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Spatial Requirements of the Antagonist Binding Site of the NK_2 Receptor

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Abstract—Computer-aided modelling has been used to identify a putative antagonist binding site in the tachykinin NK_2 receptor. In order to validate the implied spatial requirements for this region, a series of compounds, based on the potent antagonist GR 149861 have been synthesised and their binding affinities established. Our findings suggest the presence of a large hydrophobic cavity in the putative binding crevice of GR 149861.

The neuropeptides substance P (SP), neurokinin A (NKA) and neurokinin B elicit their biological effects via the tachykinin NK_1, NK_2 and NK_3 receptors, respectively.[1] These integral membrane proteins belong to the heterotrimeric G protein-coupled receptor (GPCR) superfamily which all possess a heptahelical intramembranous bundle interconnected by extracellular and intracellular hydrophillic segments.[1–3] The physiological and pathological roles of tachykinin receptor activation emphasise the need for highly specific, bioavailable antagonists. Hence the majority of efforts in the field of tachykinin receptor research are focused on the design of specific non-peptide antagonists.

Currently there are two main classes of NK_2 receptor-specific non-peptide antagonists (Fig. 1); the benzamide piperidine antagonists SR144190 (**1a**) and SR 48968[4–7] (**1b**) and those derived from indolium-piperidines such as GR 149861 (**2a**) and GR 159897[8] (**2b**). Data generated from the pharmacological characterisation of site specific receptor mutants suggest that these two classes of ligands bind to non-identical receptor sites which overlap partially near the extra-cellular portions of helices 6 and 7.[9–11] In the absence of X-ray crystal structural data for the tachykinin receptors, a 3D molecular model has been developed[12] which utilises the projection structure of the light-activated GPCR rhodopsin as a template.[13] The resultant model accommodates data derived from sequence analysis algorithms for the putative transmembrane segments and indicates the relative orientation of amino acid residues within each α helix.

Here we describe the dissemination of the NK_2 receptor antagonist binding site. The automated molecular docking program AUTODOCK[14] was used to dock SR 48968 **1b**, and GR 149861 **2a**, into the 3D receptor

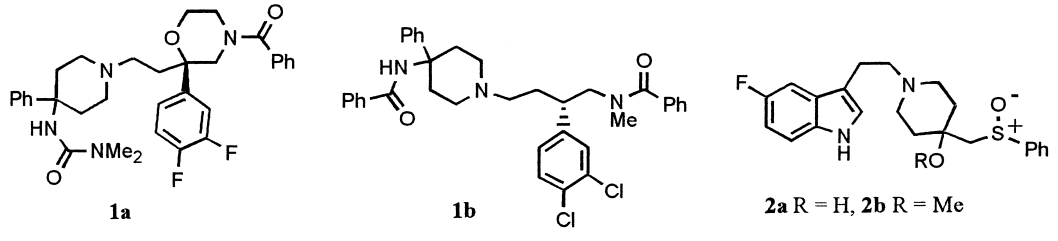

Figure 1. Known inhibitors of the NK_2 receptor.

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model. This approach identifies possible cavities within a static protein and proceeds to generate a large number of conformers for each bound antagonist. With the protein held static throughout the simulation, the antagonist molecule is manipulated to 'fit' inside the cavity, largely by applying a random displacement to each of the degrees of freedom, i.e., translation of its centre of gravity, orientation and rotation around each of its flexible internal dihedral angles. These manipulations result in new conformations of the antagonist molecule whose energy is evaluated using a grid interpolation procedure.

The results of these studies demonstrate a remarkably consistent mode of antagonist–receptor interaction; for both **1b** and **2a**, a broadly cylindrical region spanning the space enclosed by helices 3, 6 and 7 was identified. The possible contributors to binding suggested by these docking studies are to be Asn 86 in helix 2, Ala 116 in helix 3, His 198 in helix 5, Tyr 266 in helix 6 and Trp 294 in helix 7 (Fig. 2). Supportive of these findings His 198, Tyr 266, and Trp 294 are known antagonist binding residues.[9–11]

In the NK_1 receptor, the Asn 87 residue (helix 2) has been shown to be involved in the interchange between the active and inactive conformations of the receptor rather than participating in direct interactions with the agonist.[15] Asn 86 may play a similar role in the NK_2 receptor. Neurokinin binding and specificity appears to be reliant on the interplay of several discontinuous receptor epitopes near the extracellular environment rather than being attributed to interactions with specific residues within the putative binding pocket.[10,16] Nevertheless, it seems that several transmembrane residues, namely Phe 112 (helix 3), Val 201, Tyr 206 (helix 5) Phe 270 (helix 6) and Leu 292 (helix 7), may contribute to the selective binding of NKA to the NK_2 receptor[9,10] whereas other residues in these regions (Gln 109, Thr 115, His 198, Ile 202, His 267,Gly 273, Met 297), seem to be important for the binding of radiolabelled NKA to receptors in the active conformation or determine the ability of NKA to compete for antagonist-indicated receptor sites.[9,10,17]

Our data suggest that His 198 also participates in SR 48968 binding.[9] Huang et al.,[10] however, have reported His 198 as a determinant of NKA but not SR 48968 binding. Despite this discrepancy, which may be due to differences in assay technique, this residue is important for the binding of GR 159897 and of the dual antagonist MDL 103 392.[18,19] Similarly, Tyr 266 has been shown to influence the binding of both NKA and SR 48968 as well as that of the bicyclic peptide antagonist MEN 11420,[10,11] whereas Phe 270 (which resides one helical turn away) appears only to influence peptide ligand binding[11] and Tyr 289 (near the extracellular side of helix 7) contributes solely to nonpeptide antagonist binding.[10,11,19] This implies that high affinity binding of nonpeptide antagonists appears to depend predominantly on elements in the fifth to seventh transmembrane segments whereas the peptide ligands appear to make a larger number of contacts within the helical bundle. Helix 7 is completely conserved in the tachykinin receptors apart from two positions near the extracellular environment. In the NK_1 receptor, substitution of one of these positions (Ile 290-NK_1 receptor numbering) appears to simulate species differences in antagonist selectivity.[20] In the absence of data for other species homologues of NK_2 receptors, it is unclear whether this is also true for the NK_2 receptor. However, it seems from our work that the corresponding side chain (Leu 292) in the NK_2 receptor also influences non-antagonist binding.[21]

According to our docking study the aromatic residues present in both **1b** and **2a** are located in regions of high hydrophobicity with a particularly large cavity accommodating the phenyl amide group of **1b** and the phenyl sulphoxide moiety of **2b**, respectively.

The validity of this proposed binding region was investigated by preparing a number of modified ligands based on compound GR149861 **2a** (Fig. 3). Ligands **3** and **4** were prepared in order to test for the existence of a large hydrophobic cavity in which the phenyl sulphoxide group of **2a** is predicted to reside. Such a cavity would allow **3** and **4** with their sterically more demanding sulphoxide residues to retain high binding affinity to the NK_2 receptor (Scheme 1).

Additionally, due to the broadly linear shape of the putative binding cavity, conformationally constrained ligand **5** was prepared for which modelling studies predicted a very poor fit into the proposed cavity due to the rather angular nature of **5**.

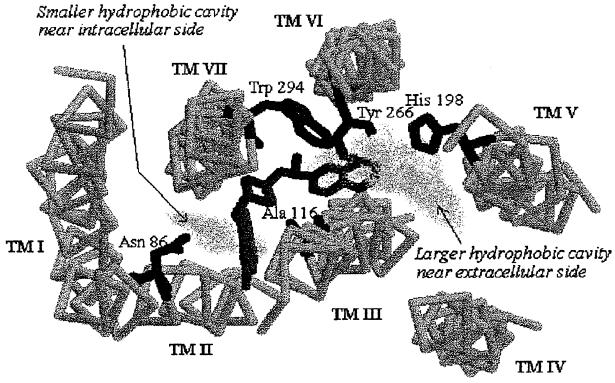


Figure 2. Extracellular view of receptor–ligand **3** interactions.

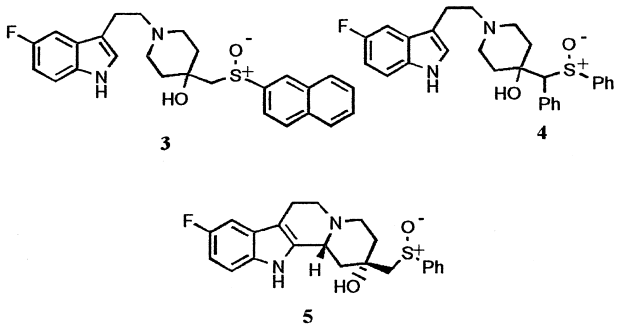
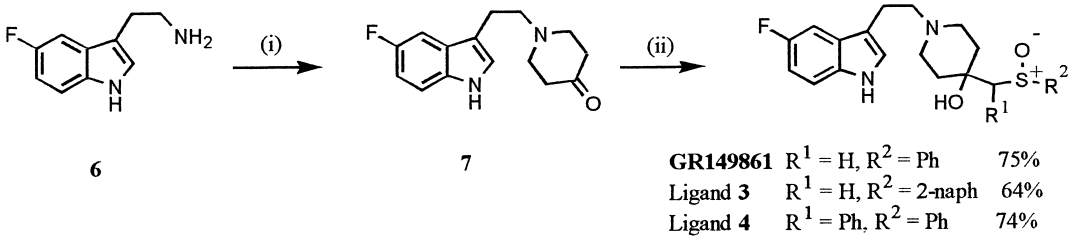
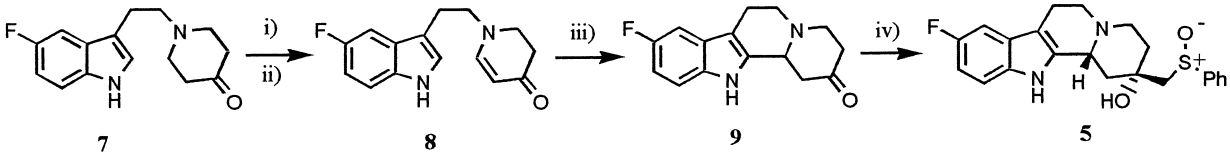


Figure 3. Modified ligands based on GR 149861 (**2a**).



Scheme 1. Synthesis of aryl-sulphoxide analogues of GR149861. Reagents: (i) *N*-methyl-4-piperidone methiodide, K_2CO_3, ethanol (65%); (ii) $R^1CH_2SOR^2$, LiHDMS, THF −70 °C.



Scheme 2. Synthesis of ligand **5**. Reagents: (i) MCPBA; (ii) TEA/Ac_2O, (65%); (iii) 10% H_2SO_4 (60%); (iv) LiHDMS, methyl phenyl sulphoxide, THF at −70 °C (61%).

The sulphoxides **3**, **4**, **5** and also **2a** were prepared via the condensation of 5-fluoro-tryptamine **6** with *N*-methyl-4-piperidone methiodide to give the amino ketone **7** in good yield. This was then followed by the addition of the appropriate sulfur stabilised anion. The 5-fluoro-tryptamine **6** was prepared following a procedure described for the synthesis of tryptamine by Pelchowicz and Bergman.[22]

Polonovsky oxidation[23,24] of the amino ketone **7** gave the required vinylogous amide **8** in good yield which was then cyclised with 10% sulphuric acid resulting in the tetracyclic amino ketone **9**. Addition of the sulphoxide derived anion then yielded **5** (Scheme 2).

It is interesting to note that this addition takes place from the convex face of ketone **9** as revealed by the single crystal X-ray structure of sulphoxide **5** (Fig. 4).

The ability of **1**–**5** to compete for ^{3}H-SR 48968 binding sites was determined by competition analysis (Table 1).[27]

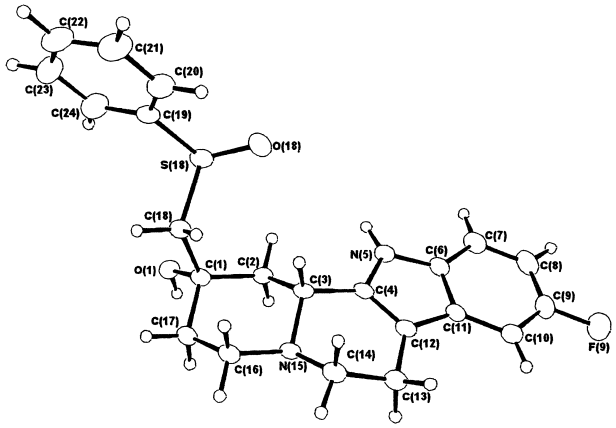


Figure 4. X-ray crystal structure of ligand **5**.

Clearly the affinity of analogue **3** indicates that the presence of a naphthyl moiety still allows near wildtype binding to the NK_2 receptor. It is interesting that a similar result has been reported[25] for an analogue of **1b** in which the *N*-benzoyl moiety was replaced by an *N*-naphthyl group. This lends strong support to our proposal that the phenyl amides of **1a** and **1b** and also the phenyl sulphoxide of **2a** and **2b** reside in the same hydrophobic cavity. The presence of a phenyl residue in the α position to the sulphoxide as in **4** results in a 5-fold reduction in receptor affinity. This suggests that in this case, the bulky phenyl ring partially hinders the interaction of the neighbouring hydroxyl group with a nearby receptor side-chain residue. The 9-fold higher affinity of **2b** as compared to **2a**, which is consistent with previously reported affinities of these two compounds,[8] suggests that the addition of the methyl group optimises the fit of **2b** into a putative binding crevice. This crevice is unlikely to be large enough to fully accommodate the additional phenyl residue of **4**. The proven poor receptor affinity of the conformationally constrained analogue **5** strongly supports the proposal that the antagonist region in the NK_2 receptor is broadly linear and as such **1**–**5** appear to bind in conformations energetically near to their global minima when calculated in vacuo.[26] This structural information paves the way for the rational design of completely new NK_2 receptor antagonists. Studies directed towards this goal will appear elsewhere.

Table 1. The affinities of **1a**, **1b**, **2a**, **2b**, **3**, **4** and **5** for heterologously expressed human NK_2 receptors

Compound	IC_{50} (nM) ± SEM
±**1a**	6.0 ± 0.9 (4)
±**1b**	14.3 ± 1.0 (4)
±**2a**	22.0 ± 3.1 (13)
±**2b**	2.5 ± 0.3 (4)
±**3**	13.4 ± 3.5 (4)
±**4**	111 ± 28.9 (4)
±**5**	25342 ± 11947 (5)

Acknowledgements

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27. IC_{50} values are means of three experiments, standard deviation is given in parentheses.

The wildtype NK_2 receptor cDNA (provided by Glaxo Wellcome) was subcloned into pFASTBAC 1 for subsequent expression in sf9 insect larval cells. 20 μg of crude membranes prepared by homogenisation were incubated for 60 min at rt with 0.5 nM ^{3}H-SR 48968 in the presence or absence of unlabelled competitor. Nonspecific binding was determined using 2.5 μM SR 144190. Samples were then harvested by GF/B fibre filtration for scintillation counting. Data were fitted by computerised nonlinear regression using Graphpad Prism v 2.0 (San Diego, CA). Compounds **1a** and **1b** were gifts from Sanofi Recherche and **2a** and **2b** from Glaxo Wellcome.