Pharmacophores Incorporating Numerous Excluded Volumes Defined by X-ray Crystallographic Structure in Three-Dimensional Database Searching: Application to the Thyroid Hormone Receptor

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In the present study we investigate whether augmentation of pharmacophores with excluded (ligand-inaccessible) volumes can condense the lengthy unspecific hit lists often obtained in 3D-database searching. Our pharmacophores contained hydrophobic features defined by the hormone, hydrogen bond donor and acceptor features of the liganded rat THR-α X-ray structure, and excluded volumes located at the positions and scaled according to the sizes of atoms delineating the binding cavity. We now show, for the first time, that it is perfectly feasible with the Catalyst software to search, in 1−2 h, medium-sized databases such as Maybridge (with 5×10^5 compounds registered as multiple conformers) with pharmacophores containing numerous ($\sim 10^2$) excluded volumes. The excluded volumes did not slow the search significantly; for pharmacophores containing more features they also reduced the size of the hit list the most. For example, with a 7-feature pharmacophore, the Maybridge hit list shrank from 4 to 1. The single remaining compound was subsequently shown to bind to THR-α with an IC_{50} of 69 μM. Thus, we conclude that structure-based pharmacophores augmented with numerous excluded volumes can effectively prune and focus hit lists. The performance of multiple excluded volume-supplemented structure-based pharmacophores in 3D-database mining as implemented with the Catalyst software compares very favorably with other published procedures, with respect to speed, specificity, and ease of use.

Introduction

The principal physiological effects of the thyroid hormones, 3,3′,5,5′-tetraiodo-L-thyronine (T_4) and its 5′-deiodinated congener T_3 (Figure 1), are associated with energy metabolism, the cardiovascular system, and lipid metabolism.[1] Thus, thyromimetics and antithyroid hormone drugs have significant therapeutic potential for the treatment of obesity, hypercholesterolemia,[2] thyrotoxicosis, and cardiac arrhythmia.[3] The actions of thyroid hormones are mediated by soluble thyroid hormone receptors (THRs). There are two subtypes of THR, designated as α and β. THRs belong to the superfamily of nuclear receptors that have a zinc-finger DNA-binding motif.[4] This motif enables the ligand-activated THR to bind to response elements (TREs) situated on DNA upstream of THR-regulated genes. The transcription of these genes is then up- or downregulated in response to the hormone. The IC_{50} for binding of T_3 to THR is approximately 0.2 nM, and T_4 binds with ∼10% of T_3's affinity. In vivo, T_4 is converted to T_3 by deiodinases.

The X-ray crystallographic structures of the liganded rat THR-α was recently published.[5] The structure was solved in the presence of three different ligands, namely, T_3, Dimit (a T_3 isostere where the 3- and 5-iodine atoms are replaced by methyl groups and the 3′-iodine atom is replaced by an isopropyl group), and $IpBr_2$ (an analogue in which the 3-, 5-, and 3′-iodine atoms of T_3 are replaced by two bromine atoms and an isopropyl group, respectively). In these complexes the ligand binding cavity is completely enclosed by the protein. A combination of experimental, quantitative structure−activity relationship (QSAR), and computational chemistry studies[6−9] conducted prior to the determination of the 3D-structure of THR-α[5] has resulted in a number of predictions of important features for ligand binding to thyroid hormone receptors. Below we describe how these predictions (i−v) are borne out in the X-ray crystallographic structure:

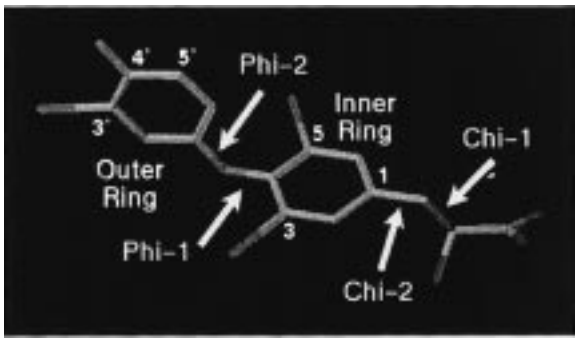

Figure 1. Structure of the thyroid hormones, T_3 ($R_5' = H$) and T_4 ($R_5' = I$), depicting the torsional angles χ_1, χ_2, ϕ_1, and ϕ_2. The natural hormone T_3 is shown in the distal conformation.

(i) The 4′-hydroxyl group of the ligand acts as a hydrogen bond donor.[6,8,10] In the X-ray structure the

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4′-phenolic hydroxyl group of T_3 is engaged in a hydrogen-bonding interaction with the His-381 imidazole Nϵ2 atom, though it is not clear if the hydroxyl group acts as a hydrogen bond acceptor or donor.

(ii) The 3′-pocket of the receptor appears to be hydrophobic since introduction of hydrophilic substituents at the 3′-position of the ligand markedly decreases affinity.[6] The pocket seems limited in depth to the length of the 3′-iodine atom of the natural hormone[6,9] but has sufficient width to accommodate a phenyl or cyclohexyl ring.[6] Analogues of T_3 with smaller substituents than an iodine atom at the 3′-position do not appear to completely fill this hydrophobic pocket since they bind THR with lower affinity than T_3.[6] A ligand's binding affinity is decreased by the presence of a 5′-substituent larger than a hydrogen atom, possibly due to steric limitations.[7] In thyronine derivatives substituted with the same substituent at positions 3 and 5, diiodo substitution produces maximal receptor affinity, and the only non-halogen substituent at these two positions which confers significant affinity is the methyl group.[7] Consistent with this SAR, pockets of limited dimensions exist in the crystallographic structure of THR-α for the 3-, 5-, and 3′-substituents, but not for 5′-substituents much larger than a hydrogen atom.[5]

(iii) The diphenyl ether oxygen atom of thyronines can be replaced by a methylene group or sulfur atom without loss of affinity for the THR.[7] In the X-ray structure of THR-α, the ether oxygen atom is buried within the hydrophobic core of the receptor and is not involved in hydrogen bonding.

(iv) The carboxylate anion of the position 1 side chain of thyroid hormones is most probably involved in an electrostatic interaction with a charged amino acid in the receptor,[10] since removal of the carboxylate leads to a large drop in THR affinity.[7] On the other hand, removal of the amino group increases the receptor affinity slightly.[7] In the X-ray structure, the carboxylate and amino groups of the position 1 side chain of Dimit form direct hydrogen bonds with Arg-228 and the backbone amide hydrogen atom of Ser-277, respectively.[11] Additionally a number of water-mediated hydrogen bonds are made with the carboxylate.

(v) A perpendicular orientation of the planes of the aromatic rings [ϕ_1 (C5−C4−O−C1′) = 90° and ϕ_2 (C4−O−C1′−C6′) = 0°] of 3,5-diiodothyronines minimizes unfavorable steric interactions between the bulky iodine atoms at the 3- and 5-positions with the hydrogen atoms at the 2′- and 6′-positions. In this 90°/0° conformation (Figure 1), the 3′- and 5′-positions are not equivalent, and the 3′-substituent could therefore be oriented either distal or proximal to the inner ring. In the distal conformation, the 3′-substituent is directed away from the inner ring, but in the proximal orientation, it is directed toward the inner ring. Studies of conformationally restricted analogues have shown that the distal conformers of THR ligands are the most active.[7] In the crystallographic structure the ligands adopt a distal conformation in the receptor complex, as the inner and outer rings in the THR-α crystallographic structure appear nearly perpendicular, with the 3′-substituent of the outer ring projecting down and away from the inner ring.[5]

Taken together, the structure−activity relationships and the 3D-structural features of the THR-α ligand binding site presented above clearly indicate that the receptor imposes strict steric requirements on ligand binding. Therefore we have included in our structure-based pharmacophore model building information about regions sterically forbidden to the ligands by the receptor. Coordinates of atoms that are essential for binding in the T_3/THR-α crystallographic complex were used to construct pharmacophore models[12] for database searching. In addition to pharmacophoric points derived from ligand atoms, we used the atomic coordinates of the T_3/THR-α crystallographic complex to define numerous ($\sim$100) excluded volumes which the ligand is not allowed to penetrate. In all previously published Catalyst pharmacophore models, either no excluded volumes have been employed or only a few have been selected which were regarded as being important.[13] The performance of such pharmacophores were evaluated by 3D-database searching with the Catalyst software.

Experimental Procedures

Catalyst Pharmacophore Construction and Database Searching. A training set of molecules and their binding affinities may be used by the program to automatically generate a number of pharmacophore models which specify the relative alignments and active conformations of the ligands consistent with their binding to a common receptor site. Alternatively, the features of the pharmacophore may be placed manually, guided by a receptor X-ray structure as done in the present study. These models may in turn be used to perform 3D-QSAR analyses or as database queries to search 3D-coordinate databases for structurally novel ligands. A pharmacophore model (in Catalyst called a hypothesis) consists of a collection of features necessary for the biological activity of the ligands arranged in 3D-space,[12] the common ones being hydrogen bond acceptor, hydrogen bond donor and hydrophobic features.[13] Hydrogen bond donors are characterized by the availability of an electropositive hydrogen atom (e.g., −OH, −SH, and −NH).[13] Any nitrogen, oxygen, or sulfur atom with at least one available lone pair of electrons is considered to be an acceptor atom. Within the program, hydrogen bond donors are defined as vectors from the donor atom of the ligand to the corresponding acceptor atom in the receptor. Hydrogen bond acceptors are analogously defined.[13] Hydrophobic features are located at centroids of hydrophobic ligand atoms.[13] The location of features in the pharmacophore query was defined by the crystallographic coordinates of atoms in the complex. Hydrophobic features were placed at the centroids of the phenyl rings and the atomic positions of the three iodine atoms of T_3 in its receptor-bound conformation. Similarly the 4′-phenolic oxygen atom of the ligand and the imidazole Nϵ2 atom of His-381 defined the end points of a hydrogen-bonding acceptor or donor vector. Because the side-chain amino group of the T_3 hormone does not appear to be important for binding,[7] it was not incorporated in the pharmacophores. The carboxylate of the position 1 side chain of T_3 was used to define hydrogen bond-accepting features. The remaining atoms delineating the binding cavity of the THR-α were represented as excluded volumes (space which the ligand is not allowed to occupy). For the present study, we have used 1 order of magnitude more ($\sim 10^2$) excluded volumes than used in any other previously reported Catalyst pharmacophore. The excluded volumes were defined within a spherical-based cutoff of 6 or 8 Å from T_3 which corresponds to the atoms, 160 or 319, respectively, delineating the binding cavity of THR-α. The spherical-excluded volumes were scaled to a given percentage of the van der Waals radii of the corresponding atom so as to increase the effective size of the binding cavity in order to partially compensate for the flexibility of both the receptor

protein and ligand and to adjust the hit rate. The values for the van der Waals radii were taken from Pauling[14] (i.e., 1.4, 1.5, 1.7, and 1.85 Å for O, N, C, and S, respectively).

Catalyst "features" are associated with position constraints which consist of the ideal location of a particular feature in 3D-space surrounded by a spherical tolerance.[13] To be retrieved as a hit, a candidate ligand must possess appropriate functional groups which can simultaneously reside within the respective tolerance spheres of the pharmacophoric features. Each feature is associated with a weight (a measure of its proposed importance to the pharmacophore as a whole), and the better the overall superimposition of functional groups of the molecule to the appropriate features of the pharmacophore, the higher the score of the fit.[15] The minimum requirement a pharmacophore model must fulfill is that it successfully identifies T_3 as a hit when included in a database.[13] It is possible to interactively view the fit of the molecules to pharmacophores in Catalyst 2.3. Default parameters were used throughout unless otherwise stated. Database searching was performed with the *Best Flexible Search* method which manipulates the conformers so as to minimize the distances between pharmacophore features and mapped atoms in the molecule.[16] We searched the Maybridge 3D-coordinate database as supplied by MSI with Catalyst version 2.3 with the crystallographic structure-based pharmacophores. In a Catalyst-formatted database, compounds are stored as multiple conformers which are representative of their available conformational space.[17,18] For the in-house test database, conformers were generated with the "best quality" option. Between 20 and 200 conformers were allowed depending upon the number of rotable bonds in a molecule, and a maximum conformational energy of 15 kcal/mol above the lowest-energy conformation was permitted. This number of conformers corresponds to a resolution of the conformational model of 1.5 Å. (The resolution of a conformational model can be defined in terms of the maximum expected rms distance of an arbitary low-energy conformer extracted from an exhaustive set, from a conformation in the model.[17,18])

In Vitro Assay. Compounds matching pharmacophoric queries from the Maybridge 3D-coordinate database were ordered from Maybridge Co. Ltd., Trevillett, Tintagel, Cornwall PL3 OHW, U.K. The affinity of a compound was determined by its ability to compete with [^{125}I]triiodothyronine ([^{125}I]T_3) (New England Nuclear #NEX 110X) for binding to the human THR-α_1 (hTHR-α_1). The receptor protein was obtained from nuclear extracts of sf-9 cells infected with recombinant baculovirus encoding for the hTHR as described previously.[19] The compounds to be tested were dissolved in dimethyl sulfoxide (Sigma #D8779), and serial dilutions of them were prepared in the same solvent. A 4-μL aliquot of the compound solution was mixed with [^{125}I]T_3 (final concentration = 200 pM) and hTHR (final concentration = 20 pM) diluting to a total volume of 204 μL with an aqueous buffer containing 400 mM KCl, 17 mM K_2HPO_4, 3 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mM EDTA, 8.7% glycerol, 6 mM monothioglycerol, and 0.5% unprogrammed rabbit reticulocyte lysate (Promega #L415) and incubated for 18−20 h at 4 °C. The incubation was terminated by separation of THR-bound [^{125}I]T_3 from free [^{125}I]T_3 on Sephadex G-25 columns as described previously,[19] and the THR-bound [^{125}I]T_3 was measured in a γ-counter and plotted versus the concentration of the compound in a competition curve. IC_{50} values were determined for each compound as the concentration of compound required to inhibit 50% of the binding of [^{125}I]T_3 to hTHR.

Results and Discussion

3D-Database searching with typical pharmacophore queries containing a minimal number of features usually generates large hit lists.[20] These hits may be screened for biological activity and/or receptor binding in the laboratory. If the hit list is larger than desired, it can be pruned by increasing the specificity of the pharmacophore by the inclusion of more features. However, if the pharmacophore is made too specific, active ligands present in the database may be missed. Therefore, a compromise with respect to the specificity of the pharmacophores has to be made that produces a hit rate tailored to demands of the particular project. Methods of adjusting the specificity of a Catalyst pharmacophore for this purpose have previously been evaluated by Sprague and Hoffmann,[20] but the influence of excluded (ligand-inaccessible) volumes on pharmacophore specificity was not at all considered. To this end, we have studied structure-based pharmacophores with various numbers of features in the absence or presence of excluded volumes, to assess their effect on the performance of the database queries with respect to the specificity of the hits and hit list reduction potential.

The effects of adjusting the number or sizes of the excluded volumes (models 1a−d, 2a−d, 4a, 5, and 6a) were also studied, as well as the influence of feature tolerances on pharmacophore performance (model 4a vs model 4b). In addition, the interactions of feature tolerances with the feature weights (models 6a−d) were examined.

Influence of Excluded Volumes on Specificity. Model 1a consists of five hydrophobic centers which correspond to the iodine atoms at positions 3, 5, and 3′ and the inner and outer rings of T_3, plus two hydrogen bond-accepting features which map the 4′-hydroxyl group and position 1 side-chain carboxylate groups of T_3, respectively. Table 1 lists the features included in each of the pharmacophores as well as the associated weights and tolerances, the scaling of the excluded volumes, and the cutoff. Figure 2 is an illustration of pharmacophore 1 (model 1d). Model 2 was derived from model 1 by deletion of the hydrogen bond-accepting feature corresponding to the position 1 side-chain carboxylate group as well as the hydrophobic feature equivalent to the 3′-iodine atom of T_3 (Table 1). To obtain estimates of the ligand specificity of the pharmacophores, we searched a test database constructed from a subset of our in-house THR ligands and a number of literature compounds (18 agonists, 1 partial agonist, and 32 antagonists). The antagonists generally had lower affinities than the agonists. Out of the possible 51 compounds contained in the test database, models 1a and 2a retrieved 12 and 32 compounds, respectively (Table 2). All of model 1a's hits and one-half of model 2a's hits were agonists, consistent with the fact that the antagonists are generally of lower affinity than the agonists. Additionally, several modified pharmacophores (models 1b−d and 2b−d) were derived from the two original pharmacophores (models 1a and 2a) in order to determine the influence of the number and size of excluded volumes on pharmacophore specificity (Table 2). These modified pharmacophore models contain excluded volumes scaled to 25% or 50% of their respective van der Waals radii and centered about receptor atoms within 6 or 8 Å of any T_3 hormone atom (Table 2). The number of hits is modulated by the vdW scaling factor used, while the inclusion of receptor atoms beyond the 6-Å cutoff does not decrease the number of hits obtained.

Table 1. Summary of the Features,[a] Their Associated Weights and Tolerances (Å), Scaling of Excluded Volumes (% vdW radii), and Atomic Cutoff (Å) of the Binding Site from the Ligand Contained in Models 1−6

	features (weights and tolerances)							volume scaling	cutoff
model	3-I	5-I	inner ring	outer ring	3′-I	4′-OH	$-CO_2^-$	(% vdW radii)	(Å)
1d	H	H	H	H	H	A	A	50	8
	(0.15, 1.0)	(0.15, 1.0)	(0.28, 1.5)	(0.28, 1.6)	(0.15, 1.0)	(0.52, 1.0, 1.0)	(0.42, 1.0, 1.0)		
2d	H	H	H	H		A		50	8
3			H	H	H	A	A	50	8
4a	H	H	H	H		D	A (2.0, 1.5, 1.5)	25	6
	(0.72, 1.5)	(0.72, 1.5)	(1.37, 1.5)	(1.37, 1.5)		(3.02, 1.6, 2.2)	A (2.5, 1.6, 2.2)		
4b[b]	H	H	H	H		D	A	25	6
							A		
5	H	H	H	H		D	A	25	6
6a	H	H	H	H		D		25	6
6b	H	H	H	H		D		25	6
	(0.15, 1.0)	(0.15, 1.0)	(0.28, 1.5)	(0.28, 1.6)		(0.52, 1.0, 1.0)			
6c	H	H	H	H		D		25	6
	(0.72, 1.0)	(0.72, 1.0)	(1.37, 1.5)	(1.37, 1.6)		(3.02, 1.0, 1.0)			
6d	H	H	H	H		D		25	6
	(0.15, 1.5)	(0.15, 1.5)	(0.28, 1.5)	(0.28, 1.5)		(0.52, 1.6, 2.2)			

[a] H, *h*ydrophobic region; A, hydrogen bond *a*cceptor; and D, hydrogen bond *d*onor. Where no weight or tolerance value is given, it is equal to the value of the equivalent feature of the pharmacophore from which it is derived. Models 2d and 3 are derived from model 1d; models 5 and 6 are derived from model 4a. [b] Model 4b differs from model 4a in that tolerances are not defined for the hydrophobic features 3-I and 5-I; thus their tolerances appear infinite and can be satisfied by any suitable functional group of a molecule.

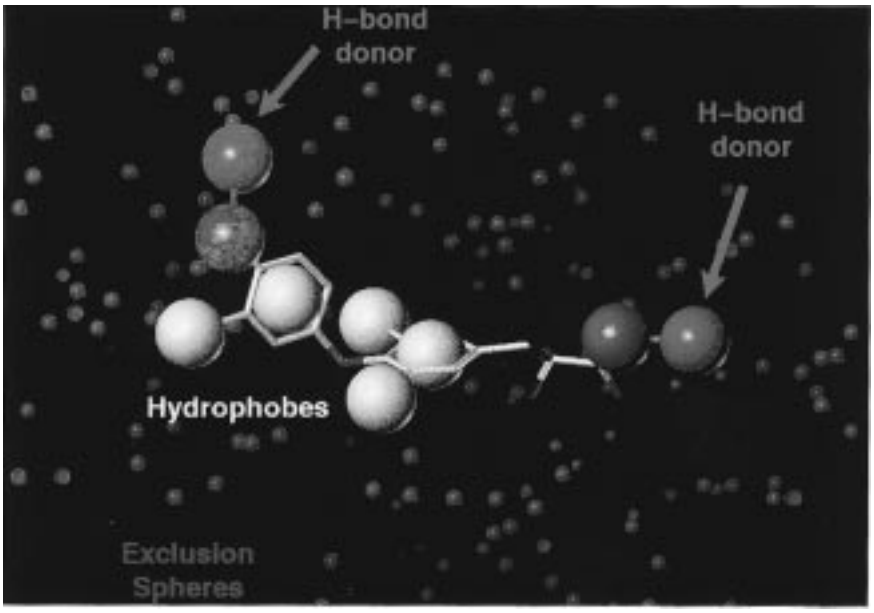


Figure 2. Illustration of the fit of T_3 to model 1d. Five hydrophobic features (yellow spheres) correspond to the inner and outer phenyl rings and the three iodine atoms of T_3. Additionally, two hydrogen bond acceptor features (red spheres) are equivalent to the 4′-hydroxyl group and a carboxylate oxygen atom of the position 1 side chain of T_3. The blue spheres correspond to the respective hydrogen bond-donating residues in the receptor. The excluded (ligand-inaccessible) volumes (green spheres), corresponding to the atoms delineating the binding cavity of THR-α, have been scaled down to a uniform size for the sake of clarity.

Significantly more hits are obtained by models 2a−d than by models 1a−d. However, the number of "active" hits is of more interest than merely the number of total hits. At threshold values for activity of $IC_{50} \leq 1\ \mu M$ and ≤ 10 nM, model 1 (7 features) returns fewer false positives (database hits which are inactive) than does model 2 (5 features), but model 2 returns fewer false negatives (active compounds missed by the query) than model 1. While the pharmacophores without excluded volumes, models 1a and 2a, retrieve up to twice as many compounds as compared to their respective pharmacophores with excluded volumes, their hit lists include 2−5 times as many false positives. That the hit lists of pharmacophores supplemented with excluded volumes have fewer false positives must be offset against the fact that they also include 2−3 times as many false negatives.

While it has previously been demonstrated that the number of features included in a pharmacophore can be used to control the number of database hits,[20] we have additionally established that supplementing pharmacophores with excluded volumes not only reduces the number of hits but also decreases the number of false positives obtained. This may be especially advantageous if one intends to purchase or request from internal sample collections all database hits as the larger the hit list the greater the expense involved. Also, if only a certain number of compounds from a hit list are selected for purchase and assay, the longer the hit list, the greater the time for postprocessing involved.

Table 2. Summary of Searching with Models 1a−d and 2a−d, of a Test Database of 51 In-House Compounds: Effect of Cutoff (Å) of the Binding Site from the Ligand and van der Waals Scaling of Excluded Volumes (% vdW radii)

				screening results					
				10 nM cutoff			1 μM cutoff		
model	cutoff (Å)	% vdW radii	no. of database hits	no. of actives[a]	no. of false[b] positives	no. of false[c] negatives	no. of actives[d]	no. of false positives	no. of false negatives
1a	0	N/A[e]	12	7	5	1	12	0	12
1b	6	25	8	6	2	2	8	0	16
1c	6	50	6	5	1	3	6	0	18
1d	8	50	6	5	1	3	6	0	18
2a	0	N/A[e]	32	8	24	0	19	13	5
2b	6	25	23	8	15	0	15	8	9
2c	6	50	20	8	12	0	14	6	10
2d	8	50	20	8	12	0	14	6	10

[a] A compound is considered to be active if it has an IC_{50} value of ≤10 nM. Two threshold IC_{50} activity values were considered, ≤10 nM and ≤1 μM. [b] False positive, a database hit which is inactive. [c] False negative, an active compound missed by the database query. [d] A compound is considered to be active if it has an IC_{50} value of ≤1 μM. [e] N/A, not applicable.

Effect of Excluded Volumes on Search Times. It is known that the receptor bound conformation of ligands frequently differs from the minimum-energy conformation of ligands.[21] This presents a problem in database searching since it will not be known a priori what conformation(s) of the ligand will bind. There are two alternative solutions to this problem.[22] The first, which has been implemented in the ISIS/3D,[23,24] UNITY,[25] and DOCK[26] software packages from MDL, Tripos, and UCSF, respectively, is to dock the ligand as flexible structures, using, for example, the directed tweak algorithm.[25] However the disadvantage of this method is that it is very time-consuming since such on-the-fly conformational searches must be repeated for each pharmacophoric search query. Furthermore, without bump-checking, many of the matched conformations produced by the directed tweak search are impossibly strained. For the ISIS/3D software the timings increase 7-fold when bump-checking is turned on.[27] With the UNITY/Tripos software the timings increase by 2−20 times when vdW bump-check is included.[28]

The other solution, which is implemented in the ChemDBS-3D/Chemical Design,[29] Catalyst/MSI,[20,30] and Merck Flexibase[31] databases, is to generate a number of energetically reasonable representative conformations for each structure at the time of compound registration and to store these conformers in the database. This latter approach requires a sufficient coverage of the available conformational space such that there exists at least one conformation of each active compound which is sufficiently close to the active conformation to be considered a hit by a database query. The Maybridge 3D-coordinate database supplied with Catalyst 2.3 contains 47 045 compounds. Timings for searches of the Maybridge database on a Silicon Graphics Extreme workstation (MIPS R-4000 CPU) are given in Table 3. Despite the large number of excluded volumes included in models 1d and 4b (7 features), it is feasible to search (<3 h) large databases with the Catalyst software, on a moderately fast workstation. The inclusion of the excluded volumes leads to an approximately 70% increase in the search time but may markedly reduce the number of false positives. Using a 6-Å (160 receptor atoms) instead of 8-Å (319 atoms) cutoff for the excluded volumes for model 1 reduced the search time by 25%. However, a much longer search time (∼48 h) is required for the pharmacophores containing fewer features (models 2 and 6, 5 features) when excluded volumes are employed (Table 3).

Table 3. Summary of the Results of Searching with Models 1, 2, 4b, and 6 of the Maybridge Database of 47 045 Compounds: Comparison of Search Times and Number of Hits With and Without Excluded Volumes

		no. of hits		search time (h)	
model	no. of features	with excl vol	without excl vol	with excl vol	without excl vol
1d	7	1	4	0.7	0.4
2d	5	2140	5138	<48	1.0
4b	7	52	155	2.5	1.5
6a	5	2350	3642	<48	1.0

The database queries of the programs FOUNDATION[32] and 3Dsearch[33] also consist of pharmacophore models supplemented by steric (ligand-inaccessible) constraints defined from protein crystallographic coordinates. However, only a single conformation of a ligand is considered, and FOUNDATION[32] only examines a single orientation of a ligand. The LUDI[34] software package has been applied to 3D-database mining, but restricted to single conformations of small rigid molecules.

Unfortunately, the experimental validation of hits obtained have been reported for only a few docking methods,[26] most notably for the DOCK suite of programs,[35−38] which have been successfully applied to identify micromolar inhibitors for several enzymes of therapeutic interest.[39] Typically 10% of the tested compounds show micromolar inhibition of the enzymes. Normally DOCK is used to search single-conformation molecule databases where the stored conformation is either a low-energy, computer-built, or crystal structure. Both ligand and receptor are assumed to be rigid, though methods of including flexibility have been explored but until recently had not been implemented for database searching.[26] The ligand and receptor cavity are defined as sets of spheres; thus to dock the ligand, sphere sets with compatible properties are matched together. Due to the large size of the hit lists generated from database searches, hits are usually selected for binding/biological assay by time-consuming visual inspection. The programs CLIX[40] and FLOG[41] essentially use the DOCK approach, though in FLOG,[31] multiple conformations of each compound are explicitly stored in a database. A database consisting of approximately 8000 compounds was searched against human immu-

Table 4. Summary of the Results of Searching with Models 1d, 2d, 3, 4a,b, 5, and 6a of the Maybridge Database of 47 045 Compounds

model	no. of hits	hit rate[a] (%)	no. screened	no. active[b]	false positives[d] (%)
1d	1	0.002	1	1	0
2d	2140	4.5	0	N/A[c]	N/A
3	110	0.23	6	0	100
4a	4	0.009	2	1	50
4b	52	0.11	12	4	67
5	31	0.065	3	1	67
6a	2350	5.0	0	N/A	N/A

[a] The hit rate is defined as the number of compounds retrieved as a percentage of compounds in the database. [b] A compound is considered to be active if it has an IC_{50} value of ≤ 200 μM. [c] N/A, not applicable. [d] False positive, a database hit which is inactive.

Table 5. Summary of the Results of Searching with Models 1d, 2d, 4a,b, 5, and 6a−d of a Test Database of 51 Compounds

	functional effect		
model	agonist	partial agonist	antagonist
1d	6	0	0
2b	14	1	8
2d	13	0	7
4a	6	0	0
4b	10	0	0
5	12	1	0
6a	14	1	8
6b	14	1	2
6c	13	0	3
6d	14	1	7
total	18	1	32

nodeficiency virus type I requiring 30 h on a cluster of four RS/6000 model 580 workstations.[42] Known high-affinity inhibitors scored high in the hit list.

In contrast to the above methods, recent software packages such as that by Welch et al.[43] and Makino and Kuntz[44] explicitly take into account ligand flexibility. A fragment-based approach is employed to dock flexible ligands into a macromolecular binding site. The former program, Hammerhead,[43] required approximately 5 days, using a variety of SGI Indigo and Challenger processors (four processors running at any given time), to complete a screen of a database of 80 000 compounds against streptavidin. The authors believe their scoring function to be reliable thus obviating the need for human selection and reducing the number of false positives selected for assay. The latter program[44] was used to screen a database of 15 000 molecules against a dihydrofolate reductase (DHFR) structure in approximately 5 days on a Silicon Graphics Indigo2 workstation with a 200-MHz R4400 processor. Known DHFR inhibitors were successfully extracted.

Effect of Excluded Volumes on the Hit Rate. The hit rates (Table 4) we achieved from searching the Maybridge database with models 1d, 3, 4a,b, and 5 are substantially less than the typical hit rate of 1−10%.[26] In general, a pharmacophore containing excluded volumes whose coordinates correspond to the atomic positions of atoms delineating the ligand's binding site produces fewer hits than an equivalent pharmacophore without excluded volumes (see, for example, Table 3, models 1, 2, 4b, and 6). Intriguingly, the reduction in the hit list size is more pronounced for the detailed pharmacophores (Table 3, models 1 and 4b) than for the reduced pharmacophore representations (models 2 and 6). This may be because a pharmacophore with fewer features imposes less stringent demands on the conformation of a molecule required to map to the pharmacophore and also avoid clashing with excluded volumes.

Complexity of the Model versus Performance. Tables 4 and 5 respectively summarize the results of searching the Maybridge and test databases with several pharmacophores. With respect to the Maybridge database, compounds were selected for purchase and screening based on their having high "best fit" scores to the pharmacophores and simultaneously a relatively low conformational energy required to achieve this fit. It can be concluded from examination of Tables 1, 4, and 5 that as features are omitted from a pharmacophore, an increase in the number of hits is obtained (Tables 4 and 5) but at the expense of an increase in the percentage of false positives (Table 4). The false positive rate for binding is defined here as the number of compounds having an IC_{50} value of >200 μM as a percentage of the total number of compounds screened.

For example, the false positive hit rate is 0% for model 1d which contains all essential pharmacophore features (i.e., only one compound was retrieved and it was found to bind to the THR-α) but 100% for model 3 where, in contrast to model 1d, position 3 and 5 hydrophobic centers are omitted from the pharmacophore (Table 1). The ligand 3′-T_1, an analogue of T_3, which is unsubstituted at positions 3 and 5 is of low affinity ($IC_{50} \approx 10$ μM),[45] though a similarly unsubstituted 3′-isopropyl-1-oxamic acid derivative exists which is of reasonable affinity ($IC_{50} \approx 11$ nM).[2]

Since the carboxylate group of the position 1 side chain of T_3 is involved in an intricate network of hydrogen-bonding interactions including water molecules and it is not known whether the 4′-hydroxyl group is an acceptor or donor, two possible alternative hydrogen-bonding networks were investigated in models 1 and 4a. In contrast to model 1d, model 4a treats the 4′-hydroxyl group as a hydrogen bond donor, requires that the moiety that corresponds to the position 1 side chain is capable of accepting two hydrogen bonds, and has a 6-Å cutoff of excluded volumes scaled to 25% of their respective atomic radii. Significantly, this model does not require a molecule to have a hydrophobic group that maps to the 3′-iodine of T_3, and its false positive hit rate is 50%. (When a hydrophobic feature corresponding to the 3′-iodine of T_3 was included in the pharmacophore, no hits were obtained; data not shown.) Model 5 differs from model 4a in that it has only one hydrogen bond-accepting function instead of two, to simulate the interactions of the position 1 side chain with the receptor. Its false positive hit rate is 67%. Sprague and Hoffmann[20] have previously investigated the effect of reducing the number of features included in a pharmacophore upon the number of hits obtained. They too noted an increase in hit rate with reduction in the number of pharmacophore features; however, the hits were not analyzed with respect to the number of false positives.

Effect of Feature Tolerances on False Positive Hit Rate. Larger tolerances for the position 3 and 5 hydrophobic centers of model 4b as compared to model 4a led to an increase in the number of hits (Tables 4

and 5), but also in the false positive hit rate (67% for model 4b). If one does not explicitly assign a tolerance to a feature in Catalyst version 2.3, its tolerance appears infinite and can be satisfied by any suitable functional group of a molecule. Thus, model 4b requires that a ligand, in addition to hydrophobic groups that map to the hydrophobic features equivalent to those mapped by the inner ring and outer rings of T_3, possesses two other hydrophobic groups. However, these need not occupy positions equivalent to the 3- and 5-iodine atoms of T_3. Sprague and Hoffmann[20] similarly noted an increase in the false positive hit rate upon increasing feature tolerances of an angiotensin II antagonist query used to search the Derwent World Drug Index. For a given cutoff and scaling of excluded volumes, a balance between the number of pharmacophoric features and their tolerances must be found to maximize the number of true positives and minimize false positives. This finding is consistent with the test database results obtained with models 1 and 2 (Table 2).

Ability of Less Stringent Pharmacophores To Identify THR Antagonists. The conformation of the receptor when bound to an agonist versus antagonist is likely to differ[5,46] as has been demonstrated for the estrogen receptor,[47] and consequently the pharmacophores are expected to be best suited for thyroid hormone agonists. In support of this notion, only the less stringent pharmacophores with fewer features (models 2 and 6) are able to retrieve a significant number of THR antagonists from the test database, probably because these are less restrictive with respect to the allowed binding modes (Table 2, 16 of model 2a's hits are antagonists).

Influence of Feature Tolerances versus Weights on Hit Rates. Model 6a differs from model 2b (Table 1) in that His-381 is assumed to be a hydrogen bond donor instead of an acceptor and its features are associated with larger tolerances and weights. However, both hypotheses contain an equal number of antagonists in their hit lists. To determine how altering model 6a's feature weights and tolerances to those of model 2 would affect its hit rate, three variants of model 6a (6b−d, Table 1) were constructed. Model 6b differs from model 6a in that the feature tolerances and weights are the same as those of model 2. As compared to model 6a, model 6c has the same feature tolerances, and model 6d has the same feature weights as model 2. The results of searching with models 6c,d against the test database (Table 5) clearly indicate that the feature tolerances have more influence than the feature weights on determining how many hits are obtained, particularly with respect to how many antagonist structures are retrieved. This interpretation is supported by the greater similarity of the database searching results between models 6b,c (which share the same feature tolerances but have different feature weights) than between models 6b,d (which share the same feature weights but have different feature tolerances).

Experimental Validation of the Pharmacophores. Model 1d: As a 3D-database search query, model 1d produced one hit from the Maybridge database (**1**) which was purchased and subsequently shown in a competitive binding assay to bind to the THR-α with an affinity of 69 μM (Table 6). Interestingly, while the pharmacophore definition is based on T_3 in its receptor-bound conformation, the structure of **1** differs markedly from T_3. Its superimposition onto T_3 is shown in Figure 3. To assess the ability of the pharmacophore to discriminate against closely related inactive ligands, we performed a substructure search based on the core of **1**, and six analogues were found (Figure 4) that were not picked up as hits by model 1d. None of these displayed significant affinity for THR (IC_{50} > 200 μM) in competitive binding assays, demonstrating that model 1d is able to successfully discriminate against inactive compounds with a high level of structural similarity to **1** (Figure 4).

Table 6. IC_{50} Values of Molecules Retrieved from the Maybridge Database That Bind to Thyroid Hormone-α

model	ligand (Maybridge code)	IC_{50} (μM)[a]
4b	**24** (BTB-04128)	0.93
4b	**26** (BTB-04667)	39
4a,b, 5	**15** (BTB-04668)	1.8
4b	**27** (JP-000639)	18.1
1	**1** (KM-07385)	69

[a] IC_{50} values from the second out of two titration experiments for each compound. The difference between the two determinations was less than 0.2 log unit.

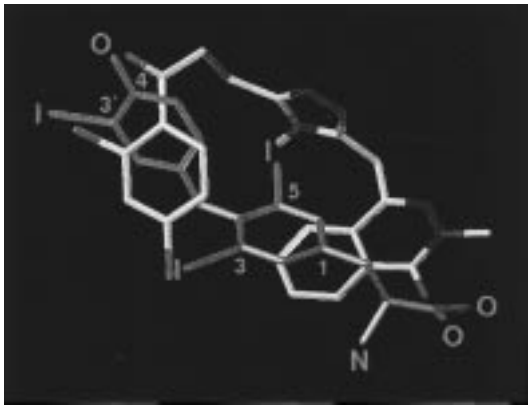
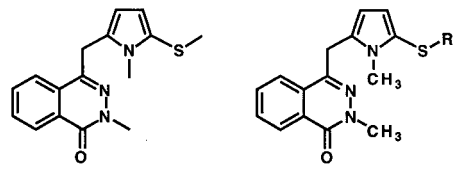


Figure 3. Superimposition of **1** (KM-07835) onto T_3 resulting from their fits to model 1d. The 2,4-dichloroacetophenone moiety of **1** (KM-07385, IC_{50} = 69 μM) overlays the position 3- and 3′-iodine atoms, the 4′-hydroxyl group, and the outer ring of T_3, the *N*-methyl group of the 1,2,4-triazole ring maps onto the 5-iodine atom, and the phthalazinone ring and its carbonyl group map onto the inner ring and the position 1 side chain carboxylate of T_3, respectively.

Model 3: When used to search the Maybridge database, model 3 produced 110 hits which can be grouped into several structural classes, the principle ones being steroids (21), diphenyl ethers (14), biaryls (13), and long-chain aliphatics or molecules with aryl groups separated by an aliphatic chain length of ≥3 (14). Of these, two steroids (**8** and **9**), two diphenyl ethers (**10** and **11**), and two biaryl ethers (**12** and **13**) were selected for testing (Figure 5). None of the six selected compounds bound with significant affinity to THR-α.

Models 4a,b: In searches of the Maybridge database, model 4a retrieved 4 compounds whereas model 4b retrieved 51. Two of the model 4a hits are diphenyl ethers (**14** and **15**), while the other two are steroids (**16** and **17**) (Figure 6). With respect to mapping to the pharmacophore, one of the steroids was scored as a "borderline" hit, as it was only barely able to satisfy the search query, and the second was only able to do so by adopting a high-energy conformation (21 kcal/mol).



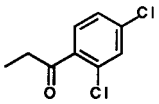
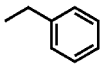
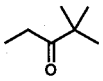
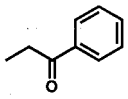
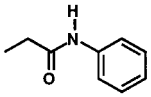
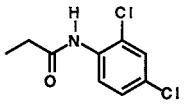
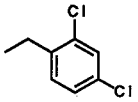
Ligand	R
1 (KM-07385)	
2 (KM-07382)	
3 (KM-07383)	
4 (KM-07384)	
5 (KM-07388)	
6 (KM-07389)	
7 (KM-07378)	

Figure 4. Structure of the Maybridge database hit compound retrieved by model 1d (**1**, KM-07385) plus the six inactive analogues (**2**−**7**) found by substructure searching of the same database (top left structure is the search query). The replacement of the 2,4-dichloroacetophenone moiety of the active analogue **1** by a benzyl group in **2**, a methyl *tert*-butyl ketone group in **3**, an unsubstituted acetophenone in **4**, and an *N*-phenylacetanilide moiety in **5** has the effect that none of these inactive analogues possesses a biosteric functional group that can mimic the 3- or 3′-iodines of T_3. While the analogue **6** contains all the functional groups required for THR binding, the insertion of an NH group between the ketone and 2,4-dichorophenyl groups increases the intramolecular distance between the chlorine atoms and the carbonyl groups and therefore this analogue is no longer able to overlay closely with the equivalent functional groups of T_3. The dichlorobenzyl group of **7** is also probably unable to do so.

Thus, these latter two compounds were not further considered. Model 4b's hits include the two diphenyl ethers **14** and **15** retrieved by model 4a, plus seven analogues (Figure 6). These nine compounds were purchased and assayed, although **18** had a poor fit to the pharmacophore and **19** must adopt a high-energy conformation (20 kcal/mol) in order to fit to the pharmacophore. The selections of **20** and **21** were based on their having high "best fit" scores to the pharmacophore

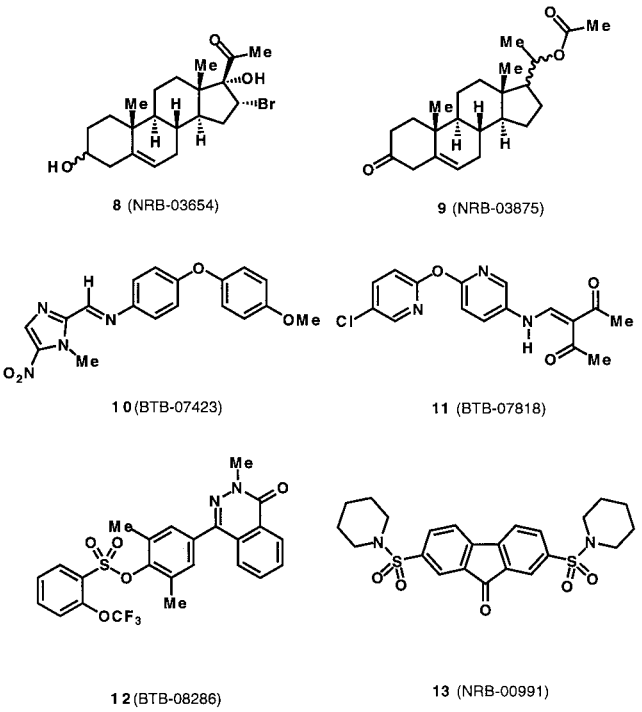
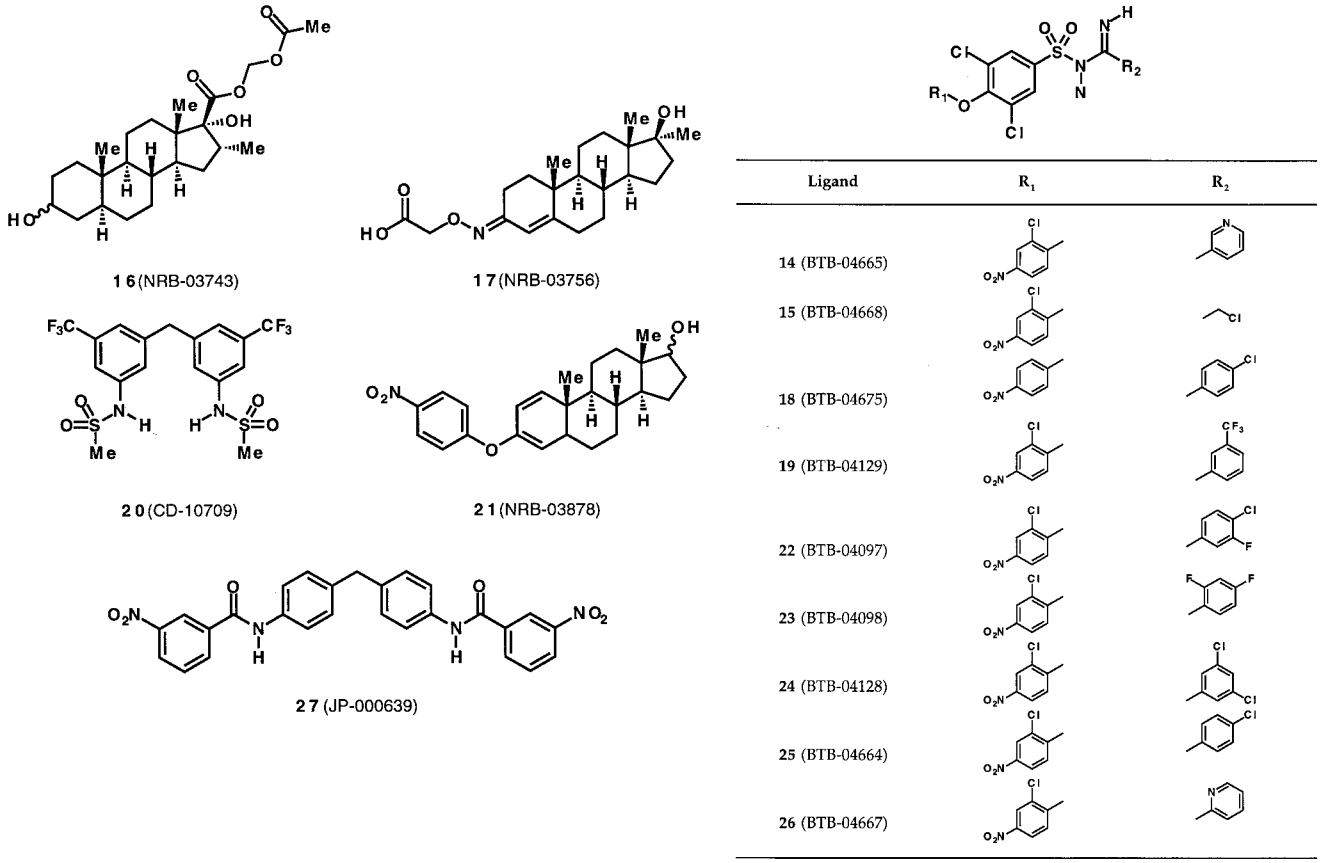


Figure 5. Structures of the six tested compounds retrieved by model 3: steroids **8** and **9** (top), diphenyl ethers **10** and **11** (middle), and biaryls **12** and **13** (bottom).

and a simultaneously relatively low conformational energy when achieving this fit. Other compounds whose scores were lower or required a higher conformational energy to fit to the query were eliminated. Compound **27** was chosen as it was considered to bear some structural resemblance to the BTB series of compounds (Figure 6). Of the purchased compounds, four were found to bind to the THR-α. Their IC_{50} values for binding are listed in Table 6. Of the compounds with negligible binding affinity (IC_{50} > 200 μM), **22** and **23** have in common a para-substituted aniline moiety, unlike the sub-micromolar binder **24**. Thus their low binding affinity may be in part due to unfavorable steric interactions with residues of the receptor, suggesting that scaling the excluded volume radii to only 25% of the corresponding atomic radii is too permissive. However a limitation of the vdW scaling approach to address the problem of receptor flexibility is that the receptor binding site is not equally flexible overall. Therefore, uniform vdW scaling will underestimate the flexibility in some parts and exaggerate it in others. Thus, it is expected that some false hits will be picked up and true positives discarded at a given van der Waals scaling factor when using a rigid pharmacophore. Unfavorable steric interactions between ligand and receptor may also occur in the case of the inactive database hits **14**, **18**, and **25** and would also contribute to the low binding affinity of **26**. That **21** was retrieved as a hit but fails to bind to the THR-α is probably because model 4b does not require that the ligand fills the hydrophobic pockets of the binding site which are normally occupied by the 3- and 5-iodine atoms of T_3.

Model 5: With the Maybridge database, a total of 31 hits were obtained with model 5, 25 of which are steroid structures and 3 (**14**, **15**, and **20**) are molecules which have previously been identified by model 4a and/or 4b and submitted for testing. Two of the remaining three



Ligand	R_1	R_2
14 (BTB-04665)		
15 (BTB-04668)		
18 (BTB-04675)		
19 (BTB-04129)		
22 (BTB-04097)		
23 (BTB-04098)		
24 (BTB-04128)		
25 (BTB-04664)		
26 (BTB-04667)		

Figure 6. Structures of compounds retrieved from Maybridge by models 4a,b. All of them were assayed for THR-α binding affinity with the exception of the top two compounds (NRB-03756 and NRB-03743).

compounds were reported as being borderline fits to the pharmacophore, and it was not possible to produce a mapping for the remaining compound. None of the hits from this pharmacophore were therefore submitted to the assay given the fact that no steroids have been reported in the literature to bind to the THRs with high affinity and given our previous negative assay result for the steroids **8**, **9**, and **21**.

Conclusion

Traditionally two separate approaches to 3D-database searching have been used. The first method involves the docking of ligands to a receptor and the second the inference of a pharmacophore from a series of compounds acting via the same target, but in the latter method, steric factors are less well-accounted for or completely neglected. However, our implementation of structure-based pharmacophores supplemented with excluded volumes in Catalyst combines pharmacophore and structure-based methods. The present work constitutes the first publication in which a database with multiple stored conformers of ligands is efficiently searched with respect to both time and specificity, with a pharmacophore incorporating a large number of excluded volumes ($>10^2$). We have shown that pharmacophores supplemented with excluded volumes can reduce the number of false positives in a hit list by a factor of 2−5. In combination with increasing the number of features included in a pharmacophore and/or reducing their tolerances,[20] the size of a hit list is reduced, but at the same time the likelihood that the selected compounds display the desired affinity is increased. In addition, this methodology constitutes an alternative to diversity analysis for the decision of which compound library to purchase, when the objective is to acquire more ligands for a particular receptor.

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