Investigation of 5-HT_4 agonist activities using molecular field analysis

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Determination of the structural properties of the 5-HT_4 agonist binding site is the major aim for developing specific selective agonists. These agonists are of potential therapeutic value for treating irritable bowel syndrome. In an attempt to deduce structural properties of the 5-HT_4 agonist binding site, a pharmacophore model and the molecular structural properties of known agonists have been compared with respect to biological activity. The pharmacophore model was developed using seven known active agonists, indolecarbazimidamide, 3-*N*-isopropylbenzimidazolone amide, 3-*N*-ethylbenzimidazolone amide and benzamide, (*R*)-zacopride, 5-CT and metoclopramide. Extensive study of the X-ray structures of these agonists, or closely-related compounds, identified important interactions constraining the conformational flexibility of the sidechains of the agonists. This knowledge, together with molecular mechanics optimization methods, allowed us to deduce the likely binding conformations of the agonists at the 5-HT_4 agonist binding site. Superimposition of the agonists was carried out using atom-by-atom fit with respect to 5-HT. This alignment was used to develop CoMFA models of the likely 5-HT_4 agonist binding site. The models were highly predictive with cross-validated q^2 values of 0.26–0.51 and final r^2 values of 0.96–0.99. Steric, electrostatic and lipophilic properties of the agonist were all found to be important. The final model identified regions in 3D property space which were important for modulating the activity of agonists.

Introduction

5-Hydroxytryptamine (5-HT) is a neuromodulator and neurotransmitter involved in controlling an array of functions in humans.[1] The actions of 5-HT are mediated by a number of specific receptors,[2,3] which have been classified on the basis of experimental data using selective agonists and antagonists. This led to the definition of four main subgroups, 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4.[4]

Recent interest has focused on the role of 5-HT_3[5–7] and 5-HT_4[8] sub-types in disease processes and also in their structural similarity.[9–11] This similarity generally translates into similar affinity values of the present generation of 5-HT_3 and 5-HT_4 ligands in binding experiments. However, functional data has revealed major differences, in that for instance, BIMU 8 and cisapride have been shown to be antagonists at 5-HT_3 receptors but full agonists at 5-HT_4 receptors.[12] Several computational studies[7,13,14] have led to the definition of an accepted three-dimensional model of the 5-HT_3 interacting site. The 5-HT_4 receptor X-ray crystal structure has not been elucidated, nor has the structure specificity of the agonist site been fully characterised. Therefore, our aim in this investigation was to characterise the agonist binding site using active known ligands,[15–17] establish an initial pharmacophore model and develop a receptor map without prior knowledge of the receptor geometry. These data will be utilized for the design of more potent, and with additional information on the 5-HT_3 site, more selective 5-HT_4 agonists.

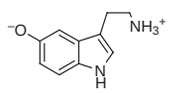
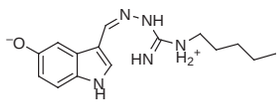
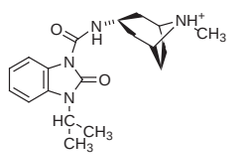
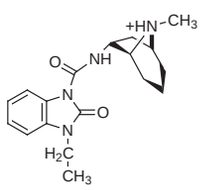
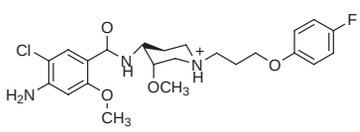
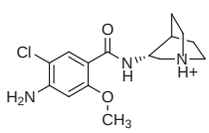
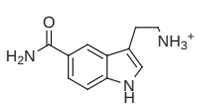
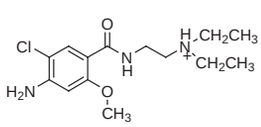
We have selected seven 5-HT_4 agonists from several chemical classes to investigate the topographical requirements of the target 5-HT_4 agonist binding site. There is relatively sparse information concerning the activity of the agonists at receptors other than 5-HT_3 and 5-HT_4 sites. (*R*)-Zacopride would appear to be amongst the most selective of the chosen compounds, but cisapride displays moderate affinity at 5-HT_{1A}, 5-HT_{2A}, D_1-adrenoceptors and dopamine D_2 receptors.[18] We used the comparative molecular field analysis (CoMFA) method to map these features. It is anticipated that the design of selective 5-HT_4 receptor agonists will lead to the development of specific drugs for treating the irritable bowel syndrome.[19]

Results and discussion

The ability of 5-HT to act at several receptor subtypes is mainly due to: a) the flexibility of the β-aminoethyl group and b) the structural diversity of its protein receptor binding sites. By exploring these aspects it should be possible to deduce some structural features which could lead to greater knowledge of the structural complementarity of host–guest specific affinity. Indeed the X-ray crystal structure of the target 5-HT_4 receptor is the choice for designing selective ligands. However, in the absence of this data, topological information about the binding site may be obtained from its known active ligands[16–18] as has been undertaken in our present research program.

The eight agonists, which include 5-HT itself, chosen for this study have biological activity quantified in terms of potency values at enhancing twitch responses of the electrically stimulated guinea pig-ileum or peristaltic reflex preparations. Both of these preparations are considered to be standard and selective functional screens for detecting 5-HT_4 agonist activity. The only other possible drug class that could produce the same effect on these tissues is the anticholinesterase inhibitors. However, there are no reports in the literature of 5-HT_4 agonists having this activity. The potency values were obtained from the literature and are expressed in potency relative to 5-HT (1.00). The

Table 1 The identities, structures and relative 5-HT_4 activities of the agonists (5-HT agonist activity = 1). State of ionization at pH 7.4 shown in structure. Measured log *P* values of uncharged compounds are in parentheses

No.		Rel. activity	log relative activity	Structure	log *P* (pH 7.4)	log *P* (neutral)
1	5-HT	1	0.00		−2.0	0.01 (0.21)
2	Indolecarbazimidamide	87	1.94		1.4	3.84
3	BIMU 8	6.02	0.78		1.3	3.96
4	DAU 6236	1.89	0.28		0.32	3.91
5	Cisapride	0.13	−0.89		2.52	4.49
6	(*R*)-Zacopride	0.007	−2.15		−1.6	2.37
7	5-CT	0.008	−2.10		−3.8	−1.9
8	Metoclopramide	0.0009	−3.05		−0.5	2.65 (2.62)

chosen agonists are members of several chemical classes and span a wide range of activities (Table 1).

Conformational properties of analogues

Cursory examination of the structures of the eight agonists suggests they may exhibit considerable conformational flexibility. However, intramolecular interactions in some cases, and extended conjugation in others, restrict considerably the conformational space of almost all agonists. Examination of the Cambridge Crystallographic Database[20] (CCD) finds examples of benzimidazolidones like **3** and **4**. In all of these examples an intramolecular hydrogen bond exists between the amide NH (donor) and the ureido carbonyl oxygen (acceptor). This locks the amide sidechain in a conformation which is coplanar with the benzimidazolidone rings.

There are also numerous examples (*e.g.*, refs. 21–23) in the CCD of amidoanisoles like **5**, **6** and **8**. In all of these examples (except a few where additional substituents block this interaction) there is an intramolecular hydrogen bond between the amide NH (donor) and the anisole oxygen. As in the previous case, this locks the amide sidechain into a conformation coplanar with the anisole ring. In the receptor environment (with relative permittivity ~4–5) it is very likely that these intramolecular interactions will be significant and will strongly influence the conformational preferences of the sidechains.

The two compounds serotonin (5-HT, **1**) and the analogue 5-CT (**7**) appear to be more flexible. The X-ray structure of Thewalt and Bugg[24] shows that ethylamino sidechain can adopt a conformation in which some of its torsion angles are *gauche*. However the crystal structure of Amit *et al.*[25] shows 5-HT adopting an extended ethylamino sidechain with an all-*trans* conformation. This conformation positions the ethylamino group in the same plane as the indole ring.

The remaining agonist, **2**, contains the carbazimidamide moiety. Inspection of the crystal structures of related compounds (*e.g.* ref. 26) shows that tautomerism of this moiety, and extended conjugation to the π system of the indole ring, results in a conformation in which the carbazimidamide moiety is coplanar with the indole ring. These restrictions on side

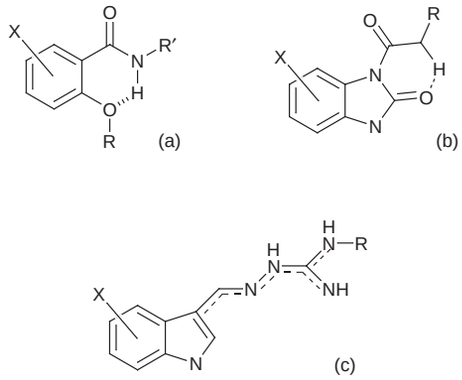


Fig. 1 The intramolecular hydrogen bond formation in the agonist structures a) benzamide b) benzimidazolone and c) carbazimidamide.

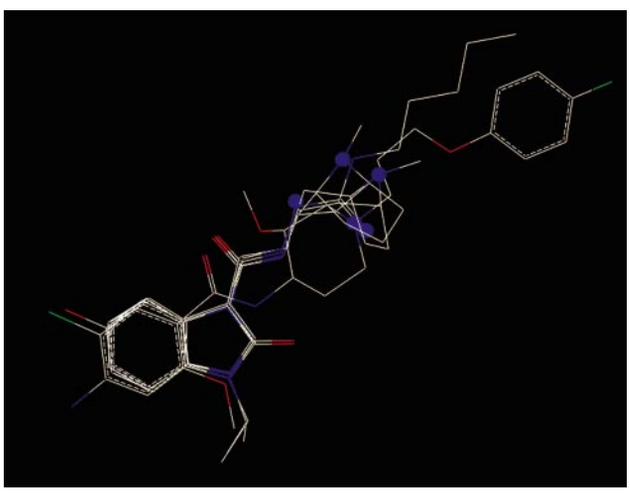


Fig. 2 Superimposition of 5-HT and the four agonists **2**–**5**. The positions of the basic nitrogens are denoted by blue spheres.

chain conformation are illustrated in Fig. 1. The influence of this tautomerism, and to a lesser extent the intramolecular hydrogen bonds which affect the accessible conformations of the other agonists, must be carefully considered when conformational analyses of these compounds are undertaken.

Pharmacophore model of 5-HT$_4$ binding site

The development of the pharmacophore models from molecular superimposition methods[17] have found use in structure–activity studies in explaining the topography which the receptor presents to the ligand. Four of the seven most active agonists; indolecarbazimidamide (**2**),[27] BIMU 8 (**3**),[28] DAU 6236 (**4**)[21] and cisapride (**5**)[29] (Table 1) were used to produce the pharmacophore model of the 5-HT$_4$ agonist binding site. Essentially planar conformations of these four compounds together with 5-HT were used. These were derived from the X-ray structures after geometry optimization using the Tripos force field and appropriate tautomeric or intramolecular hydrogen bond constraints (donor–acceptor distances constrained to 2.7 Å using a force constant of 200 kcal mol^{-1} Å^{-2}). In all cases the minimum-energy conformer chosen for the superimposition was virtually identical to its crystal structure. The superimposition of the four agonists (Fig. 2) was carried out by least squares fit of the rigid portions of the molecules, essentially the rings and sidechains up to the point one bond past the constraints using the FIT algorithm in Sybyl. The basic nitrogen atoms in the side chain of each of the four agonists occupied a small region (radius ~1 Å) approximately 8 Å from the centroid of the benzene rings (superimposed). This pharmacophoric grouping is illustrated in Fig. 3 from which it is suggested that the four agonists could exert their pharmacological

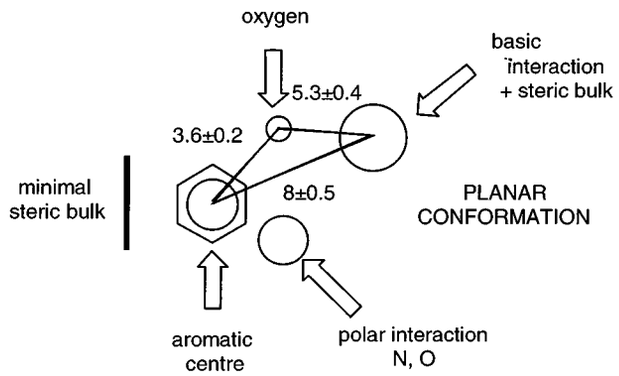


Fig. 3 Pharmacophore model derived from superimposition of the 5-HT$_4$ agonists **2**, **3**, **4** and **5** and 5-HT.

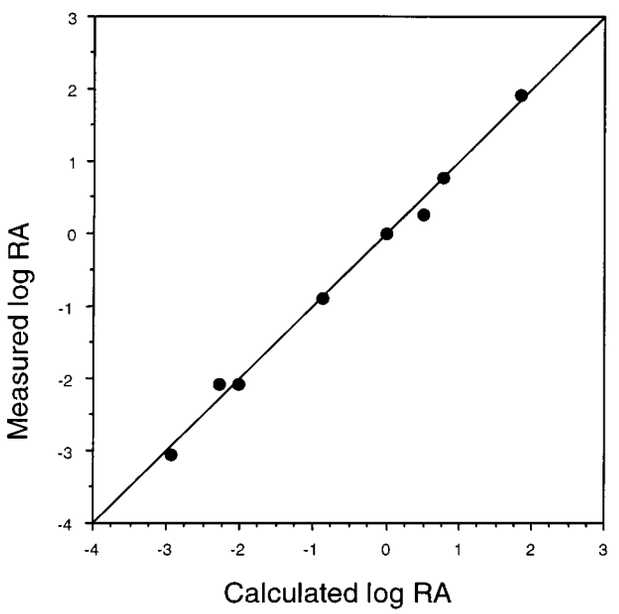


Fig. 4 Observed *versus* predicted log relative activity for best CoMFA model 2 - steric, electrostatic fields and log *P*.

effects by interacting with the same binding site with common binding points on the 5-HT$_4$ receptor.

CoMFA analyses[30]

In this part, three less active agonists [(*R*)-zacopride, 5-CT and metoclopramide][31] were included with the four active agonists (Fig. 2) to widen the range of the agonist activity with respect to structural variation and to establish a more reliable structure–activity model. Prior to the CoMFA analyses, we took the planar, X-ray structures (or modified closely related crystal structures) and carried out geometry optimization using molecular mechanics and the Tripos force field. The optimized geometries were superimposed using the Sybyl Fit routine, in which the RMS error in pairs of atoms are minimized using a least squares criterion. Gasteiger–Hückel charges were calculated for each agonist. CoMFA analyses were carried out using the default values (grid spacing of 2 Å, and a methyl probe with a +1 charge, standard CoMFA scaling). Leave-one-out (LOO) cross validation was used to select the number of principal components and to calculate the cross-validated statistics. The final CoMFA model was generated using no cross validation and the number of components suggested by the LOO validation run. Seven models were derived using the molecular fields in combination. The statistical properties of the models are summarized in Table 2. The quality of the CoMFA models is illustrated in Fig. 4, which show plots of observed log relative

Table 2 Statistical properties of PLS models using various CoMFA fields and log *P*. The LOO cross-validated r^2 value is denoted q^2; SEP is the standard error of prediction; *n* is the number of components used in the PLS analysis; r^2 is the non cross-validated value; SEE is the standard error of estimation; *F* is the *F*-statistic for the analysis; and contributions gives the relative contributions of the fields or log *P*

Model	1	2	3	4	5	6	7
Components	steric electro lipo	steric electro log *P*	steric electro	steric log *P*	steric lipo	electro lipo	electro log *P*
q^2	0.409	0.570	0.443	0.394	0.431	0.218	0.547
SEP	1.99	1.47	1.67	3.48	1.69	1.98	1.35
n	4	3	3	6	3	3	2
r^2	0.999	0.994	0.995	0.999	0.987	0.993	0.936
SEE	0.083	0.168	0.157	0.070	0.259	0.190	0.507
F	720	235	271	671	98	183	36
Contributions							
steric	0.325	0.352	0.488	0.732	0.487		
electro	0.358	0.419	0.512			0.519	0.698
lipo	0.317				0.513	0.481	
log *P*		0.228		0.268			0.302

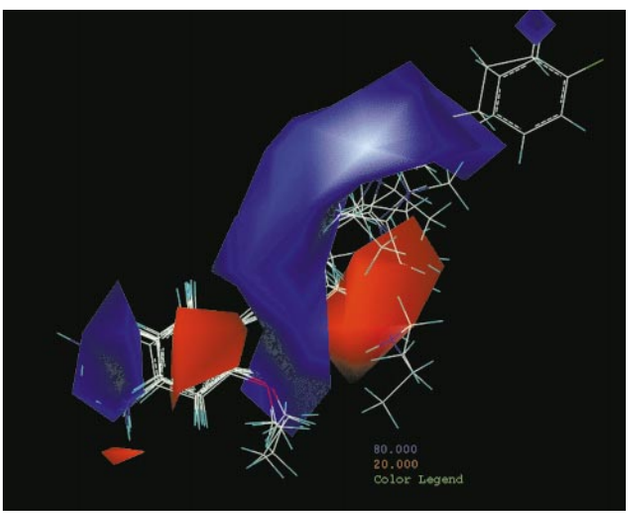


Fig. 5 Electrostatic CoMFA map for model 2 showing contributions to 5-HT_4 activity. Red denotes regions where positive charge is detrimental to activity and blue denotes regions where positive charge enhances activity. The superimposed agonists are also shown for reference.

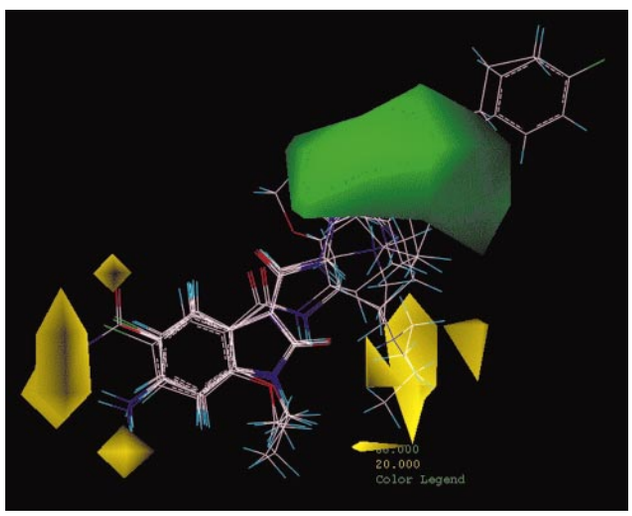


Fig. 6 Steric CoMFA map for model 2 showing contributions to 5-HT_4 activity. Yellow denotes regions where steric bulk is detrimental to activity and green denotes regions where steric bulk enhances activity. The superimposed agonists are also shown for reference.

activities *versus* log relative activities predicted from the best CoMFA model (model 2), using steric and electrostatic fields and log *P*. This model had the highest cross-validated q^2 value, best SEP and used a modest number of PLS components. CoMFA maps from other models were qualitatively quite similar to those of model 2. Model 4 was discarded as it had the worst SEP and a large number of principal components compared with the number of compounds.

Interpretation of the CoMFA studies

The steric, electrostatic and lipophilic fields were highly significant, as was the log *P* value of the agonists. The log *P* values gave a better model than the lipophilic fields in some cases (see Table 2). As the lipophilic fields and log *P* were providing similar information, they were not used together.

The electrostatic map (Fig. 5) shows a region where positive charge enhances activity (blue) near the basic nitrogen pharmacophore region. This region extends down to the region involved in intramolecular hydrogen bonds. There is a small region between these (red) where positive charge is detrimental. This probably reflects the presence of the methoxy group of cisapride, whose activity is substantially weaker than that of 5-HT. The red shaded region in the centre of the aromatic rings probably reflects the electron density in the π systems while the blue shaded region to the left of these rings is indicative of the detrimental effect of electronegative amino substituents in this region in agonists such as metoclopramide, 5-CT and (*R*)-zacopride.

The steric map illustrated in Fig. 6 shows a favourable (green) region of the terminus of the sidechain, with sterically unfavourable (yellow) regions lying near the phenol hydroxy group of 5-HT and opposite the keto groups of agonists **3** and **4**. This latter region reflects the possibly detrimental effect of the methoxy substituent of cisapride on activity.

Details of the pharmacophore model

The pharmacophore model (Fig. 3) was used to identify important structural features of agonist–receptor interaction. The key features of the pharmacophore model are: a) an essentially planar agonist structure; b) the presence of at least one aromatic ring; and c) the presence of a basic nitrogen atom in the ring plane and approximately 7.5 Å from the ring centroid (Fig. 6).

The aromatic centre appears to be essential for binding. There appears to be a requirement for an electronegative atom [the indole ring nitrogen of the 5-HT (or equivalent), or the anisole ether oxygen] next to the ring, and the distance of this atom from the basic sidechain nitrogen (or equivalent) may be important for the interaction with the 5-HT_4 receptor, or for stabilizing the pseudo planar structure of the positively charged sidechain though intramolecular hydrogen bonding (the low activity of **5**–**8** reflects presence of unfavourable structural features such as an aromatic amino group). The distance of the

base moiety nitrogen from the benzene ring centroid may contribute to selective agonist activity as it was consistent in all four agonists and 5-HT within 2 Å. The activity of the four most potent agonists also hints at a requirement for lipophilic interaction in the sidechain. Specifically, indolecarbazimidamide (**2**) and cisapride (**5**), which possess high agonist activities, have characteristic lipophilic groups, *n*-pentyl and γ-(*p*-fluorophenoxy)propyl respectively, and they appear at the lipophilic region of the pharmacophore model. These pharmacophoric aspects were used in our work as a template to design compounds from new chemical classes that may bind at the 5-HT_4 receptor subtype. The phenolic group in the structure of 5-HT is an essential binding group to all 5-HT receptors, but little is known about its contribution to 5-HT_4 agonist activity. Indolecarbazimidamide, the only active agonist with a phenolic group, will be a target for QSAR in our studies. It is not known whether the indole nitrogen (or equivalent) needs to be unsubstituted or alkylated (*i.e.* lipophilic[32]). The investigation of more analogues for QSAR may establish its contribution towards selectivity at the 5-HT_4 receptor.

Comparison with related 5-HT pharmacophore models

Two recent papers have discussed 5-HT_3 pharmacophore models and possible differences between 5-HT_3 and 5-HT_4 receptor binding requirements. Capelli and co-workers[33] employed QSAR methods using molecular orbital-derived descriptors, to find correlations between structure and 5-HT_3 activity. Their three-component pharmacophore model was similar to our 5-HT_4 model (Fig. 3) in specifying limited tolerance to substituents on the benzene ring, an aromatic interaction, and polar interaction with the protonated sidechain nitrogen. However, the position of the sidechain protonated nitrogen atom was much lower than in our 5-HT_4 model. Lopez-Rodriguez *et al.*[7] used the active analogue approach to study the binding requirements of 5-HT_3 and 5-HT_4 receptors. Their pharmacophore models also reflected this difference in position of the sidechain protonated hydrogen between the receptor subtypes. Their 5-HT_4 model also incorporated an additional hydrophobic pocket beyond this nitrogen atom, in the same position as the large sterically favoured green region in Fig. 6, into which the pentyl chain of indocarb and the methyl group of BIMU 8 and DAU 6236 fit. The main difference between the 5-HT_3 and 5-HT_4 ligand binding requirements are therefore in the positions of the sidechain nitrogen atoms, and the degree of tolerance for substituents attached to this nitrogen atom.

Methods

Molecular modelling was performed using the SYBYL6.4 software package on a Silicon Graphics O2 workstation. This was used for the structural search, superimposition, determination of RMS deviation and establishing the pharmacophore model. Gasteiger–Hückel charges were added and the structures minimised using the MAXIMIN2 molecular mechanics method using Tripos force field supplied within SYBYL with the convergence criterion set at 0.05 kcal $Å^{-1}$ mol^{-1}). CoMFA analyses[30] were carried out using a Csp^3 carbon atom probe with a positive charge. Standard grid size of 2 Å, steric cut-off of 30 kcal mol^{-1} and CoMFA scaling was used in all analyses, not just where non field (log *P*) values were included. The leave-one-out (LOO) cross validation was used to select the optimum number of parameters for the final PLS analysis. The lipophilic fields were calculated using the HINT! program[34] using explicit hydrogens, polar proximity effects through bonds, and e^{-r} disance function. HINT! was also used to calculate the log *P* values, as it was able to reproduce the measured log *P* values[35] for 5-HT (0.21) and metoclopramide (2.62) with acceptable accuracy. State of protonation for each agonist was inferred from the pK_a values for the heteroatoms as calculated using the software package pK_a Calculator version 3.5 (Advanced Chemical Development Software). The calculated pK_a values for metoclopramide and 5-HT were consistent with their measured values.[36,37] CoMFA analyses were carried out on the ionized form existing at physiological pH.

Conclusions

This investigation provides important structure–activity information which will be considered in our research program on the design and synthesis of selective and potent 5-HT_4 agonists. This was achieved in spite of the small number of agonists available. The essential design features derived from the agonist study are summarized below.

Intramolecular hydrogen bonding. It appears that the hydrogen atom of the amide bond plays a significant role in determining the agonist activity by forming intramolecular hydrogen bonds (Fig. 1). This has also been reported earlier in the literature.[23] These amide nitrogen atoms, in the presence of an accessible hydrogen bond acceptor (*e.g.* imidazolidone oxygen in example **3** and **4** or anisole oxygen in example **5**), lock the agonists in an essentially planar, conformationally restricted conformation. In new analogues this could be replaced by quinoline or benzopyran, or reduced versions of these.

Orientation of the basic nitrogen atom. The position of the basic side chain nitrogen atom with respect to that of the benzene ring centroid approximately 8.0 Å apart for all agonists, appears to be important as a common structural property.

Lipophilic properties of the agonists. The lipophilic properties may contribute to selectivity. The log *P* values were significant properties in the SAR and lipophilic fields were significant in the molecular field analyses. The increase in both hydration energy and log *P* may lead to higher agonist effect. It was evident that a large lipophilic substituent[25] may be a contributing factor to the specificity of the agonists with highest activity (indolecarbazimidamide, BIMU 8, DAU 6236 and cisapride).

Aromatic substitution. The introduction of isosteric or bioisosteric substituents on the aromatic moiety (or indolyl group) may increase recognition by 5-HT_4 agonist site. The CoMFA model gives us a quantitative basis for evaluating substituent, and heterocycle replacement, strategies.

Acknowledgements

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