given in Table 1. Ligand binding data were fitted to a single-affinity state inhibition curve to determine IC_{50} which was subsequently converted to K_i using the method of Cheng and Prusoff [61]. Inhibitory potency of GTPγS is given as IC_{50} instead of K_i given that the effect of guanine nucleotide is not competitive [41]. The [^{125}I]sauvagine K_d used in this calculation was 19, 19, 16, 17 and 16 pM for wild-type, M276I, M276V, M276L and M276A receptors, respectively (Table 2). All ligands fully displaced [^{125}I]sauvagine binding, except GTPγS (maximal inhibition values of 88 ± 1, 91 ± 3, 89 ± 2, 91 ± 1 and 88 ± 1% for WT, M276I, M276V, M276L and M276A, respectively, which are not significantly different—$p = 0.34$, single-factor ANOVA). Data are mean ± S.E.M. (n = 3–17). Figures highlighted in bold font represent a >3-fold reduction of affinity produced by the receptor mutation.

[a] $p < 0.001$, two-factor ANOVA followed by Bonferroni post-test comparing wild-type with each of the four mutant receptors.

mutations significantly affected astressin or sauvagine affinity (Fig. 3A and B; Table 3). Finally, we tested the effects of the guanine nucleotide analogue GTPγS, which binds the α-subunit of G-proteins, uncoupling receptor from G-protein and consequently reducing the binding of agonists [41]. The extent to which the guanine nucleotide reduces radiolabeled agonist binding can be used to assess the extent of receptor–G-protein coupling in the cell membranes. GTPγS reduced binding of [^{125}I]sauvagine to the WT receptor by 88% with an IC_{50} of 0.3 nM (Fig. 3C; Table 3), consistent with previous studies [42]. None of the M276 mutations significantly affected the maximal extent of inhibition or the IC_{50} for GTPγS (Fig. 3C; Table 3). Taken together these results indicate that peptide binding is not appreciably affected by M276 mutations, implying no dramatic alteration of receptor structure and that M276 is not a determinant of peptide interaction with the CRF_1 receptor.

3.2. *Effect of M276 mutations on standard non-peptide ligand interaction with the CRF_1 receptor*

Binding affinity of the monocyclic non-peptide antagonist NBI 27914 was reduced by M276I mutation [30]. We first tested the effect of this mutation on a wide range of ligands that possess all the chemical structural features of the standard CRF_1 receptor topology [12–15] (the monocyclic NBI 27914 and aryltriazole-(*S*)-71; bicyclic antalarmin, NBI 30775 and DMP696; and tricyclic NBI 34041 and NBI 35965; Table 1). These compounds are classified here as 'standard' CRF_1 receptor antagonists (Table 1). The affinity of all these compounds was reduced by M276I mutation (from a 5.0-fold reduction for NBI 30775 to a 75-fold reduction for NBI 35965; Fig. 3; Table 3). This finding indicates that the M276I mutation impairs binding of standard CRF_1 receptor antagonists.

We hypothesized that the molecular basis of the M276I mutation's effect was related to the rotational constraint of the side chain of isoleucine compared to methionine. Isoleucine possesses a branched β-carbon (Fig. 1) that limits the rotational freedom of its side chain when in an α-helical environment [43,44]. The unbranched side chain of methionine (Fig. 1) is not rotationally constrained. We substituted methionine 276 for valine, which is β-branched and rotationally constrained similarly to isoleucine (Fig. 1) [44]. The M276V mutation reduced affinity of all standard CRF_1 receptor antagonists, from a 4.2-fold decrease for NBI 30775 to a 61-fold decrease for NBI 27914 (Fig. 3; Table 3). The reduction of non-peptide ligand affinity for each ligand closely matched the reduction observed for the M276I mutation (Fig. 3; Table 3). We next tested leucine at residue 276. Leucine is a structural isomer of isoleucine but is not rotationally constrained in an α-helix because it is branched at the γ-position rather than the β-position (Fig. 1) [44]. Strikingly, the M276L mutation did not appreciably affect non-peptide ligand binding (Fig. 3; Table 3). Binding of only one non-peptide ligand was affected by M276L mutation (NBI 27914, 4.1-fold reduction of affinity; Fig. 3D; Table 3). This finding strongly supports the hypothesis that rotational freedom of the residue 276 side chain is a key determinant of non-peptide ligand binding to the CRF_1 receptor. Finally, M276 was mutated to alanine, to determine the extent to which hydrophobic bulk of the methionine and leucine side chains contributes to non-peptide ligand binding affinity (Fig. 1). M276A mutation did not appreciably affect affinity of non-peptide ligands (Fig. 3; Table 3), except NBI 27914 for which the affinity was reduced minimally (3.5-fold; Fig. 3D; Table 3). These data indicate that the methionine side chain is not directly involved in standard non-peptide ligand binding, suggesting that hydrophobic bulk at residue 276 is not required for interaction of standard non-peptide ligands with the CRF_1 receptor.

3.3. *Effect of M276 mutations on non-standard non-peptide ligand interaction with the CRF_1 receptor*

The findings above imply that rotationally constrained side chains at residue 276 of the CRF_1 receptor interfere with binding of standard non-peptide antagonists. We used non-peptide ligand SAR to investigate the determinants of the ligand that underlie this effect. The reduced ability of rotationally constrained side chains to efficiently bind standard antagonists suggests interference of a restricted component of the ligands. The heterocyclic core of standard non-peptide antagonists is in most cases constrained in a planar configuration [12–15,33] so we speculated that binding of this relatively rigid region of the molecule would be affected by M276I and M276V mutations. Supporting this hypothesis, all the non-peptide ligands affected by the mutations possess a planar core group (Table 1). We also tested the affect of these mutations on an acyclic CRF_1 receptor antagonist, termed arylamidrazone-51 (compound 51 of ref. [38]; Table 1). This compound conforms to the topology of CRF_1 receptor antagonists, with the exception that the putative hydrogen bond acceptor nitrogen atom is contained within an acyclic core [12,38] (Table 1). Binding affinity of arylamidrazone-51 was not significantly affected by M276I or M276V mutations (Fig. 3K; Table 3), consistent with the hypothesis that this less rigid compound can better adapt to more constrained amino acid side chains at residue 276. Binding of this compound was also not significantly affected by M276L and M276A mutations (Fig. 3K; Table 3).

The heterocyclic core group of standard antagonists bears a small aliphatic substituent, typically a methyl group, adjacent to the putative hydrogen bond acceptor nitrogen atom [12–15,33] (Table 1). We investigated the effect of this substituent on the effect of M276 mutations using desmethyl-NBI 34041, an analogue of NBI 34041 that lacks the core methyl substituent (Table 1). Binding affinity of this compound was reduced by M276I and M276V mutations to the greatest extent of all compounds tested (200-fold by both mutations; Fig. 3L; Table 3). This effect was considerably greater than that for the parent compound NBI 34041 (18-fold for M276I and 7.2-fold for M276V; Fig. 3I; Table 3). Binding affinity of desmethyl-NBI 34041 was also significantly reduced by M276L and M276A mutations (3.5- and 5.5-fold, respectively; Fig. 3L; Table 3), whereas binding of NBI 34041 was not significantly affected by these mutations (Fig. 3I; Table 3). Taken together these data indicate the core methyl substituent is a determinant of the effect of M276 mutations, providing additional support to the hypothesis that the mutations modulate interaction of the heterocyclic core with the CRF_1 receptor.

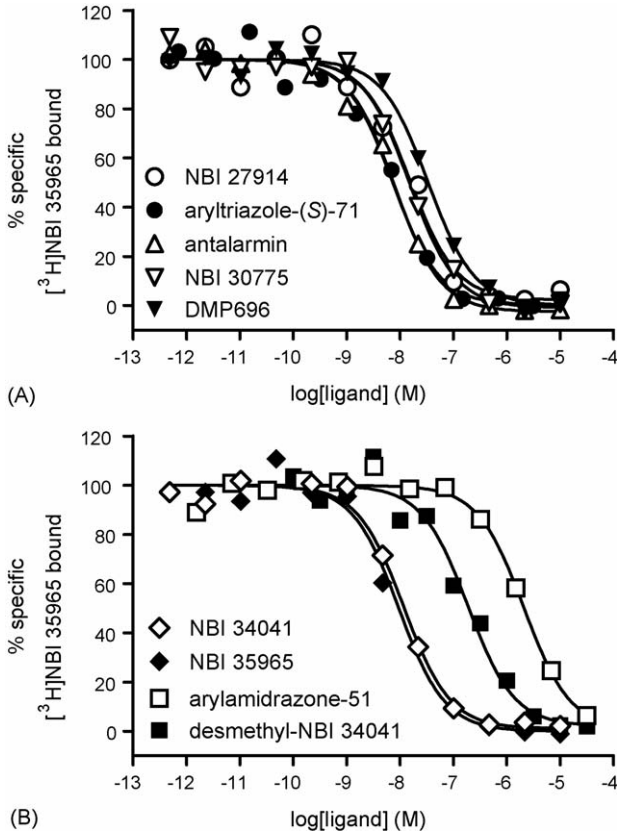


Fig. 4 – Displacement of [^{3}H]NBI 35965 binding to the wild-type CRF_1 receptor. Inhibition of [^{3}H]NBI 35965 binding to wild-type and mutant receptors was measured as described in Section 2 for monocyclic and bicyclic ligands (A), and tricyclic and acyclic ligands (B). Curves are fits to a single-affinity state inhibition equation. Data points are single determinations. Data are from single experiments representative of 3–10 independent experiments.

3.4. *Non-peptide ligand displacement of [^{3}H]NBI 35965 binding to the wild-type CRF_1 receptor*

In the characterization of non-peptide ligand binding to mutant receptors, compound affinity was measured by displacement of a labeled peptide agonist ([^{125}I]sauvagine). This approach was used because peptide ligand affinity was not affected by the mutations, allowing large reductions of non-peptide ligand affinity to be measured that would not be easily measurable using a radiolabeled non-peptide ligand. However, ligand binding data suggest the binding sites for peptide and non-peptide ligands are at least partially distinct [30,40,45]. To validate the [^{125}I]sauvagine displacement data, non-peptide ligand affinity determined using this radioligand was compared with affinity measured by competitive displacement of [^{3}H]NBI 35965 binding to the wild-type CRF_1 receptor. This experiment was also performed to measure the maximal displacement of [^{3}H]NBI 35965 binding by the various non-peptide ligands, to provide an assessment of the extent to which the non-peptide ligands bind an overlapping binding site on the CRF_1 receptor.

[^{3}H]NBI 35965 bound the wild-type CRF_1 receptor in CHO cell membranes with an affinity of 0.48 nM (Table 2), consistent with previous studies [40,46]. The B_{max} for this non-peptide antagonist (26 pmol/mg, Table 2) was similar to the B_{max} for the peptide antagonist [^{125}I]astressin (24 pmol/mg, Table 2). All the non-peptide antagonists tested displaced [^{3}H]NBI 35965 binding to the CRF_1 receptor (including arylamidrazone-51), with the maximal extent of inhibition exceeding 90% (Fig. 4; Table 4). This finding is consistent with all ligands binding a common overlapping binding site on the receptor. The K_i value of compounds in displacing [^{3}H]NBI 35965 binding was not significantly different from K_i in displacing [^{125}I]sauvagine binding (compare data in Tables 3 and 4, statistical analysis in legend to Table 4). This finding confirms that [^{125}I]sauvagine is a suitable ligand for reliably

Table 4 – Affinity and maximal inhibition of non-peptide ligand displacement of [^{3}H]NBI 35965 binding to the CRF_1 receptor in CHO cell membranes

Ligand	pK_i (K_i, nM)	Maximal inhibition (%)
'Standard' antagonists		
NBI 27914	9.02 ± 0.16 (0.96)	99 ± 1
aryltriazole-(S)-71	8.84 ± 0.04 (1.4)	98 ± 1
antalarmin	8.90 ± 0.03 (1.3)	101 ± 1
NBI 30775	8.60 ± 0.06 (2.5)	101 ± 1
DMP696	8.22 ± 0.04 (6.1)	101 ± 1
NBI 34041	8.62 ± 0.04 (2.4)	101 ± 1
NBI 35965	8.67 ± 0.04 (2.1)	100
'Non-standard' antagonists		
arylamidrazone-51	6.67 ± 0.15 (220)	92 ± 3
desmethyl-NBI 34041	7.19 ± 0.14 (64)	100 ± 2

Displacement of [^{3}H]NBI 35965 binding to the CRF_1 receptor was measured as described in Section 2 for 'standard' and 'non-standard' antagonists (Table 1). Data were fitted to a single-affinity state inhibition equation and K_i determined from IC_{50} using the method of Cheng and Prusoff [61]. Maximal inhibition values were calculated by dividing the binding inhibited by compound by that inhibited by unlabeled NBI 35965 (included as a control). Differences of pK_i between displacement of [^{3}H]NBI 35965 binding and [^{125}I]sauvagine binding (see Table 3) were tested statistically by two-factor ANOVA. This analysis reported no significant difference between the data for the two radioligands ($p > 0.05$), no significant interaction effect ($p > 0.05$), and the anticipated significant difference of pK_i between different compounds ($p < 0.001$). Data are mean ± S.E.M. (n = 3–10).

measuring receptor binding affinity of the unlabeled non-peptide antagonists used in this study.

4. Discussion

Molecular interactions between the transmembrane region of the CRF_1 receptor and non-peptide ligands are not well understood. Previously, mutation of the methionine to isoleucine at residue 276 has been shown to reduce the binding affinity of NBI 27914. In this study we investigated the mechanism of this mutation's effect on non-peptide ligand binding. The principle findings are: (1) M276I mutation reduced affinity of all standard non-peptide ligands tested; (2) Rotationally constrained aliphatic side chains at residue 276 (of isoleucine and valine) reduced non-peptide ligand affinity whereas substitution with the less-constrained leucine had little effect; (3) a compound that lacks a planar core was unaffected by any of the M276 mutations and core demethylation altered the affect of the mutations; (4) none of the mutations affected peptide ligand affinity. Taken together, these data suggest the rotationally constrained side chains act as a steric barrier for localization of the rigid core of the compounds in the vicinity of residue 276. This residue was not a determinant of peptide ligand interaction, consistent with the hypothesis of at least partially non-overlapping binding sites for peptide and non-peptide ligands for the CRF_1 receptor.

Retrospectively, the examination of rotational constraint of hydrophobic side chains at residue 276 indicates that side chains that are unconstrained are tolerated for binding (methionine and leucine), whereas residues that are constrained impair the binding of standard non-peptide antagonists (isoleucine and valine) (Table 3). Side chain rotational constraint has been shown previously to dictate protein–ligand interaction [47–49] and intramolecular specificity of protein interactions [50]. For example, M626I mutation in TM6 of the G-protein-coupled thyrotropin receptor has been shown to produce constitutive receptor activity via steric hindrance, resulting in repulsive separation of TM3 and TM6 [51]. For the CRF_1 receptor, one possible explanation for the effects observed in this study is that rotational restriction constrains the aliphatic side chains such that they act as a steric block to non-peptide ligand binding. Specifically, our findings suggest the flexible side chains of methionine and leucine have the capacity to be positioned away from the non-peptide ligand in an energetically favorable orientation that cannot be adopted by the constrained side chains of isoleucine and valine. It is unlikely that the flexible aliphatic side chains of methionine and leucine have a direct role in non-peptide ligand binding because M276A mutation had no appreciable effect on non-peptide ligand affinity.

The simplest mechanism of steric block by isoleucine and valine mutations is that a rigid region of non-peptide ligand is localized to this region when bound to the receptor. According to this mechanism, eliminating the rigid group in the ligand that is sterically blocked should eliminate the effect of M276I and M276V mutations. The heterocyclic core of non-peptide ligands is generally the largest rigid portion of the compounds tested and is present in all affected compounds (Table 1), so we examined the effect of M276 mutations on binding of an acyclic compound, arylamidrazone-51. Two observations suggest this compound binds the same region of the CRF_1 receptor as standard non-peptide antagonists. First, the compound retains critical chemical structure features of standard non-peptide ligands (the lower ortho-substituted aryl group, separated by a two-atom spacer from a potential hydrogen bond-accepting nitrogen atom; Table 1 [12,38]). Second, arylamidrazone-51 effectively inhibits [^{3}H]NBI 35965 binding to the CRF_1 receptor (Fig. 4B; Table 4). Binding of arylamidrazone-51 was unaffected by M276I and M276V mutations (Fig. 4K; Table 3), consistent with the hypothesis that the rotationally constrained side chains at this position sterically hinder the presence of the heterocyclic core in the vicinity of this residue. We also investigated the effect of core methylation on sensitivity to the mutations using desmethyl-NBI 34041, which lacks the core methyl substituent (Table 1). Removing the core methyl group increased sensitivity to the M276I and M276V mutations by an order of magnitude and introduced sensitivity to M276L and M276A mutations (Table 3). This finding indicates the core methyl group is a determinant of sensitivity to M276 mutations, consistent with spatial proximity between the core and M276. The precise molecular mechanism of this effect remains to be determined.

The putative positioning of non-peptide ligands within the vicinity of M276 could be useful in the generation of a molecular model of non-peptide ligand binding to the CRF_1 receptor. However, at present there is little information regarding the detailed tertiary structure of the CRF_1 receptor or Family B GPCR's in general [26] on which to base a genuinely predictive model that could further aid and enhance non-peptide ligand development. Family B GPCR's share little if any significant primary sequence homology with Family A GPCR's, for which homology models have been developed using the X-ray structure of rhodopsin [52].

None of the M276 mutations significantly affected peptide ligand binding (Figs. 2 and 3A and B; Tables 2 and 3) indicating M276 is not a determinant of peptide ligand binding. Peptide binding determinants have been identified in the extracellular regions of the CRF_1 receptor (Fig. 1) [27–30,53–60]. The identified amino acid determinants of peptide binding are distant from M276, which is located within TM5 (Fig. 1). These considerations suggest the binding sites for peptide and non-peptide ligand are at least partially distinct. This hypothesis is consistent with mathematical modeling of ligand binding data, which imply non-peptide ligand allosterically modulates peptide ligand binding, and vice versa [40,45,46].

In conclusion, we have identified a putative molecular mechanism for the effect of M276 mutations on non-peptide ligand binding to the CRF_1 receptor. The mutant binding data are consistent with steric hindrance between constrained side chains at residue 276 and the rigid heterocyclic core of the compounds, localizing non-peptide ligand within the vicinity of M276. This residue is distant from peptide binding determinants implying allosteric regulation of peptide binding by non-peptide ligands. These findings enhance the present understanding of the molecular basis of non-peptide ligand binding, and the mechanism by which the compounds inhibit peptide binding to elicit their antagonist effect.

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