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SLATE: A method for the superposition of flexible ligands

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Summary

A novel program for the superposition of flexible molecules, SLATE, is presented. It uses simulated annealing to minimise the difference between the distance matrices calculated from the hydrogen-bonding and aromatic-ring properties of two ligands. A method for generating a molecular stack using multiple pairwise matches is illustrated. These stacks are used by the program DOH to predict the relative positions of receptor atoms that could form hydrogen bonds to two or more ligands in the dataset. The methodology has been applied to ligands binding to dihydrofolate reductase, thermolysin, H_3 histamine receptors, α_2 adrenoceptors and 5-HT_{1D} receptors. When there are sufficient numbers and diversity of molecules in the dataset, the prediction of receptor-atom positions is applicable to compound design.

Introduction

Molecular superposition

A prerequisite for many molecular-similarity studies is the optimal alignment of the ligands in question. This, in turn, requires the relevant binding conformation for each ligand to be ascertained. In the majority of cases, this is a non-trivial problem because most ligands are highly flexible. The sub-problems that need to be solved are categorised below.

1. There are two general strategies for tackling the molecular similarity and superposition problems. Firstly, a method could be developed in which each trial alignment/superposition is followed by a calculation of molecular similarity, which is then optimised. Secondly, an alignment-independent method could be used for determining the molecular similarity first, and once the similarity is optimised the molecular superposition can be performed. The first strategy would place the molecules onto each other by translation and rotation [1]. The second strategy would use a method for molecular similarity that is positionally invariant, such as distance-matrix procedures, but would require a final step for superposing the molecules [2].
2. A method has to be chosen to define molecular similarity. Available methods range from atom-based approaches [2–4] to those that characterise molecular properties on surfaces [1, 5–10].
3. A decision has to be taken whether to use whole molecule molecular similarity (as the majority of methods do), or partial molecular similarity [11]. The utility for which the superposition process is needed will determine the choice between whole and partial similarity. If the similarity study were aimed at gleaning information about possible drug-receptor interactions, then partial molecular similarity would be desired.
4. A decision has to be made about the molecular properties to be included in the matching, care being taken to exclude problems of multi-collinearity.
5. If multiple properties are to be included in the molecular similarity measure, how are the properties to be weighted in the objective function?

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6. With flexible molecules, should a set of predetermined conformations be used [1, 8, 9], or should conformational analysis be performed on the fly [5]?
7. What is an appropriate method for handling flexibility and weighting conformations to be matched? Energy cut-offs tend to be used, but would it be better to use a method based on Boltzmann probability distributions?
8. If the final use of the superposition procedure is to gain a picture of how the superposed molecules match in the site, then a receptor-centric approach is needed to cope with subtle directional equivalencies in the site. For example, a carbonyl oxygen atom in the site can accept a donor hydrogen atom from two distinctly different positions from a pair of ligands.
9. A decision has to be taken for handling a set of molecules. Should they be treated pairwise, or could a method be developed for the set of molecules as an ensemble? Pairwise methods have the advantage that they can be used later in cluster analysis for partitioning the molecular similarity results. However, the disadvantage of this procedure is that the molecular similarity and subsequent superposition of the whole set may not be a true consensus.

The inclusion of completely rigid molecules in the dataset greatly simplifies the superposition, though not all datasets possess rigid compounds. Methods for the unbiased superposition of flexible, dissimilar molecules often rely on either conformational sampling [5–7], in which conformational space is explored in discrete increments, or superposition of representative conformers [1, 8, 9], which assumes that the conformers are numerous and diverse enough to span the whole of conformational space sufficiently. It is only recently that methods have been developed that can cope with flexing more than one molecule simultaneously during the course of the superposition [5].

Superposition of molecules on the basis of atom position [2] is only successful if the molecules are ostensibly similar because atom centres do not define the properties of the ligands as perceived by a binding site. Most methods carry out superposition using molecular properties such as sterics [1], hydrogen-bonding [6, 8], electrostatics [10] or a combination of these [5, 7, 9] because these provide a picture of the ligand from the perspective of the binding site. In each case, the molecules are reduced to a more simple representation for which similarity between the two can be quantified,

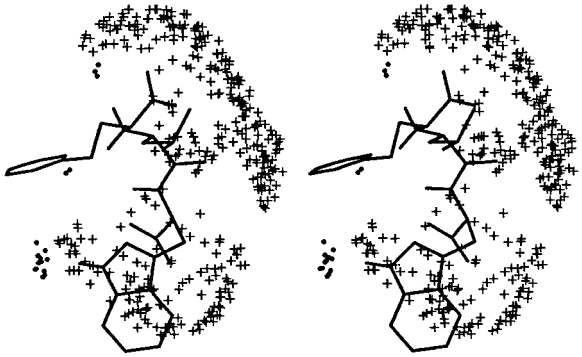

Figure 1. Stereoview of the hydrogen-bond map for the thermolysin ligand CCT. As with all other figures, only those hydrogen atoms bonded to heteroatoms are displayed. Points arising from acceptor groups are labelled as crosses and those from donor groups as dots.

and therefore maximised. In this study, each molecule is reduced to a representation of its hydrogen-bonding groups and aromatic rings to generate superpositions. This reduction in the amount of information allows the optimisation algorithm to deal with the additional complexity created by the flexibility of the molecules.

Hydrogen-bonding and molecular superposition

Previous work has shown that superposition of hydrogen-bonding atoms can be inappropriate because ligand hydrogen-bonding atoms do not need to be superposed in order to interact with the complementary receptor atoms [12]. A receptor-based approach, whereby ligand hydrogen-bond maps (representing all the feasible positions of complementary receptor atoms given a ligand structure) were superposed, was found to be time-consuming [12]. A more common approach is to reduce hydrogen-bonding maps to maximum probability points and to use these as a basis for superposition [5, 6, 8].

The hydrogen-bonding maps [12] about a molecule are typified by that of the thermolysin ligand *N*-{1-(2(*R*, *S*)-carboxy-4-phenylbutyl)-cyclopentylcarbonyl}-(*S*)-tryptophan, (CCT), shown in Figure 1. Regions about acceptor groups are diffuse because lone-pair directionality is not absolute, whereas regions about donor groups are focused because hydrogen bonds tend towards linearity, a trend noted by others [13, 14].

Consequently, the acceptor atoms of different ligands in the same site would be expected to occupy the same positions in space, i.e., be contained within the small hydrogen-bonding region about the receptor donor group. However, the acceptor groups would not be expected to be oriented similarly because almost

regardless of the orientation, the receptor donor group would still lie within the diffuse region generated by the ligand acceptor atom. There are therefore two potential problems with representing acceptor groups on the ligands by their lone-pair positions. First, it is possible that the acceptor atoms are not superposed at all because there is possible rotation about either the single lone pair position or, if there are two lone pairs, the axis joining the lone-pair positions. Secondly, if the atoms are superposed in addition to the lone pairs, the superposition is biased towards orienting acceptor groups (e.g., carbonyl groups will have their C and O atoms superposed) when this is not necessary for both groups to bind to the site. The method described in this paper therefore represents hydrogen-bond acceptor groups of ligands solely by the position of the acceptor atom.

The hydrogen-bond donor groups of different ligands in the same site would all be expected to project (along the X–H bond) to the receptor acceptor atom. The direction of the projection (i.e, the position of the heavy donor atom) is irrelevant because it could occur at any position in the diffuse region surrounding the acceptor atom on the receptor. Hence, in this study, ligand donor groups are represented by the optimum position of the receptor acceptor atom, a projection along the X–H bond.

Strategy

In the work presented in this paper, a program, SLATE, has been written to superpose a pair of flexible molecules according to their hydrogen-bonding properties, aromatic rings and any other user-defined atoms. The superposition problem is combinatoric in nature and requires an optimisation method to obtain the final solution. Numerous minimisation techniques have previously been used in molecular-similarity work, including branch-and-bound searching [3], genetic [4, 5] and other evolutionary algorithms [15]. In this work, simulated annealing has been used because it has been shown to work for similar problems [12]. Other methods would be expected to work equally well.

Multiple-molecule alignment is achieved by searching through all pairwise alignments for a conformation of one of the molecules present in alignments with all others. Procedures that superpose multiple molecules simultaneously would be expected to be quicker than those amalgamating the results of multiple pairwise matches because only a single run is required (rather than ${}_nC_2$ pairwise matches for n molecules). However, they could suffer from the disadvantage that they rely on the presence of the same pharmacophoric features in all of the molecules in the set. Features present in only a subset of the ligands are ignored. Consider three ligands, A, B and C. In the worst case, a superposition would not be possible if ligand A spanned two binding-site pockets that were individually occupied by ligands B and C. In the current method, although no apparent superposition is found between B and C, a conformation of A should exist that aligns well with B and C separately.

In this work, the resultant superposition is used to predict the positions of receptor hydrogen-bonding atoms. These positions can be used to build up or validate a receptor model, which could be used to rationalise binding data for compounds. Alternatively, they could form the input for a database query or a *de novo* design program in the search for molecules binding to the same site.

Like most molecular-similarity methods, this work relies on the assumption that the binding site exists in the same conformation for each ligand. It has been tested with a number of examples from the PDB, in which the superposition and receptor-atom positions can be verified using binding sites known not to change significantly with the introduction of different ligands, namely thermolysin and dihydrofolate reductase (DHFR). It was then used in the validation and modification of a model for the binding site of H_3 antagonists, leading to the design and synthesis of novel H_3 antagonists [16]. Finally, the procedure was applied to α_2 and 5-HT_{1D} agonists. Agonists were chosen in preference to antagonists because they are more likely to (a) bind to exactly the same site on the receptor and (b) draw the receptor into the same conformation and therefore present the same site pharmacophore.

All CPU times are given for a single run on one processor of a Silicon Graphics Power Challenge R10000, compiled with optimization level 2 and mips2 flags.

Methods

Critical-point generation

Each molecule is reduced to a small number of critical points to simplify the superposition of a pair of molecules for a given pair of conformations. A hydrogen-bond acceptor group is represented by the position of

its acceptor atom. A donor group is represented by the idealised position of the receptor acceptor atom, which is therefore assumed to occupy the same position for each ligand. This position is a projection along the X–H bond by the optimum hydrogen-bond length as determined from crystal-survey data [14]. Aromatic rings are represented by two points positioned above and below the ring such that their midpoint lies at the centroid and the vector joining them is perpendicular to the ring. The length of the vector determines the importance of the relative orientation of the rings in a superposition. Low values only require the centroids to superpose whereas larger values also force the planes to align. A default vector length of 2 Å was used, though the user can vary this value if required.

Objective function for minimisation

Any given conformation of a molecule is represented by the distance matrix of its critical points. The difference between two molecules is quantified as the sum of the elements of the difference distance matrix (DDM), the matrix created by calculating the magnitude of the difference for all corresponding elements of the matrices [2]. This objective function value varies according to the points selected from each molecule and the correspondence between the points. The selection of points determines which rows and columns are included in the matrix and the correspondence determines the relative ordering of the rows and columns. The superposition of a pair of molecules is achieved by determining the configuration with the lowest objective function value. The program MATFIT [16] is used to generate the superposition from the resultant optimum correspondence. The total number of possible configurations for superposing two flexible molecules A and B is the product of the

- number of conformations of molecule A: this number is effectively infinite because each torsion angle can have any real value
- number of conformations of molecule B
- number of combinations of donor, acceptor and aromatic points from A: for example, if molecule A contains 3 donor, 1 acceptor and 2 aromatic points and 3 points are required for superposition, there could be 3 donor points (1 possible selection), 2 donors and 1 acceptor (3), 2 donors and 1 aromatic (6), 1 donor and 2 aromatics (3), 1 of each (6) or 1 acceptor and 2 aromatics (1), giving 20 possible combinations of points
- number of combinations of donor, acceptor and aromatic points from B
- number of correspondences between selected points of A and B: for example, if there are 2 donors and 1 acceptor point from each ligand being matched, there are 2 ways of matching them (donors can only correspond with donors, acceptors with acceptors and aromatics with aromatics).

The DDM method has the advantage that it is rotation and translation invariant, removing 6 potential degrees of freedom. There are, however, two disadvantages inherent in the method. First, for a single trial, the number of points for a given superposition must be constant since lowering the number of points improves the value of the objective function because the number of elements of the DDM has reduced. Therefore separate runs must be carried out for each feasible number of matching points. This is dealt with in terms of null correspondences [3]. A run matching the maximum possible number of points (the lower of the number of points from the two molecules) contains no null correspondences. A reduction in the number of points to be matched is carried out by increasing the number of null correspondences. The user therefore specifies the total number of points to be matched; the algorithm decides how many of these points are donor, acceptor or aromatic. For example, if there are 10 critical points in molecule A and 9 in molecule B and if there are no null correspondences, 9 points are matched because all points from B have a correspondence in A. If there are 3 null correspondences, 6 points would be matched. Secondly, DDM methods cannot distinguish mirror images, which have identical distance matrices but do not superpose. Post-processing of the matches reveals such discrepancies because the rms separation of the superposed points is high.

Minimisation of objective function

The simulated annealing algorithm is very similar to that used to superpose hydrogen-bonding regions [12], and is summarised in Figure 2. At each step, the torsion angle of a random rotatable bond is changed by a random amount. Although no conformational energy calculations are carried out, each new conformation is checked for steric clashes. The correspondence between the critical points is optimised by brute force or using simulated annealing if the number of possible correspondences exceeds 1000. However, if the correspondence does not change for 10 successive updates of the global minimum, this correspondence is as-

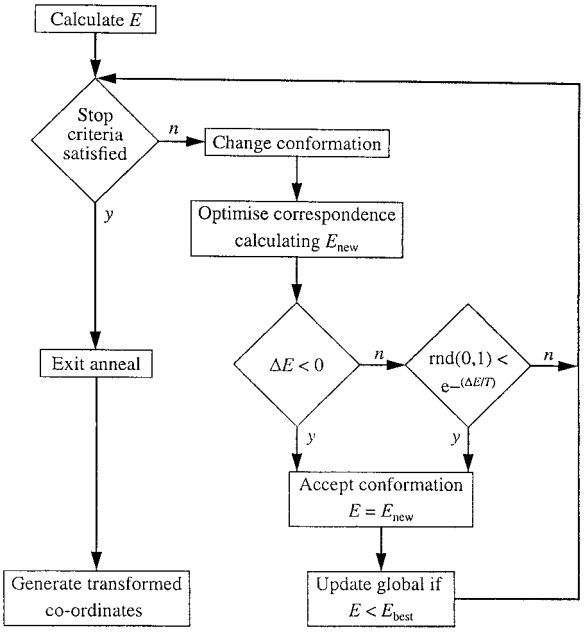

Figure 2. Flow chart illustrating the annealing algorithm from SLATE. E is the objective function value, ΔE the change in value of E and T the annealing temperature.

sumed to be optimal and is retained for all future conformations, allowing the algorithm to concentrate its efforts on obtaining the final conformations. If the new value of the objective function is lower than the current value, the change is unconditionally accepted. Otherwise the Metropolis test is used to decide whether the new conformation is accepted. More positive values of ΔE are less likely to be accepted as the annealing temperature is reduced. The default Markov chain length (number of conformational changes before altering the temperature) is 6000 and the maximum possible number of Markov chains is 500. A dynamic cooling schedule is used [2], allowing the rate of cooling to be dependent on the current state of the system. The stop criteria for the algorithm are: (a) a perfect match is obtained i.e., the objective function value is zero; (b) 500 Markov chains are completed; (c) the proportion of changes accepted falls below a certain value, default 0.004; (d) 40 Markov chains pass without an update of the global minimum; (e) the maximum change in E for one change in conformation is equal to the difference between the highest and lowest values observed during the Markov chain (the Huang stop criterion [18]).

The program MATFIT [16] is used to generate the superposition from the correspondence output by the annealing algorithm. The DDM method requires that the number of points to be superposed remains fixed. If this number of points is too high, then all the correct points would be superposed but any extra pairs would raise the rms separation value. To correct for this, if any pair of superposed points are further than a user-defined distance (default 1 Å) apart in the final superposition, that pair of points are deleted from the correspondence and MATFIT is run again on the new correspondence. This procedure is run iteratively until all points superpose to within the threshold distance. It prevents badly superposed points driving the rms-based superposition of MATFIT away from the correct overlay.

Further options/features

The assumption made in carrying out the superposition with SLATE is that the equivalent hydrogen-bond acceptor atoms of the different ligands occupy the same positions in space, and the hydrogen-bond acceptor atoms of the receptor do not alter their positions when bound to different ligands. In reality, there is little energetic penalty to be paid by deviating from these ideal positions in space by a small amount because of the known variation in hydrogen-bond geometry [14], i.e., some degree of tolerance is more realistic than requesting an exact superposition. To this end, if two corresponding distance matrix elements are within a user-defined threshold value (default 0.5 Å) of each other, the appropriate DDM element is set to zero. This increases the likelihood of perfect matches being obtained (according to the objective function) but could be at the expense of accuracy if the points can be superposed perfectly (i.e., for molecules in a congeneric series).

The user can control the program in a number of other ways. First, the rotatable bonds can be defined. By default, the program spins all single bonds, with the exception of phenol C–O and amide C–N bonds, which are flipped such that the torsion angle is $\pm 180°$. Any bonds can be specified as freely rotatable or completely rigid. Secondly, the correspondence between pairs of atoms can be forced beforehand. This can include non-hydrogen-bonding atoms, so for example, lipophilic atoms can be superposed, which is useful for tidying up superpositions. Although this introduces bias into the superposition, it is a useful way of increasing the efficiency of the program for ostensibly similar molecules. Finally, certain hydrogen-bonding groups can be ignored by the program if requested, for example if they are known not to play a role in ligand binding.

Assessment of matches

For any pair of molecules, SLATE obtains different solutions each time it is run because (a) torsion space is sampled continuously and (b) simulated annealing cannot always obtain the global minimum, merely good local minima. Hence, multiple matches are generated, each overlapping a certain number of hydrogen-bonding critical points with a certain rms value. These values alone are not sufficient to discriminate between superpositions, since there is limited steric information contained within these data. Hence, for each superposition obtained, a steric score is calculated by the program PLM [1], which determines the degree of overlap (on a scale of 0–1) of the molecular skins of the two ligands using a grid-based method. Good superpositions require good steric scores (ζ values) in addition to low rms values for the critical points.

Generation of multiple-molecule overlays from multiple pairwise matches

When superposing flexible molecules, different runs would be expected to give different superposing conformations of the same pair of ligands, especially if the ligands are derivatives of each other. Narrowing down the superposition to a single conformation requires the incorporation of more than one other ligand into the superposition although in the simplest case, when one of the ligands in the dataset is rigid, all the other ligands can be superposed onto the rigid base. However, in cases of datasets with no rigid molecules, the bioactive conformation of a ligand can be narrowed down to the subset of conformations that superpose with all the other active ligands. The incorporation of multiple diverse ligands into the dataset ideally would reduce this subset to a single bioactive conformation.

The method for determination of the bioactive conformation is illustrated for three molecules A, B and C. In the first instance, conformations of A are sought that are replicated in matches with B and C. If no such conformations exist, a conformation of B that superposes with A and C is found, and so on. The conformations of a ligand obtained by superposing with two other ligands are compared by computing the atomic rms separation over all non-hydrogen atoms. This calculation is only carried out for atoms of relevance to the pair of superpositions being considered. For example, if molecule A has an alkyl chain involved in the superposition with molecule B but not with molecule C, the alkyl atoms would not have been considered in generating the match AC and therefore its atoms need not be considered in calculating the rms between an AB conformation and an AC conformation.

Generation of receptor-atom positions

From the structure of a ligand, it is possible to output a hydrogen-bond probability map, a representation of all the possible complementary receptor atom positions [12]. When ligands are superposed, the regions where their maps overlap describe the positions of receptor atoms that could form hydrogen bonds to more than one ligand. The introduction of more molecules into the superposition would be expected to focus large diffuse regions down to localised clusters of points, giving a more confident prediction of the positions of the complementary receptor hydrogen-bonding atoms. The program DOH has been written to determine such overlapping hydrogen-bonding regions.

Three-dimensional crystal-survey information [14] is used to plot a hydrogen-bond map of each ligand onto the same grid. Gridpoints arising from more than one molecule represent potential receptor atom positions. This is similar to the previous approach to overlapping hydrogen-bonding regions [12], except that rather than searching for regions where random clouds overlap (which requires a judicious choice of threshold distance to take into account the density of the points, and also takes time because numerous distance values are calculated), coincident gridpoints are used. Preliminary studies have shown that merely outputting these gridpoints produces too many points to give useful predictions. Many of these points are redundant and can be removed. If regions X, Y and Z overlap, the points where only X and Y, or Y and Z overlap need not be considered since a receptor atom that can bond to all three ligands would not be used to its full potential at these points. In order to reduce the number of overlapping points further, there is the option to consider only gridpoints for which the overlapping regions are of the same type (i.e., both donor or both acceptor). This does have the disadvantage that amphiprotic groups on the receptor are not recognised unless more than one donor and more than one acceptor region overlap. Nevertheless, it has been found to filter out a great deal of noise in the data output in many examples studied (data not shown), and has therefore been used in all the experiments presented in this paper.

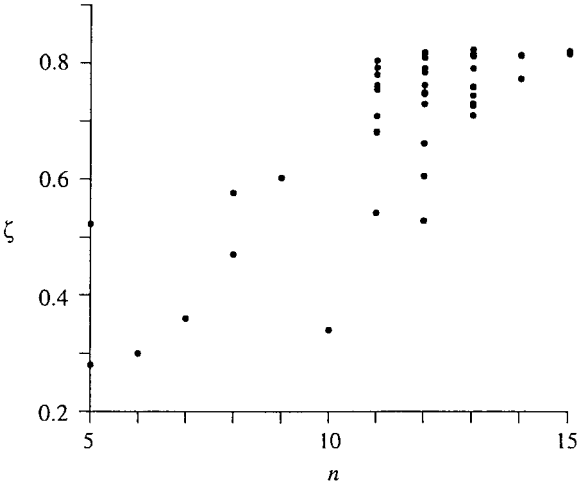

Figure 3. Scatterplot showing PLM steric score ζ against number of hydrogen-bonding points matched, *n*, for 50 matches of methotrexate and folate. For reference, the crystal overlap gave 10 hydrogen-bonding points matched and a steric score of 0.7548.

Table 1. Best and worst 5 ranked results obtained when superposing methotrexate and folate with SLATE

Nulls/	H-bonding similarity			Steric similarity		Sum of
trial	*n*	rms/Å	Rank	PLM score ζ	Rank	ranks
0/3	15	0.530	1	0.8225	3	4
0/2	15	0.559	3	0.8257	2	5
1/10	13	0.363	5	0.8275	1	6
0/1	15	0.532	2	0.8211	6	8
0/7	14	0.450	4	0.8182	8	12
3/3	8	0.457	46	0.4706	46	92
3/1	7	0.520	47	0.3601	47	94
3/2	5	0.168	49	0.5234	45	94
2/3	6	0.513	48	0.2997	49	97
1/4	5	0.435	50	0.2803	50	100

n is the number of points matched.
rms is the root-mean-square separation for the superposed points.

The resultant output is a list of points, tagged with the number of regions overlapping at that point and the type of receptor atom expected at the point. In addition, the program outputs which molecules contribute to which clusters of points. This can be useful because some clusters are only contributed to by one molecule (focusing two hydrogen-bonding groups to a common point), which can then be discarded by the user.

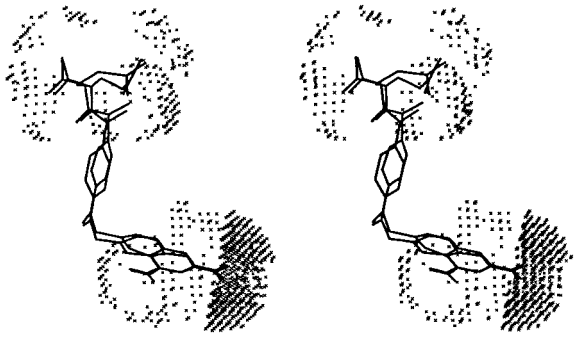

Figure 4. Stereoview of the best match obtained when superposing methotrexate and folate with SLATE, allowing both molecules to flex. The overlapping hydrogen-bonding regions are also shown.

Results

Folate and methotrexate

The co-ordinates of methotrexate and folate were extracted from the Brookhaven Protein Databank [19] files 4dfr and 1dyi, respectively. Ten trials were run for each of 0–4 null points (15–11 matching points), each run taking up to 20 min. The results are shown in Figure 3. The majority of trials produced a steric score of more than 0.6, with at least 11 points superposed. However, this example illustrates the need to run the program a number of times because occasionally, poor results are obtained. They usually occur either when the algorithm has become trapped in a local minimum, or when there are symmetry problems and MATFIT has tried to superpose mirror images. If these results were to be compared with other superpositions to build up a molecular stack, the poor matches ($\zeta < 0.6$ or $n < 11$) would be filtered out before further processing. The results are ranked according to their hydrogen-bond and steric similarities in Table 1. The match with the lowest sum of the ranks is shown in Figure 4, along with the overlapping hydrogen-bonding regions. The diffuse regions of overlap do not provide an accurate prediction of receptor-atom positions because the dataset is too small and not sufficiently diverse.

Although the result is an apparently satisfactory match, it differs greatly from the known crystal superposition of the two ligands (Figure 5). Indeed, the best rms deviation between the atoms of SLATE and crystal conformations over the 50 runs is 2.2 Å for methotrexate and 2.1 Å for folate. This is expected given that in the crystal match, only 10 hydrogen-bond points are superposed (rms 0.587 Å) and the orientation would have been ranked 42 amongst the 50 trials

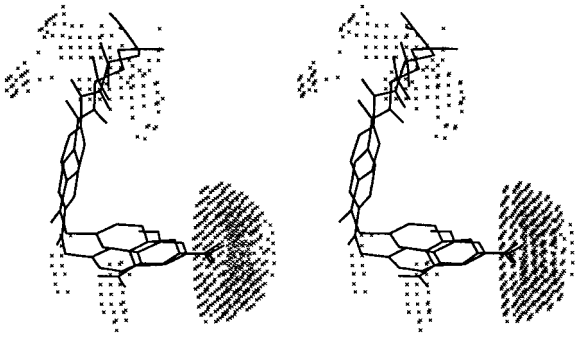

Figure 5. Stereoview of the crystal superposition of methotrexate and folate, shown with the overlapping hydrogen-bond regions.

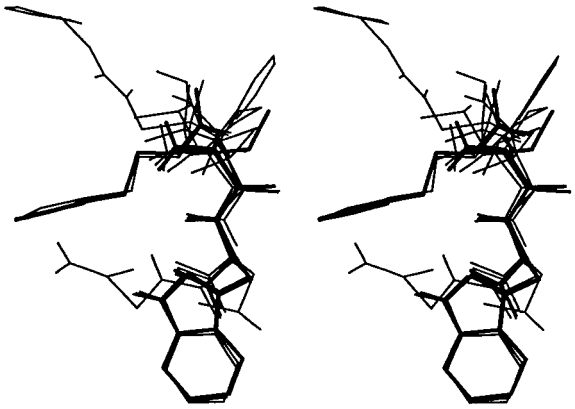

Figure 6. Stereoview of the SLATE superposition of the five thermolysin ligands onto CCT (bold).

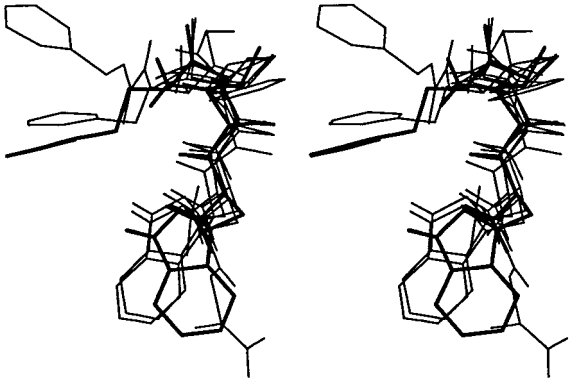

Figure 7. Stereoview of the crystal superposition of the six thermolysin ligands (CCT bold).

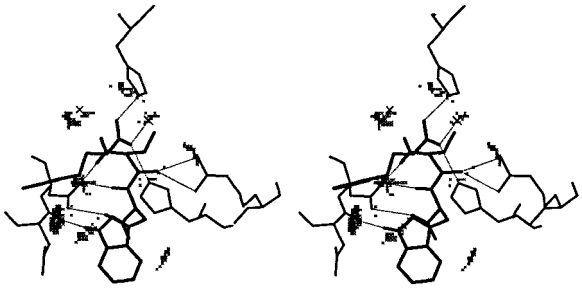

Figure 8. Stereoview of CCT in the binding site of thermolysin, shown with the predicted positions of the hydrogen-bonding atoms. The hydrogen bonds between ligand and site are shown as dotted lines. The crosses on the right and left are the active site zinc atom and a conserved water molecule, respectively.

run. The steric similarity for the crystal orientation is 0.7548, ranked 25 out of 50. These two parameters alone are not able to determine the similarity between the two molecules in the context of the DHFR binding site. This could be because features other than sterics and hydrogen-bonding properties also contribute significantly to the interactions between these ligands and DHFR. Alternatively, the ligands could possess multiple binding modes.

This example highlights three important problems. First, topologically similar molecules are superposed in an obvious fashion. In the case of the DHFR ligands, the obvious superposition provides the wrong result. Furthermore, the ligands chase each other in that there are a number of different conformations for which the superposition appears equally accurate. Which of these solutions is more appropriate requires the incorporation of more molecules (preferably dissimilar) into the superposition. Secondly, not a great deal of information has been learnt about the site if only two molecules have been superposed. The overlapping hydrogen-bonding regions are too large to provide any meaningful prediction of receptor-atom positions. Thirdly, the structure of the site has been assumed to be constant for both ligands. Although this is true for the protein atoms, the bridging water molecules mediating the hydrogen bonds to the carboxylic acid moieties are not consistently positioned, so in effect the sites are different.

Six thermolysin ligands

The structures of six thermolysin inhibitors were extracted from the PDB files 1thl, 1tmn, 2tmn, 3tmn, 5tln and 6tmn. Five of the ligands were allowed to flex freely and were each in turn superposed onto CCT (from 1thl), which was kept rigid in its crystal conformation. This mimics the scenario in which the binding conformation of one ligand is known (either because it is rigid or because there is crystal information) and the binding conformations of other ligands are required. Each match was carried out 10 times for 0–4 nulls (each run taking up to 5 min) and the

best match was chosen using the methods previously described. The resulting superposition is shown in Figure 6. When compared with the crystal superposition (Figure 7), the stack is more compact. Indeed, the steric and hydrogen-bond scores are higher than for the crystal orientations (data not shown). The overlapping hydrogen-bonding regions compare favourably with the hydrogen-bonding atoms of the site (Figure 8), the 11 clusters of overlapping points predicting the positions of 8 atoms to within 1 Å. These atoms are Asn-112 O (0.22 Å), Asn-112 $O^{\delta 1}$ (0.20 Å), Asn-112 $N^{\delta 2}$ (0.37 Å), His-146 $N^{\varepsilon 2}$ (0.37 Å), Arg-203 $N^{\eta 2}$ (0.21 Å) and His-231 $N^{\varepsilon 2}$ (0.44 Å), the catalytic zinc atom (0.80 Å) and the conserved water 362 (0.78 Å). The distance from the zinc atom was quite large because zinc does not form hydrogen bonds with the carboxylate moieties of the ligands; the contacts are shorter (up to 2.2 Å rather than the hydrogen-bond distances of up to 3.2 Å). The distance from the water atom was larger because the conservation of this position is not as great as for the protein atoms; it is held in place by hydrogen bonds rather than covalent bonds. It is notable that only 3 of the 11 clusters gave rise to false positive results, indicative of the improvement in performance seen when more ligands are incorporated into the superposition.

H_3 antagonists

Several classes of H_3 antagonists are known [20, 21], potent representatives of which are shown in Figure 9. They consist of a compulsory imidazole, which could interact with two receptor hydrogen-bonding atoms, attached to a side chain often containing a basic nitrogen atom, which could interact with a receptor acceptor atom [22]. Attachment of a lipophilic residue (tail) can significantly influence the H_3 activity. For many classes of antagonists, the most potent compounds are those with cycloalkyl or (halogenated) benzyl groups attached. However, the SAR concerning the terminal lipophilic moiety throughout the different classes of H_3 antagonists is far from unambiguous and many authors have suggested different binding sites for H_3 antagonists. For example, Stark and co-workers [25] have proposed a different, unique binding mode for the isothiourea derivatives of clobenpropit, as substitution of the isothiourea group with halogenated benzyl groups leads to far more potent compounds than substitution with cycloalkyl groups. Several authors have suggested an alternative binding mode for thioperamide and its derivatives [20, 26–28] and Vollinga et al. described a class of antagonists (represented by **8**), for which the type of lipophilic terminus has little effect on the activity [29].

Recent molecular modelling studies have proposed that H_3 antagonists interact with two distinct hydrophobic pockets of the receptor binding site, explaining the observed differences in the SAR of the different classes of ligands [30]. However, detailed information about the position and shape of these pockets could not be obtained, as the computational methods used did not allow a more accurate determination of the binding mode of the flexible H_3 ligands. SLATE has been used to reveal more information about these interactions.

The molecular co-ordinates of all the ligands shown in Figure 9 were constructed using MacroModel [31]. The basic nitrogen atoms in the side chain of the ligands were protonated with the exception of the nitrogen atoms of the urea, thiourea and carbamate groups [32, 33]. Since, at present, no indication exists about the bioactive tautomeric form of the imidazole moiety [30], the N^t-H tautomer was arbitrarily selected for all investigated compounds.

Aromatic regions were included in the matching procedure to ensure a tight superposition of the imidazole rings. Initially, the lipophilic tails of the antagonists, used to construct the pharmacophore, were truncated to methyl groups, to facilitate steric evaluation of the fits. For the structures containing a piperidine ring, all different ring conformers were generated and used as starting geometries. To this end, MacroModel [31] was used for molecular mechanics conformational analysis of the different possible ring structures (using the ring closure bond option). By rotating all bonds through 360° with increments of 10°, a large number of conformations were generated. These conformations were energy optimised using the Amber force field [34] with the program Batchmin 2.7 [31] in order to obtain low energy conformations.

The approach of pairwise matching was used, i.e., a unique conformation of a reference ligand output from superposition trials with different ligands was sought. Thioperamide was selected as the reference structure. First, thioperamide (with the piperidine ring in the chair conformation) and GT2331 were superposed by running 50 trials of SLATE, allowing both compounds to flex. The fit with the best steric score is shown in Figure 10. In total, six different conformations of thioperamide (set I) were found to match different conformations of GT2331. In a similar experiment, thioperamide and clobenpropit were

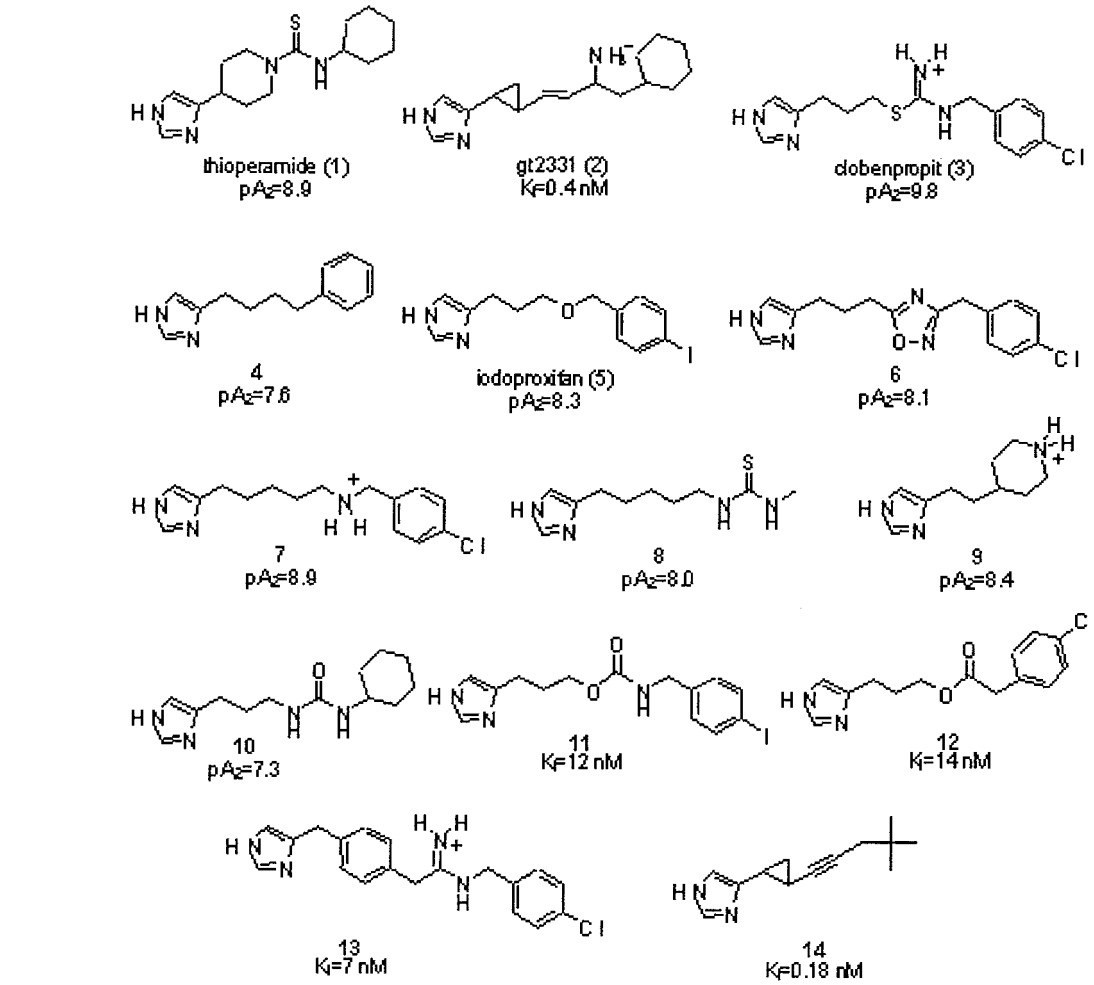

Figure 9. The H_3 antagonists studied. The biological data were determined by evaluation of the influence of the compound on electrically evoked, cholinergic contractions of guinea pig intestine preparations (pA_2) [23] or by evaluation of the influence of the compound on K^+-stimulated [^{3}H]-histamine release on rat cortex (K_i) [24].

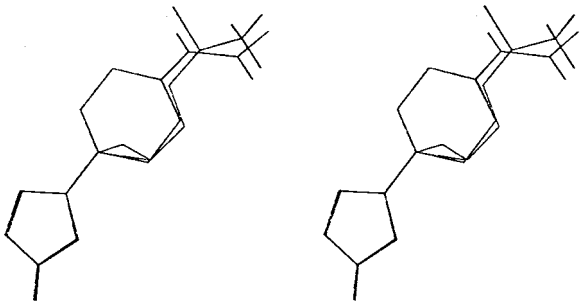

Figure 10. Construction of the pharmacophore by superimposing **1** and **2**, letting both compounds flex. The fit with the highest steric similarity is shown (stereoview).

superposed, allowing both ligands to flex, resulting in many conformations of thioperamide (set II). However, comparison of the conformations of thioperamide in set I and set II revealed that only one conformation was found in both sets. Furthermore, this unique conformation of thioperamide was found in the superpositions that had the highest steric similarity score in both sets I and II. Therefore, this conformation of thioperamide, and the corresponding conformations of GT22331 and clobenpropit, were treated as the bioactive conformations. A unique pharmacophore was only found when the piperidine ring of thioperamide was in the chair conformation. No identical conformations of thioperamide in the different sets could be found when the piperidine ring was (fixed) in a different conformation.

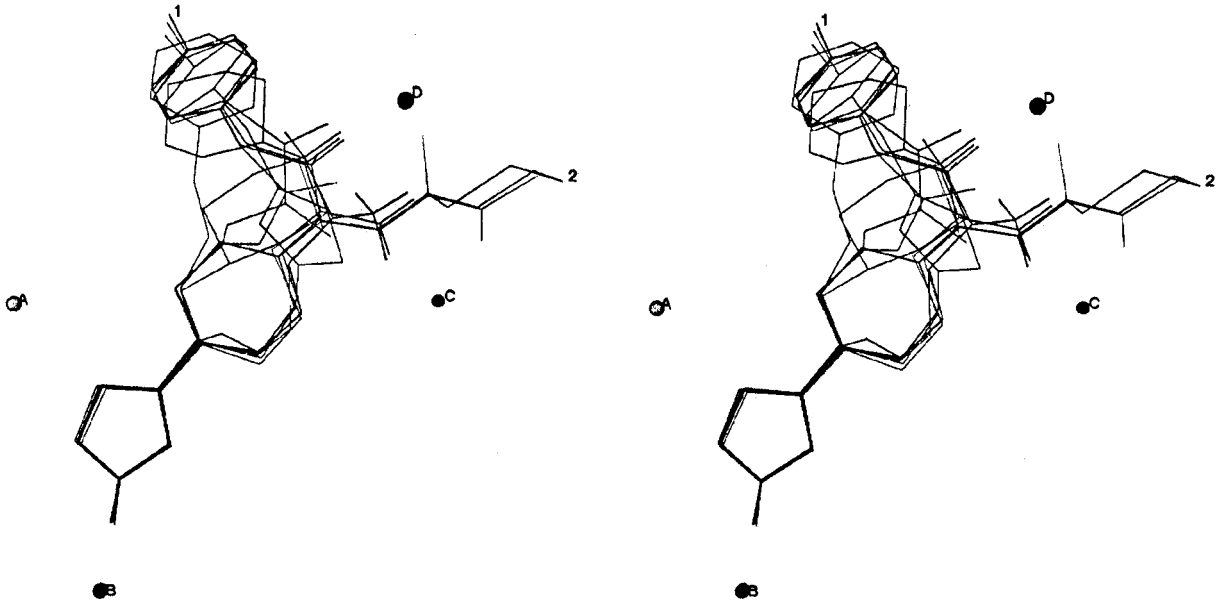

Figure 11. Stereoview of superposition of ligands **1–12** illustrating the position of the four hydrogen-bonding site points (A–D) and two lipophilic pockets (1 and 2) and giving an indication about the steric requirements of the derived pharmacophore.

As can be seen in Figure 10, the methyl groups (that represent the lipophilic tails) of thioperamide and GT2331 have different positions and orientations, indicating that this procedure finds two lipophilic pockets available for antagonist binding, thereby validating earlier findings [30]. Figure 10 also illustrates that it is not necessary to force the basic nitrogen atoms of the ligands to occupy the same position in space. A certain degree of positional freedom for these substructures is allowed to obtain an optimal position to interact with the complementary hydrogen-bonding acceptor site points, hence taking into account the directionality of the intermolecular hydrogen bond.

The lipophilic tail of thioperamide, a cyclohexyl group, was added using the program MacroModel. This substructure was energy optimised using the Amber force field [34] while the positions of all other atoms of the template were fixed. The relative position of the lipophilic tail of clobenpropit, a 4-Cl-benzyl group, was determined by superposition onto thioperamide using SLATE, again using the approach of pairwise matching. The complete flexible antagonists **4**, **5** and **6** were matched with clobenpropit, which was fixed in the proposed bioactive conformation except for the lipophilic tail and the bonds of the isothiourea group, that were allowed to flip 180°. Comparing the three sets as described before revealed the unique relative position of the lipophilic tails.

Having derived the relative position and orientation of the imidazole, the basic groups in the imidazole side chain and the lipophilic tails of these ligands, subsequent ligands were fitted onto this pharmacophore. In all cases, the fit with the highest steric score was selected.

Compounds **13** and **14** could not be fitted satisfactorily onto the pharmacophore with SLATE because the hydrogen-bonding information was not sufficient to drive the superposition. Instead, the in-house program PSEUDO [Tim Perkins, personal communication], which flexibly superposes ligands on the basis of steric similarity (using the same objective function as PLM [1]), was used to obtain the superposition. This procedure revealed another hydrogen-bonding site point.

The antagonistic binding site of the histamine H_3 receptor can therefore be described by four hydrogen-bonding site points and two lipophilic pockets (Figure 11). The imidazole moiety in all of the ligands interacts with site points A and B. Basic nitrogen atoms of the imidazole side chain of the ligands can interact with site points C and/or D. Only clobenpropit is able to interact with all four hydrogen-bonding site points, which might explain the high potency of this antagonist. In addition, it has been suggested that clobenpropit and its derivatives have a different binding mode since cycloalkyl groups as lipophilic tails are

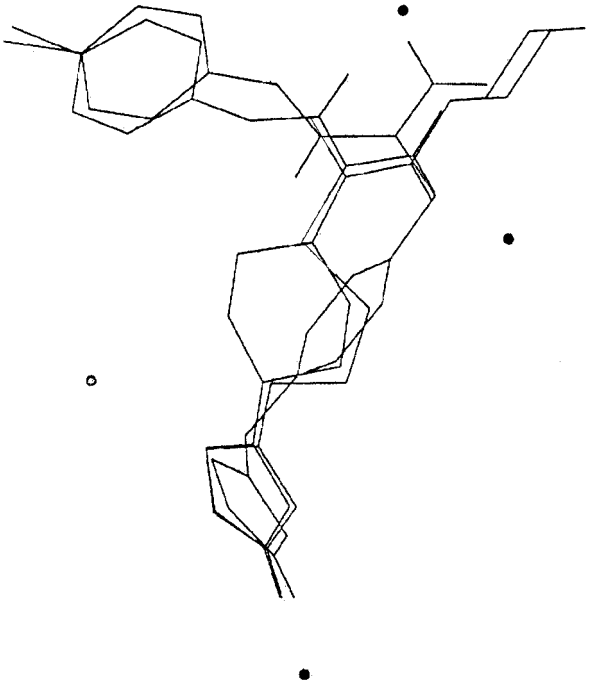

Figure 12. Superposition of **1**, **7** and **13**. Compound **13** does not interact with site points C or D.

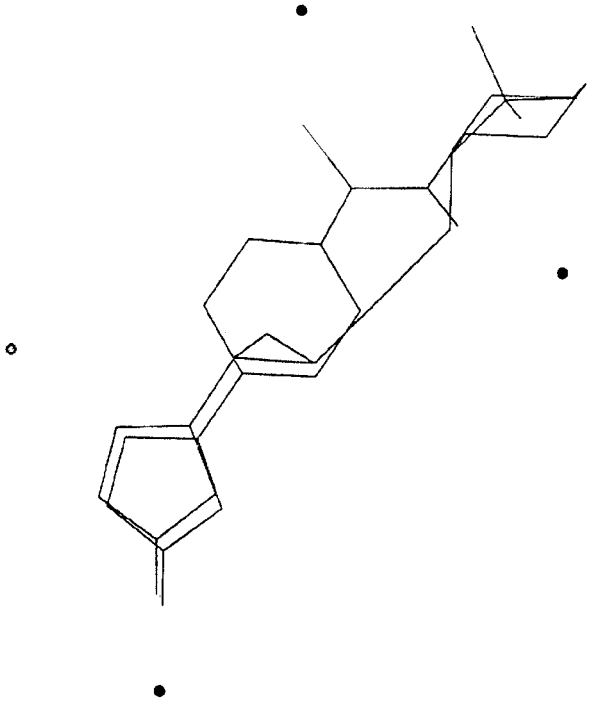

Figure 13. Superposition of **1** and **14**.

less effective than substituted benzyl groups (*vide infra*). The interaction of the isothiourea group with two hydrogen-bonding acceptor site points might benefit significantly from electron-withdrawing groups, e.g., halogenated benzyl groups. This electronic effect will be less prominent for the other classes of antagonists.

Pocket 1 is occupied by the majority of the antagonists (Figure 11), which all contain cycloalkyl and (substituted) benzyl groups. This hydrophobic region is easily accessible and (as is apparent from the SAR) with the proper lipophilic tail, a high increase in affinity can be obtained. The lipophilic tails of **1**, **8** and **14** interact with hydrophobic pocket 2 (Figure 11). The entrance to this pocket seems to be rather narrow, being enclosed by the two site points C and D. The thiourea moieties of ligands **1** and **8** only partly overlap, so the orientation, and hence the SAR, of the lipophilic tails are different.

Compound 13 could not be fitted into the pharmacophore using SLATE. Using PSEUDO under the default conditions, a conformation of **13** was derived (Figure 12) in which the amidine moiety does not interact with the hydrogen-bonding acceptor site points C or D, explaining why SLATE did not find this conformation. No data for the role of the amidine group are available, but these studies imply that this group is not involved in any hydrogen-bonding interaction with the receptor. The program PSEUDO was also used to fit compound **14**. The lipophilic tail of this antagonist, a *tert*-butylgroup, has not been replaced by other lipophilic moieties. Thus, no experimental data are available that could indicate whether the compound interacts with pocket 1 or pocket 2. This superposition (Figure 13) suggests that the *tert*-butylgroup of **14** occupies pocket 2. One of the methyl groups of this moiety is positioned just above the centre of the cyclohexyl ring of thioperamide. This is in perfect agreement with recently published data in which it was shown that replacement of the cyclohexyl ring of thioperamide (**1**) by an adamantly group results in a slightly more potent H_3 antagonist [35]. In this model, the chiral cyclopropyl units of **2** and **14** occupy exactly the same position in 3D space as might be expected when considering the stereospecifity of the H_3 receptor.

Following on from these studies, a number of ligands that interact with all four hydrogen-bonding site points and the two lipophilic pockets have been designed, synthesised, and shown to be active at the H_3 receptor [16].

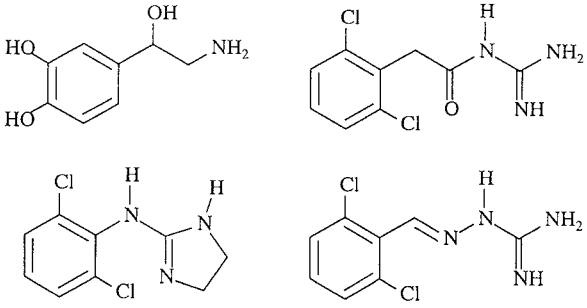

Figure 14. The four α_2 agonists used in the study (clockwise, from top left), noradrenaline, guanfacine, guanabenz and clonidine.

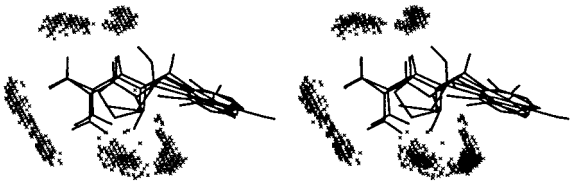

Figure 15. Stereoview of the superposition of the four α_2 agonists, along with the predicted receptor-atom positions.

Four adrenoceptor agonists

SLATE has also been used to superpose four α_2 agonists (Figure 14). The co-ordinates of clonidine and noradrenaline were extracted from the Cambridge Structural Database [36] and those of guanfacine and guanabenz were generated within Cerius2 [37]. Each possible pair of molecules was superposed (10 trials for 0–4 nulls, each taking up to 4 min), allowing complete flexibility for each molecule and including aromatic points in the match. For each ligand, a conformation was sought that appeared in good matches (at least three points overlaid with an rms separation less then 0.5 Å and a steric score greater than 0.5) with the other three ligands. Similar conformations were defined as those with an rms separation of less than 0.1 Å over all non-hydrogen atoms. Only one conformation of guanfacine satisfied these criteria, giving rise to the superposition shown in Figure 15. The resultant overlapping regions of hydrogen-bond probability predict the positions of 6 possible receptor atoms.

Five 5-HT receptor ligands

Dihydroergotamine, 5-HT_{1D} sumatriptan and 311c90 (Figure 16) are all known to possess agonist activity at the 5-HT_{1D} receptor, explaining their use in migraine therapy, whereas methysergide (Figure 17), although used prophylactically in migraine, shows no 5-HT_{1D}

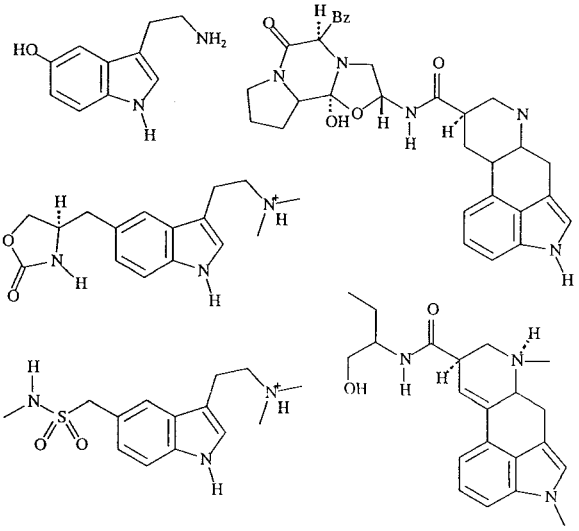

Figure 16. The five 5-HT-receptor ligands used in the study (clockwise from top left), 5-hydroxytryptamine, dihydroergotamine, methysergide, sumatriptan and 311c90.

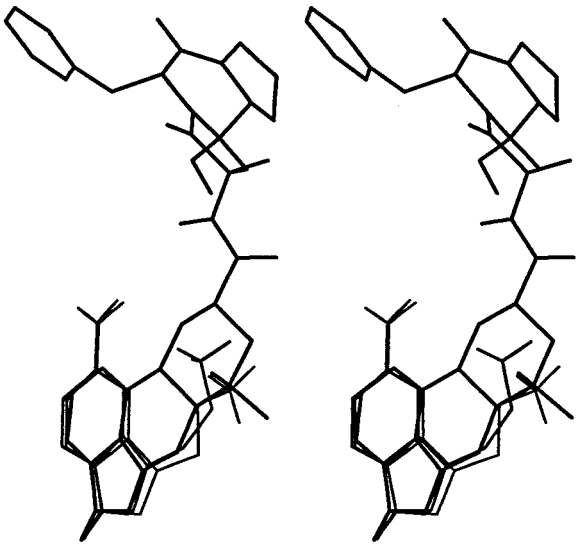

Figure 17. Stereoview of the six acceptable solutions, having run 10 trials superposing 5-HT onto dihydroergotamine (bold).

agonist activity [38]. These five compounds, the latter three of which were used as a test for GASP [5], were superposed with SLATE to identify differences between methysergide and the 5-HT_{1D} agonists.

The co-ordinates for dihydroergotamine and 5-HT were extracted from the CSD [36]. Low-energy conformations for the other molecules were built using CONCORD [39]. Preliminary tests showed that the structure for dihydroergotamine is rigid in the portion superposing 5-HT and when SLATE was used to super-

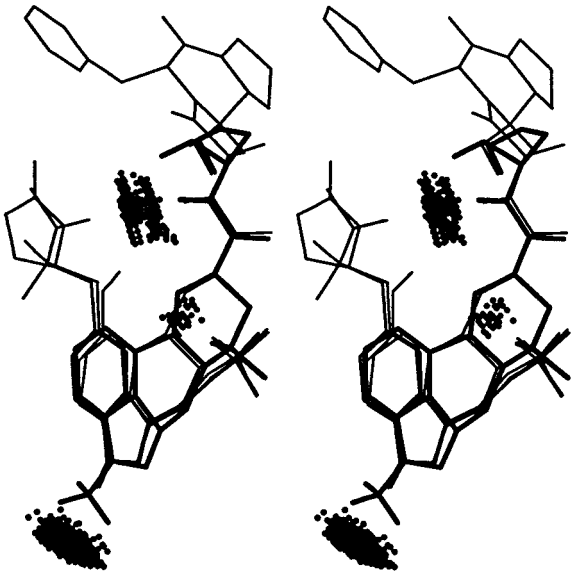

Figure 18. Stereoview of superposition of the five 5-HT receptor ligands, along with the predicted hydrogen-bonding atom positions for the 5-HT_{1D} receptor. Methysergide, known not to act as an agonist at 5-HT_{1D} receptors, is shown in bold.

pose it with 5-HT, a number of conformations of 5-HT were found to superpose equally well in terms of critical points (Figure 17). Following examination of these results, the match was tidied up by forcing the correspondence between the non-hydrogen-bonding carbon atoms between the indole and amine moieties. Dihydroergotamine was then used as a rigid base onto which 311c90, sumatriptan and methysergide were superposed, using the same methods as for 5-HT. Run times were up to 10 min for all examples.

A very tight superposition between all five molecules was obtained, as shown in Figure 18. As expected, the indole rings provide the dominant feature of the superposition because four aromatic points and a predicted receptor acceptor atom position are present in four of the five ligands. The positions of two receptor atoms that could accept hydrogen bonds from all four agonists are predicted by DOH. In addition, two other sets of overlapping hydrogen-bonding regions are identified, which could represent two more acceptor atoms on the receptor.

The superposition also illustraties the differences between methysergide and the other ligands. First and foremost, the hydrogen-bond acceptor region corresponding to the indole nitrogen atom of all four 5-HT_{1D} agonists is occupied by a methyl group from methysergide. In addition to not being able to form a hydrogen bond with the proposed receptor acceptor atom, it sterically will not fit the site as modelled here. Secondly, the indole ring of methysergide is not quite as well superposed. The alternative orientation of the aromatic rings could affect the affinity of the ligand for the site because both aromatic ring stacking and cation-π interactions are directional in nature.

Discussion

A method to superpose dissimilar, flexible molecules on the basis of their hydrogen-bonding (and other) properties has been presented and shown to work for a number of examples. The resulting superpositions can be used in the prediction of receptor atom positions by the program DOH. SLATE and DOH can therefore be used in the rationalisation of binding data for compounds binding to a site of unknown structure and in the search for ligands that would also bind to the same site.

SLATE cannot be used to generate the optimum superposition for a pair of molecules with a single run, which merely generates a single good solution. If both molecules are flexible, there will often be many possible such solutions. The incorporation of further molecules into the superposition is required to choose between alternative matches. Clearly the existence of a rigid molecule in the dataset simplifies the process, though this is not always necessary for meaningful results to be obtained.

The superposition of more than two molecules is carried out by analysing the results of multiple pairwise matches. This has the advantage over methods simultaneously superposing multiple ligands that all possible features of commonality are used, rather than those present in all the ligands, but has the disadvantage that useful results cannot be achieved with a single run of the program. Furthermore, it relies on exactly the same conformations of the same molecule being present in matches with two different molecules. Although similar conformations can often be found, the fact that identical conformations cannot be found (because the torsion angles are covered completely rather than in increments) imparts a systematic error in the procedure. A small degree of fine tuning would be required to obtain more accurate matches, i.e., to convert the similar conformations to identical conformations and to carry the changes through to the other molecules.

SLATE superposes ligands on the basis of their hydrogen-bonding properties, aided by the use of aro-

matic rings and a judicious user-defined choice of non-hydrogen-bonding atom correspondences. It relies on these features dominating the molecular recognition process for the ligands in question, which is not always the case. If the program were to be used completely generally, some way of representing the hydrophobic moieties of a ligand with a small number of critical points would have to be devised. The current approach of using atom positions (representing the hydrophobic moieties) to tidy up matches, although feasible for obviously similar molecules, would not be expected to work in the general case, because atom superposition is not a prerequisite for steric similarity.

A possible future development would be to carry out the superposition of multiple molecules such that they remain within a certain steric volume. The use of excluded steric volumes has proved a useful feature in database searching [40], and an equivalent approach in superposition could help to drive the annealing towards the global minimum, whilst building up a more thorough picture of the common binding site. It would also take some account of the steric similarity during the course of the superposition.

Although SLATE allows flexibility, it does not alter the conformations of rings. Currently, flexible rings are dealt with by generating rings in a number of low energy conformations and treating each conformation as a separate molecule. This has proved a successful approach when applied to H_3 ligands. A future development of the program could be to alter the ring conformations during the annealing, either by using a bond break/closure system as implemented in GASP [5] or by toggling between a number of prestored low energy conformations.

There are currently no energy criteria to be satisfied by any of the conformations generated by SLATE because the program is intended as an idea generator rather than a program to output absolute binding conformations. Furthermore, any energy cut-off criteria applied during the course of the annealing algorithm could hinder progress since it would be harder to leave local wells in the configuration landscape. However, intermolecular steric clashes, the main cause of poor conformational energies, are disallowed and the bond lengths and angles are retained from the input conformation. These criteria would appear to suffice since none of the results obtained yet have given rise to conformations of any ligands that could be considered as inappropriate. However, conformational energy cut-offs could be used to filter out matches in a post-processing step. It is known that ligands do not bind to sites in their global minimum conformations, though suggested cut-off values for the relative energy of the binding conformation vary [41–43]. This value depends on the energy recouped by intermolecular interactions with the receptor and the distribution of conformational energy values for the ligand in solution.

In conclusion, a novel method for the superposition of flexible, dissimilar ligands has been presented and validated. This method can be used in the generation of ligand pharmacophores or receptor models and therefore provides a potentially useful tool for ligand-based drug design.

Acknowledgements

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