CoMFA-Based Prediction of Agonist Affinities at Recombinant Wild Type versus Serine to Alanine Point Mutated D2 Dopamine Receptors†

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Agonist affinity changes dramatically as a result of serine to alanine mutations (S193A, S194A, and S197A) within the fifth transmembrane region of D2 dopamine receptors and other receptors for monoamine neurotransmitters. However, agonist 2D-structure does not predict which drugs will be sensitive to which point mutations. Modeling drug−receptor interactions at the 3D level offers considerably more promise in this regard. In particular, a comparison of the same test set of agonists across receptors differing minimally (point mutations) offers promise to enhance the understanding of the structural bases for drug−receptor interactions. We have previously shown that comparative molecular field analysis (CoMFA) can be applied to comparisons of affinity at recombinant D1 and D2 dopamine receptors for the same set of agonists, a *differential* QSAR. Here, we predicted agonist K_L for the same set of agonists at wild type D2 vs S193A, S194A, and S197A receptors using CoMFA. Each model used bromocriptine as the template. $\ln(1/K_L)$ values for the low-affinity agonist binding conformation at recombinant wild type and mutant D2 dopamine receptors stably expressed in C6 glioma cells were used as the target property for the CoMFA of the 16 aligned agonist structures. The resulting CoMFA models yielded cross-validated R^2 (q^2) values ranging from 0.835 to 0.864 and simple R^2 values ranging from 0.999 to 1.000. Predictions of test compound affinities at WT and each mutant receptor were close to measured affinity values. This finding confirmed the predictive ability of the models and their differences from one another. The results strongly support the idea that CoMFA models of the same training set of compounds applied to WT vs mutant receptors can accurately predict differences in drug affinity at each. Furthermore, in a "proof of principle", two different templates were used to derive the CoMFA model for the WT and S193A mutant receptors. Pergolide was chosen as an alternate template because it showed a significant *increase* in affinity as a result of the S193A mutation. In this instance both the bromocriptine- and pergolide-based CoMFA models were similar to one another but different from those for the WT receptor using bromocriptine- or pergolide- as templates. The pergolide-based S193A model was more strikingly different from that of the WT receptor than was the bromocriptine-based S193A model. This suggests that a "*dual-template*" approach to differential CoMFA may have special value in elucidating key differences across related receptor types and in determining important elements of the drug−receptor interaction.

Introduction

Brain D2 DA receptors play major roles in normal physiology throughout the lifespan and in both neurological (e.g., Parkinson's disease) and psychiatric (e.g., schizophrenia) disorders.[1−6] Several DA receptors belonging to D1 and D2 subfamilies have been cloned.[7] In vitro mutagenesis of many receptors, including DA receptors,[7] has provided data that can be used to investigate quantitative structure−activity relationships for the receptors. For example, agonist affinity changes dramatically as a result of serine to alanine

† Abbreviations: 7-OH-DPAT, ±-2-dipropylamino-7-hydroxy-1,2,3,4-tetrahydronaphthalene; ADTN, 2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene; APO, *R*-(−)-apomorphine; Br-APB, 3-allyl-6-bromo-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepin; Cl-APB, 3-allyl-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepin, SKF-82958; Cl-PB, 6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepin; CoMFA, comparative molecular field analysis; DA, dopamine; D1 receptor, recombinant rhesus macaque D1 dopamine receptor; D2 receptor, recombinant rat $D2_{short}$ dopamine receptor; DHX, dihydrexidine; 4-MNP-DHX, 4-MNP-dihydrexidine; EDTA, ethylenediaminetetraacetic acid; HEPES, (*N*-{2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid}); K_L, low-affinity agonist dissociation constant, determined in C6 cells in the presence of saturating GTP and sodium; NPA, *R*-(−)-*N*-*n*-propylnorapomorphine; PPHT, 2-(*N*-phenylethyl-*N*-propyl)amino-5-hydroxytetralin; QSAR, quantitative structure−activity relationship; S193A, S194A, and S197A, serine to alanine mutants at positions 193, 194, and 197 of the D_{2Short} dopamine receptor; SEE, standard error of the estimate; SEP, standard error of prediction; WT, wild-type receptor.

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Table 1. Agonist Affinities at WT and Mutant D2 DA Receptors[a]

agonist	WT	S193A	S194A	S197A
7OHDPAT	0.4 (0.2−0.6)	1.1 (0.8−1.5)	0.3 (0.2−0.4)	1.0 (0.8−1.3)
ADTN	0.21 (0.16−0.28)	17 (14−20)	0.45 (0.38−0.54)	0.12 (0.09−0.16)
APO	0.2 (0.1−0.3)	1.2 (0.3−4.6)	1.6 (0.6−4.3)	0.6 (0.5−0.7)
Br-APB	0.48 (0.26−0.87)	1.6 (1.1−2.3)	0.5 (0.3−0.9)	0.3 (0.1−0.5)
bromo	0.008 (0.006−0.010)	0.008 (0.005−0.012)	0.017 (0.012−0.023)	0.004 (0.003−0.005)
Cl-APB	0.4 (0.3−0.5)	1.6 (1.4−1.9)	0.5 (0.5−0.6)	1.1 (1.0−1.3)
DA	6.2 (3.4−11.3)	267 (212−337)	37 (21−66)	49 (18−127)
DHX	2.3 (1.9−2.6)	13 (11−15)	2.5 (2.1−3.0)	3.5 (2.7−4.4)
fenoldopam	1.3 (1.1−1.4)	13 (10−17)	2.7 (2.2−3.4)	1.9 (1.6−2.3)
lisuride	0.0006 (0.0004−0.0009)	0.0003 (0.0002−0.0004)	0.0023 (0.0019−0.0027)	0.0004 (0.0003−0.0006)
methoxytyr-3	94 (59−149)	251 (154−411)	88 (50−152)	225 (82−613)
NPA	0.009 (0.006−0.013)	0.37 (0.26−0.53)	0.52 (0.41−0.66)	0.04 (0.03−0.05)
pergolide	0.03 (0.02−0.05)	0.004 (0.003−0.005)	0.045 (0.040−0.049)	0.04 (0.03−0.05)
(−)PPHT	0.010 (0.009−0.012)	0.090 (0.071−0.113)	0.034 (0.028−0.040)	0.012 (0.011−0.014)
quin	1.9 (1.2−2.9)	3.5 (2.3−5.2)	6.7 (4.1−11)	2.3 (1.1−4.7)
SKF38393	46 (35−62)	39 (26−59)	18 (14−22)	135 (82−220)

[a] Affinities are in μM as geometric mean ± SEM.

mutations (S193A, S194A, and S197A) within the fifth transmembrane region of D2 DA receptors.[8,9] However, agonist 2D-structure does not predict which drugs will be sensitive to which mutations. Recent 3D QSAR methods facilitate a more realistic approach to drug−receptor interactions than 2D-based methods.

Direct study of those aspects of 3D drug structure which are most closely associated with particular biological target properties at a given receptor is provided by the comparative molecular field analysis (CoMFA) procedure, incorporating partial least squares (PLS) regression.[10] Previously, we have demonstrated that drug affinity data obtained at recombinant receptors may represent a significant improvement over more traditional target data obtained at endogenous brain receptors in deriving CoMFA models.[11] This initial work was extended in a paper in which drug−receptor models for a set of active (agonist) drugs with very different three-dimensional shapes were directly compared at D1 vs D2 DA receptors; a procedure termed *differential QSAR*.[12] The same drugs were used to study the binding at each receptor because the active sites of both D1 and D2 receptors appear to be similar. This made possible more direct comparisons of CoMFA models than was previously the case. CoMFA models for agonist binding to D1 and D2 receptors differed. Furthermore, formulated as 3D queries, aspects of the D1 and D2 CoMFA models also yielded different hit lists of compounds as a result of searching chemical databases. Finally, the D2 CoMFA model was assessed against direct models of drug docking to the D2 receptor (protein homology modeling), with results that supported the CoMFA model approach and extended the inferences possible from the contour maps of the receptor binding pocket that are generated by CoMFA.

The present paper represents a further extension of the first two papers in the series. Here, we have compared CoMFA models generated for the same training set of compounds at WT and three Ser-to-Ala mutant D2 receptors. There were two major purposes to the work. First, we hoped to be able to provide predictions of agonist affinity that clearly differentiated among WT and the mutant receptors, and did so with greater accuracy than was previously the case. Second, we wished to make use of a modification of the previously developed differential QSAR approach. Here, as a proof of principle, we extended that approach to a *dual template, differential QSAR* method in which standard and alternate CoMFA models were developed for the WT and S193A mutant receptor. Standard models used bromocriptine as the template for comparison of WT with all mutants. However, the alternative S193A mutant receptor CoMFA model utilized as template pergolide, a compound that showed the *largest increase in affinity* as a result of the mutation.

Methods

Measurement of Drug Affinity. C6 glioma cells stably expressing the D2 receptor[13] were grown and prepared for radioligand binding experiments as described previously.[6] Confluent cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na^+−HEPES, pH 7.0, 2 mM EDTA). After 10−20 min, the cells were scraped off the plate into centrifuge tubes and centrifuged at 17 000 rpm for 20 min. The crude membrane fraction was re-suspended with a Brinkman polytron homogenizer (setting 6 for 10 s) in assay buffer (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 1 mM EDTA, 0.025% ascorbic acid, and 0.001% bovine serum albumin). Determination of agonist affinity was carried out in an assay volume of 1 mL, including cell membranes, {^{3}H} spiperone (0.2 nM), GTP (100 μM), and test drugs. GTP was added to shift the receptors to the low-affinity agonist binding conformation, which is the relevant conformation in vivo.[14] (+)-Butaclamol (2 μM) was used to define nonspecific binding. Assay incubations were at 30 °C for 1 h and stopped by dilution with ice-cold wash buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl) and filtration with a Tomtec 96-well cell harvester. Samples were counted in a Wallace 1205 Betaplate scintillation counter. Competition curves were analyzed by nonlinear regression using Prism (GraphPad Software, Inc.). Agonist affinities as geometric means ± SEM for WT, S193A, S194A, and S197A D2 DA receptors are reported in Table 1.

Drugs and Reagents. DHX and 4-MNP-DHX were generous gifts from Dr. Richard Mailman (University of North Carolina).[15,16] ADTN, 7-OH-DPAT, bromocriptine, (+)-butaclamol, *R*-(−)-apomorphine, Cl-APB, Cl-PB, PPHT, quinpirole, SKF38393, lisuride, α-ergocriptine, and pergolide were obtained from Research Biochemicals International (Natick, MA). {^{3}H}Spiperone was from Amersham (Piscataway, NJ). Most other drugs and reagents were from Sigma Chemical Co. (St. Louis, MO).

Drug Affinities at Wild Type and Mutant D2 Dopamine Receptors. The training set consisted of a structurally diverse set of 16 agonists for which stereochemical information is known. Some of the drug affinity values have been reported previously.[9]

Computational Chemistry. Overview of the Approach. In this work, the initial conformations for alignment of the training set compounds were established from CONCORD-

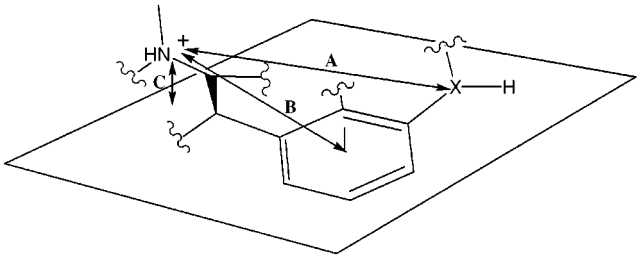
derived structures[17] and minimized using the MAXIMIN2 procedure within SYBYL.[18] The probable bound conformations of the agonists of the training set at WT and S193A, S194A, and S197A D2 DA receptors were then determined using flexible field fit methods. Agonist binding affinities at recombinant WT and mutant D2 receptors stably expressed in C6 glioma cells were obtained for all training set compounds. We related agonist affinities at the recombinant DA receptors to the CoMFA steric and electrostatic fields of the aligned structures.[11,19−21]

Templates for the alignment of the training set compounds are crucial in determining the final model. In the present experiments, we used bromocriptine, a high-affinity and conformationally constrained D2 agonist, as the standard template for the WT and mutant D2 receptor CoMFA models. This allowed a direct comparison among the models for effects of the mutations. However, this is a highly conservative approach for two reasons. First, it utilizes a compound whose affinity is *not* altered by the mutation. This is somewhat suspect from a biological perspective since one would prefer to have as a template a compound that fulfilled the normal criteria (high affinity and conformational constraint) but also was *responsive to the mutation*. Thus for the S193A mutant receptor, we utilized a *dual template* approach with pergolide as template in addition to bromocriptine. As a further control, pergolide was also used as the template for a CoMFA involving the WT receptor. As shown in Table 1, pergolide showed a substantial increase in affinity with the S193A mutation.

Pharmacophore Information. In determining relevant components of the pharmacophore map, key interactions between drug and receptor must be considered. Perhaps the single most essential feature of drug binding to dopamine receptors is the formation of the electrostatic bond between the Asp residue in transmembrane region III of the receptor and the positively charged nitrogen of the drug. While this feature does not distinguish between agonists and antagonists, it is absolutely essential for drug binding. The second type of essential features of a pharmacophore map are those which determine agonist binding, the hydrogen bonding interactions between hydroxyl or other hydrogen bonding elements of the drug and various Ser residues within transmembrane region V of the receptor. Key pharmacophoric features include the distances of the catechol hydroxyl group oxygens or equivalent from the cationic nitrogen and the height of the cationic nitrogen above the plane of the ring to which the hydroxyl oxygens or equivalent nitrogen are bonded (see Table 2). As previously discussed[11,12] such general features *do not* assume a common pharmacophore map for each CoMFA model. However, these approaches do recognize that certain portions of an agonist must be able to form electrostatic or hydrogen bonds to yield high affinity and efficacy. Pharmacophore maps were obtained from those aligned structures having high affinity at each receptor (Table 2). For ergoline compounds in the training set, we aligned the cationic nitrogen, the nitrogen on the five-member ring, and the aromatic six-member ring. Because CoMFA fields represent the enthalpic aspect of the overall free energy of the drug−receptor interaction and the relationship between free energy and the equilibrium-binding constant is logarithmic, the affinity values given in μM were expressed as $\ln(1/K_L)$.

Molecular Modeling: Initial Conformations and Template Selection. In the present study, SYBYL 6.5.2[18] was used for the modeling. Initial structures were generated using the cleanup procedure within SYBYL and minimized using MAXIMIN2. As previously discussed,[12] ligands probably do not bind to the receptor in their global minimum energy conformations. This is because some degree of torsional change or rotatable bond flexion is required to adapt the drug and receptor to electrostatic and hydrogen-bonding distances to yield a drug−receptor complex of lower energy. The "minimum" energy conformation resulting from a MAXIMIN2 procedure is, therefore, only a useful starting point for possible candidate conformations for the compound. Nevertheless, it is important to restrict the possible conformations of the drugs to those that can reasonably be obtained upon binding. Although there are no absolute rules, a ≈10 kcal/mol cutoff (difference between the local minimum and conformational energy) is considered reasonable in CoMFA studies.[22,23]

Table 2. Pharmacophoric Information for Compounds Having High Affinity at D2 DA Receptors[a]



X = O or N

compound	N−OH (meta)	N-plane	N-centroid
	Wild Type		
bromocriptine	6.0	0.45	5.225
lisuride	6.0	0.42	5.211
pergolide	6.0	0.66	5.217
PPHT (S-)	6.6	0.42	5.228
	Wild Type (PERG)		
bromocriptine	6.0	0.45	5.255
lisuride	6.0	0.28	5.212
pergolide	6.0	0.63	5.211
PPHT (S-)	6.0	0.02	5.236
	S193A		
bromocriptine	5.9	0.86	5.196
lisuride	6.0	0.32	5.238
pergolide	5.9	1.0	5.133
PPHT (S-)	6.5	1.4	5.035
	S193A (PERG)		
bromocriptine	5.9	0.86	5.196
lisuride	6.0	0.32	5.238
pergolide	5.9	1.2	5.110
PPHT (S-)	6.5	1.4	5.035
	S194A		
bromocriptine	6.0	0.45	5.255
lisuride	6.0	0.27	5.212
pergolide	6.0	0.58	5.214
PPHT (S-)	6.6	0.16	5.258
	S197A		
bromocriptine	6.0	0.45	5.255
lisuride	6.0	0.27	5.212
pergolide	6.0	0.62	5.198
PPHT (S-)	6.6	0.09	5.287

[a] Values are given in Å. In the accompanying figure, distance A represents the N−OH (meta) distance, distance B represents the N-centroid distance, and C represents the N-plane height.

Minimum energy conformations of the training set compounds served as the starting conformations for compound alignment. Determination of a particular (bound) conformation and alignment of all chosen conformations in a common orientation are necessary for pharmacophore map determination and as a prelude to the CoMFA. Selection of useful template compounds for each receptor is, therefore, essential (see above, Overview). In the present study we used *exactly* the same set of 16 agonists for several parallel CoMFAs to predict affinity at WT and mutant D2 receptors. This was crucial to achieve the goals of the present work since a major objective was to compare the various D2 receptor CoMFA models directly. In each major analysis, drug alignment was with a conformer of the template compound bromocriptine, a drug with high D2 affinity and little conformational flexibility. This yielded a unique set of alignments of the training set compounds for each CoMFA model. The CoMFA model was optimized for prediction of drug affinity at the particular WT/mutant receptor.

Alignment. Flexible field fit procedures are an improvement over static fit methods because they allow minor changes in compound conformation as a result of the field fit process.[10,11,19−21,24,25] MOPAC charges were recalculated following the flexible field fit, and the conformational energy of the compound was obtained. Following CoMFA model improvement at each receptor, the flexible field fit procedures were repeated using the CoMFA region from the previous best run and the electrostatic and steric fields for the template associated with that model. Compounds in the training set were refit to the template based on the size of the residual resulting from the previous CoMFA run. This set of procedures was repeated until the model could not be further improved. In this way the final, "optimum" CoMFA model was derived iteratively.[11,12]

CoMFA. Regression of a target property (dependent variable) {e.g., $\ln(1/K_L)$} against predictors (independent variables) calculated as steric and electrostatic components of the intermolecular interaction field forms the basis for partial least squares (PLS) regression. As with other regression methods, the model retains only those predictors that differentiate among one another in the final version. Thus, for example, although the cationic nitrogen is clearly an essential feature of drug binding to receptors for biogenic amines, it has essentially no weight in the final CoMFA model because it is a feature common to all compounds in the training set. The predictors are evaluated at the grid points of a three-dimensional lattice containing all members of the training set of aligned compound structures. In the present study, "grid boxes" were generated for alignments based on templates as follows: WT, bromocriptine and pergolide; S193A, bromocriptine and pergolide; S194A, bromocriptine; and S197A, bromocriptine. As discussed,[11,12] we have systematically investigated the effects of changing several CoMFA parameters, including dielectric (function as "1/*r*" vs constant), grid step size (1.5−2.5 Å), probe atom type (H^+, O^-, $C_{sp3}{}^+$), and the column filtering values (≈1.0−4.0 kcal/mol). We used the "leave one out" method for cross-validation.[18] In the present paper, we restricted the steric and electrostatic cutoff values to those most consistent with noncovalent interactions (steric ≥ 15 kcal/mol; electrostatic ≥ 22 kcal/mol.[26] We also optimized the CoMFA using the Region Focus feature within SYBYL, which utilizes a discriminate (or alternative) function to select only those CoMFA components from an optimal non-cross-validated PLS analysis that actually contribute to prediction of affinity.

Computational chemistry approaches to the study of drug−receptor interactions only analyze trhe enthalpic, not entropic, aspects of drug binding. However, incorporating drug hydrophobicity represents a convenient first approximation of some aspects of the entropic interaction. To determine the effect of the hydrophobicity variable on the final model, a variant of the standard hydrophobicity term (C*logP; derived from CoMSIA) was incorporated into the molecular spreadsheet and added as a predictor variable to the CoMFA. Both default and final CoMFA models incorporating the hydrophobic term were studied at all receptors. ("SYBYL 6.5.1 introduced CoMSIA (Comparative Molecular Similarity Indices Analysis) as a method for comparing molecular structures among a group of structures brought into a common alignment. It was developed by Drs. Gerhard Klebe, Ute Abraham, and Thomas Mietzner while at BASF Ludwigshafen, Germany. This technique is most commonly used in drug discovery to find the common features that are important in binding to the biologically relevant receptor. CoMSIA is an extension of the CoMFA (Comparative Molecular Field Analysis) methodology. Both are forms of QSAR (Quantitative Structure Analysis Relationships), and both are based on the assumption that changes in binding affinities of ligands are related to changes in molecular properties, represented by fields. They differ only in the implementation of the fields. The CoMSIA field types are: steric, electrostatic, hydrophobic, hydrogen bond donors, and hydrogen bond acceptors." [SYBYL 6.5.1 manual])

Although we used the Region Focus method within SYBYL to attempt to further improve the best models achievable by standard methods, we decided to use the contour maps derived from the standard models as the basis for comparison, to ensure a valid comparison among models.

Docking of Agonists into D2 Receptor Models. The protein homology model used here for docking was essentially that developed for the D2 receptor by two of the coauthors.[27,28] In brief, it was based on the experimentally obtained three-dimensional bacteriorhodopsin structure and D2 receptor ligand binding mutagenesis data. These data were used to align rhodopsin with bacteriorhodopsin. Alignment of low homology sequences was aided by establishing polar and nonpolar faces via helical wheels. From the alignment, D2 residues were substituted into the Protein Data Bank coordinates of bacteriorhodopsin to build an initial databased D2 model. Local geometry optimization, Pro replacements, and side chain rotation consistent with protein structure knowledge refined this model. Global energy minimization was not used for the final model for the reasons discussed[27] (footnote 1 of that citation).

Four D2 agonists (bromocriptine, pergolide, dopamine, and PPHT) representing D2 agonists of varied structure in the CoMFA alignment derived here, were docked into the dopamine D2 receptor homology-based models as we have previously published.[27,28] As a first approximation, the models were not changed from the model described in our most recent paper.[12] Target points for the docking were the Asp in helix III and the Ser residues on helix V. Docking was optimized in the region of the cationic nitrogen and lone pair electrons of each compound.

Since bromocriptine had only one of the hydrophilic groups (the nitrogen in the five-member ring) which corresponds to the meta-hydroxyl of dopamine, docking to only one Ser hydroxyl of the receptor was possible. The hydroxyl in PPHT was aligned with the bromocriptine nitrogen in the five-membered ring. To a first approximation, the four docking sites of the training set compounds to the WT and three mutant receptors were quite similar, despite the differences in the mutant Ser. Although docking only involved residues in the binding site, PPHT also fit into the ancillary pocket. This hydrophobic pocket was described earlier and consisted of aromatic residues F110, W358, F361, and Y380.[27,28] The influence of this pocket on the CoMFA analysis is described in the Results, Protein Homology Modeling section.

Results

Drug Affinity. We determined D2 agonist affinity in membranes prepared from C6 glioma cells expressing the recombinant receptor variants (Table 1). The assays were conducted under conditions that prevented coupling of the receptors to G proteins. Hill coefficients for competition binding curves did not differ significantly from unity (data not shown). None of the mutations markedly affected the binding of the ergolines with two exceptions. First, lisuride's affinity at the S194A receptor was decreased (Table 1). In contrast, the affinity of S193A for pergolide (alternate template) was increased compared to the affinity of the WT D2 receptor for that ergoline. The relative stability of bromocriptine binding affinity across all mutations provided a basis for the use of this compound as the standard CoMFA template. In contrast, the *increase* in affinity for pergolide at the S193A receptor constituted an ideal basis for its selection as an alternate CoMFA template (a dual CoMFA template approach).

There were some striking results from comparing the effects of Ser-to-Ala mutations on agonist affinity. First, only one compound (pergolide at the S193A receptor) showed a large increase in affinity. Second, several

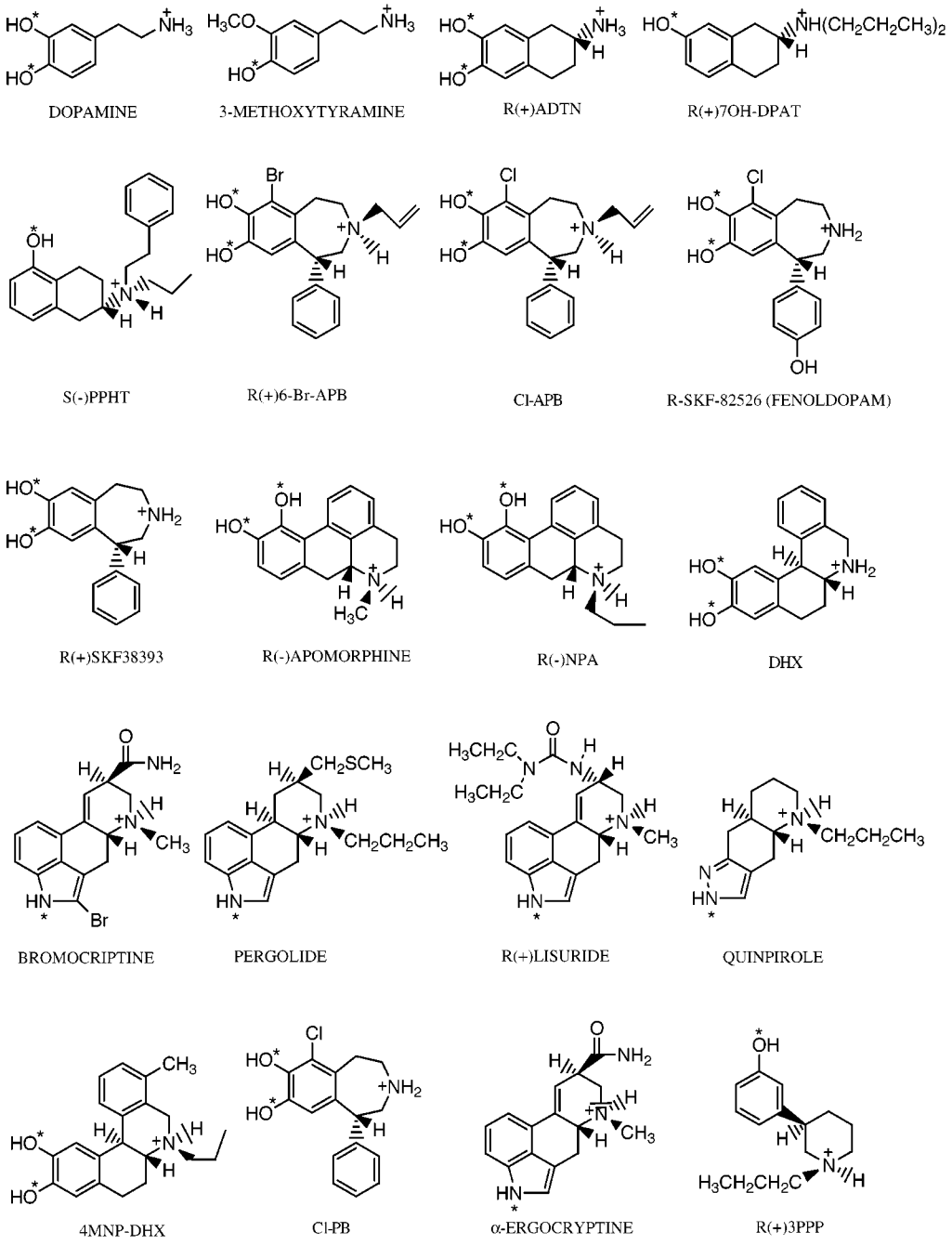


Figure 1. Structures of drugs in the training and test sets. *Refers to groups used in establishing the pharmacophore map.

compounds (ADTN, APO, DA, DHX, fenoldopam, and NPA) showed large decreases in affinity at the S193A and/or S194A receptors. Finally, DA itself showed the most consistent effects (reductions in affinity) across all mutations.

Computational Chemistry. Two-Dimensional Structures and Alignment of the Training Set Compounds. Flexible field fit procedures were used to determine the final alignments and conformations of compounds in the training sets. Final alignments for the WT and three mutant CoMFA models using the standard (bromocriptine) template were similar (Figure 2, stereoview). In all of them, the importance of the cationic nitrogen and hydrogen bonding regions is apparent.

Pharmacophore Maps for Affinity at Dopamine Receptors. A pharmacophore map and information derived from it are shown in Table 2 for WT and mutant models using the bromocriptine-based template and for the S193A receptor with both bromocriptine- and pergolide-based templates. For the compounds with highest affinity (lisuride, bromocriptine, and pergolide) at the WT and mutant D2 receptors, the distance between the carbon of the five-member ring "meta" to the cationic nitrogen and the cationic nitrogen was 5.9−6.6 Å. For the catecholamine compound PPHT, this value was 6.5−6.6 Å. The height of the cationic nitrogen above the plane of the ring associated with the hydrogen bonding is another key pharmacophoric feature. The value for this element ranged from 0.42 to 0.66 Å for the WT, to 0.32−1.4 Å for the S193A (bromocriptine), 0.32−1.4 Å for the S193A (pergolide), 0.27−0.58 Å for the S194A, and 0.09−0.62 Å for the S197A receptors.

Plane heights for bromocriptine changed from a low

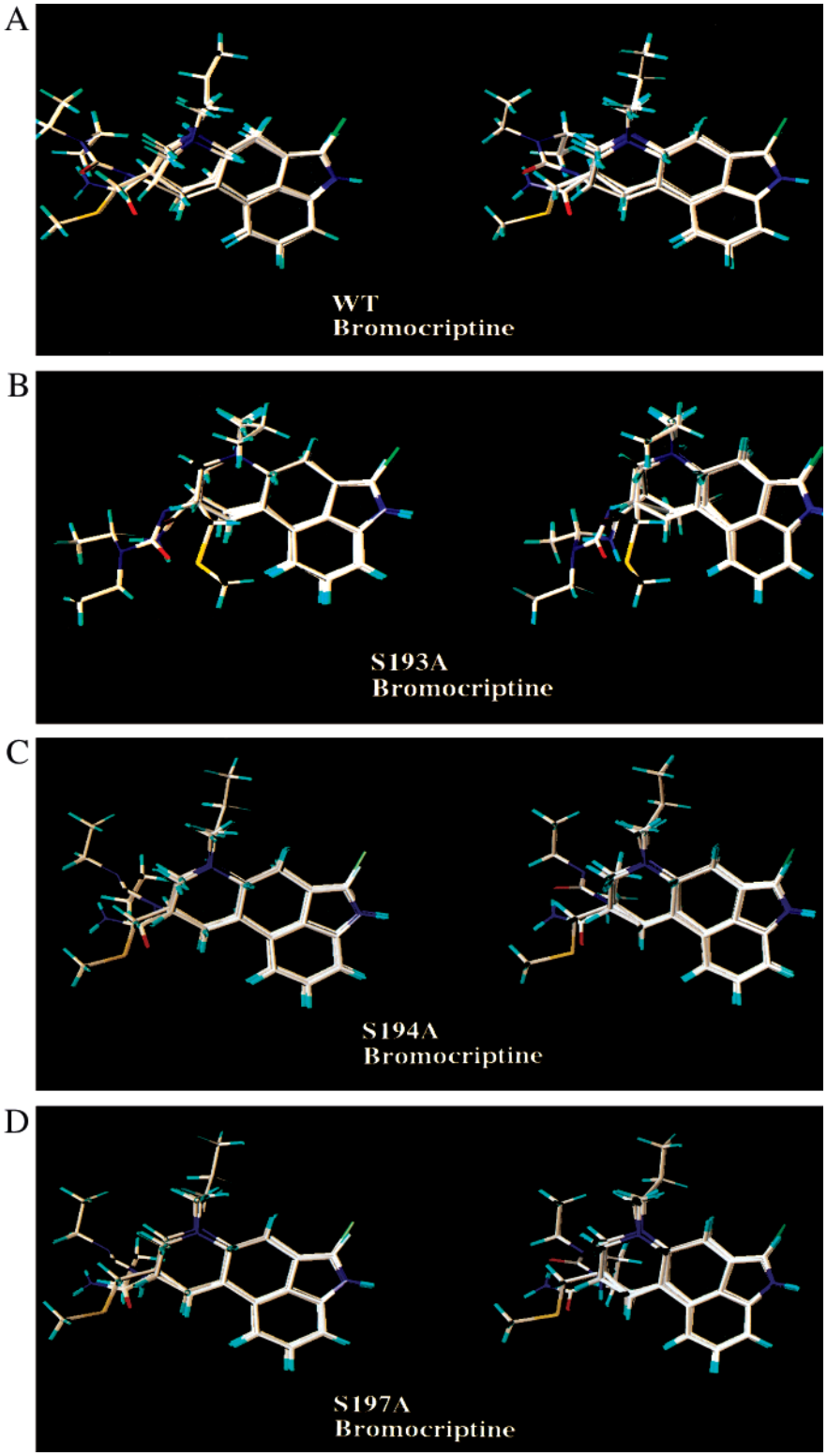


Figure 2. Structures of the high-affinity aligned drugs of the training set. All panels show bromocriptine, pergolide, and lisuride (stereoview). Panel A: alignment with bromocriptine for WT D2 receptor affinity. Panel B: alignment with bromocriptine for S193A D2 receptor affinity. Panel C: alignment with bromocriptine for S194A D2 receptor affinity. Panel D: alignment with bromocriptine for S197A D2 receptor affinity.

of 0.45 Å at WT to a high of 0.86 Å at the S193A receptor. Plane heights for lisuride were slightly reduced by all three mutations. Plane heights for pergolide varied, being maximal for the S193A receptor, particularly for the pergolide-based S193A pharmacophoric model (1.2 Å). The plane height for the catechol compound (S)-PPHT was greatest at the S193A receptor.

Comparative Molecular Field Analysis. To ensure maximal consistency among the models, the following restrictions were placed on model optimization. First, both steric and electrostatic features were used. Second, the same constraints (steric $\geq \approx 15$, electrostatic $\geq \approx 22$) were placed on all models. Third, the distance-dependent dielectric function was used (rather than set to a

Table 3. CoMFA Summary for WT and Mutant D2 DA Receptors[a]

fields	*E* cutoff, kcal per mol	dielec. funct.	CoMFA region	min. σ	grid step size, Å	probe atom type	no. columns	q^2	no. comp.	SEP	q^2 (no.) CoMFA + hydro.[b]
				Wild Type							
default (both)	S30/E30	1/*r*	default (auto)	2.0	2.0	Csp3+	284/2112	0.716	6	2.240	0.654 (8)
BEST	S25/E21.5	1/*r*	+0.5*X*, +0.2*Z*	3.7	2.0	Csp3+	168/2112	0.849	5	1.548	0.728 (7)
Region Focus[a]	S25/E21.5	1/*r*	+0.5*X*, +0.2*Z*	2.8	2.0	Csp3+	120/2112	0.911	6	1.254	
				S193A							
default (both)	S30/E30	1/*r*	default (auto)	2.0	2.0	Csp3+	265/2178	0.682	3	2.435	0.617 (6)
BEST	S33/E20	1/*r*	+0.4*X*, −1.4*Z*	3.5	2.0	Csp3+	172/2178	0.864	5	1.743	0.826 (3)
Region Focus	S33/E20	1/*r*	+0.4*X*, −1.4*Z*	2.0	2.0	Csp3+	68/2178	0.945	6	1.105	
				S194A							
default (both)	S30/E30	1/*r*	default (auto)	2.0	2.0	Csp3+	294/2376	0.604	6	2.328	0.591 (6)
BEST	S30.0/E20.0	1/*r*	+1.0*X*, +0.2*Y*, +0.1*Z*	3.7	2.0	Csp3+	166/2376	0.842	5	1.395	0.758 (8)
Region Focus	S30.0/E20.0	1/*r*	+1.0*X*, +0.2*Y*, +0.1*Z*	3.1	2.0	Csp3+	98/2376	0.939	5	0.870	
				S197A							
default (both)	S30/E30	1/*r*	default (auto)	2.0	2.0	Csp3+	290/2376	0.666	5	2.605	0.617 (6)
BEST	S16/E22	1/*r*	+0.75*Y*	2.9	2.0	Csp3+	204/2376	0.835	5	1.832	0.754 (10)
Region Focus	S16/E22	1/*r*	+0.75*Y*	2.0	2.0	Csp3+	139/2376	0.925	5	1.205	
				WT − Pergolide							
default (both)	S30/E30	1/*r*	default (auto)	2.0	2.0	Csp3+	284/2112	0.716	6	2.240	0.654(8)
BEST	S30/E22	1/*r*	default (auto)	3.9	2.0	Csp3+	164/2112	0.822	6	1.773	0.735(8)
Region Focus	S30/E22	1/*r*	default (auto)	2.6	2.0	Csp3+	128/2112	0.899	6	1.335	0.654(8)
				S193A − Pergolide							
default (both)	S30/E30	1/*r*	default (auto)	2.0	2.0	Csp3+	269/2178	0.680	3	2.444	0.706 (6)
BEST	S33/E20	1/*r*	+0.4*X*, −1.4*Z*	3.2	2.0	Csp3+	184/2178	0.849	3	1.677	0.819 (3)
Region Focus	S33/E20	1/*r*	+0.4*X*, −1.4*Z*	2.2	2.0	Csp3+	147/2178	0.905	6	1.539	

[a] Region Focusing is performed on the non-cross-validated CoMFA model which normally uses information in all columns. [b] "Hydro." refers to the hydrophobicity component (from CoMSIA); "no." refers to the number of principal components used in the PLS model.

constant value). Fourth, all final CoMFA models utilized a Csp3+ probe atom, and a 2.0 Å step size to ensure comparability among them. Various CoMFA regions were found to be optimal among the various models. The minimum σ value in the various models ranged from the default value of 2.0−4.0 kcal/mol. Steric and electrostatic cutoff energies ranged from 16 to 33 and from 20 to 30 kcal/mol, respectively. Finally, the contour maps were all displayed using the 80:20 percentile feature.

Standard Template-Based CoMFA Models. The cross-validated R^2 (q^2) values that resulted from the various CoMFA options for $\ln(1/K_L)$ as the target property are shown in Table 3 for the WT and mutant recombinant D2 receptors. Results from the initial CoMFA run using the SYBYL default settings ranged from q^2 values of 0.604 to 0.716. The results from the optimal CoMFA models for each D2 receptor are also given in Table 3. These values ranged from a q^2 of 0.835 to 0.864 without and from 0.911 to 0.945 with Region Focusing. As expected, the R^2 values for default, best, and region focused CoMFA models ranged from 0.999 to 1.000 (Figure 3). As discussed in Methods, "best" models were those yielding the highest q^2 values plus the lowest values for the SEP (standard error of prediction). These best models all utilized both steric and electrostatic features (ranging from 16 to 33), a dielectric function of "1/*r*", a grid step size of 2.0 Å, Csp3+ probe atom, and minimum σ values ranging from 2.0 to 3.7. The optimum CoMFA region involved deviation from the default region calculated by SYBYL by no more than 1.4 Å in any one direction. Overall, the models were quite robust. In some cases (WT), the default q^2 was so high that relatively little refinement needed to be done to optimize the model. In other instances (all mutant models) optimization improved the q^2 from approximately 0.7 to 0.9. The importance of region focusing was shown by the fact that this procedure increased the q^2 value for the best S197A model from 0.835 to 0.925. As noted above (Methods), an a priori decision was made to use the nonregion focused models as the basis for comparing the contour maps among the receptor variants. Region Focusing was conducted on the optimal non-cross-validated CoMFA model at each receptor. This method includes only those features of the CoMFA that optimally contribute to the model. As expected, an examination of Table 3 shows that the number of CoMFA columns used in the model resulting from Region Focusing was somewhat less than the number of columns used in the original optimal model. In summary, even the poorest of the optimized cross-validated CoMFA models accounted for > 75% of the variance in the affinity data while the best model accounted for >90% of the variance in the dependent variable.

Figure 3 shows the relationships between measured and predicted affinities for the non-cross-validated models having R^2 values from 0.999 to 1.000. None of the training set compounds had predicted affinities outside the 95% confidence intervals.

To provide an initial estimate of a possible entropic contribution of the mutations to agonist binding, a hydrophobic term (as a variant of the standard C*logP) was added as a column in each molecular spreadsheet. This was done using CoMSIA (Comparative Molecular Similarity Indices Analyses, SYBYL 6.5.2). That column was used as an *extra predictor variable* in the CoMFA analyses. This resulted in a PLS in which agonist affinity was predicted by a combination of the CoMFA column information plus the hydrophobicity term. As shown in Table 3, addition of this term to the model generally lowered the q^2 value (except for the default run for the pergolide-S193A model). (Temperature

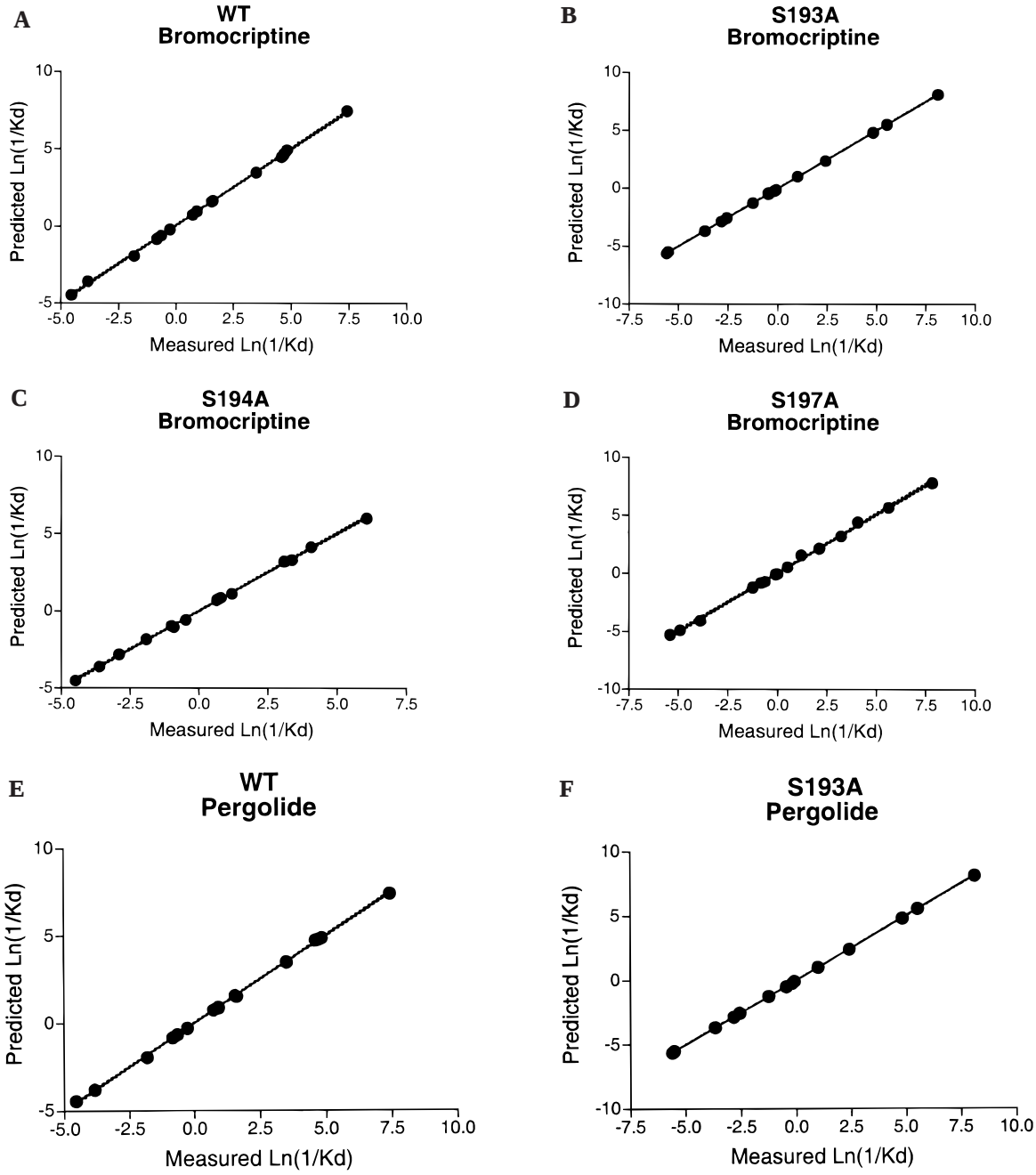


Figure 3. Correlation (for non-cross-validated partial least-squares analysis) between measured and predicted affinities for training set compounds at WT and mutant D2 receptors. Model represented in panels A−D used bromocriptine as the template. Models represented in panels E−F used pergolide as the template. R^2 values ranged from 0.999 to 1.000. Panel A: WT, bromocriptine template. Panel B: S193A, bromocriptine template. Panel C: S194A, bromocriptine template. Panel D: S197A, bromocriptine template. Panel E: WT, pergolide template. Panel F: S193A, pergolide template.

dependence of binding represents one direct means to determine possible entropic aspects of drug−receptor interactions.)

Alternate Template-Based CoMFA Models. For the WT and S193A receptors, CoMFA models were also derived using pergolide as the template. These models were virtually identical in terms of the basic features incorporated into them except in the number of CoMFA descriptor columns entering into the model. The use of pergolide as a template for the S193A model resulted in essentially no change in the optimum models in terms of predictive validity (q^2). Thus, pergolide was as good a choice as bromocriptine for the training set template, based on this objective criterion. As discussed below (contour maps), other indicators suggested that pergolide might be a superior template for the S193A CoMFA.

Contour Maps with Bromocriptine Templates. Figure 4 (panel A) shows the contour map of the final WT D2 CoMFA model, whereas panels B−D of the same figure present the contour maps for the three mutant D2 receptors (panel B, S193A; panel C, S194A; panel D, S197A). Both steric and electrostatic fields were utilized, since this improved the standard error of prediction (SEP) and the q^2 value over alternative models for both WT and mutant D2 receptors. As discussed above, incorporation of similar features in all models facilitated a direct comparison among them.

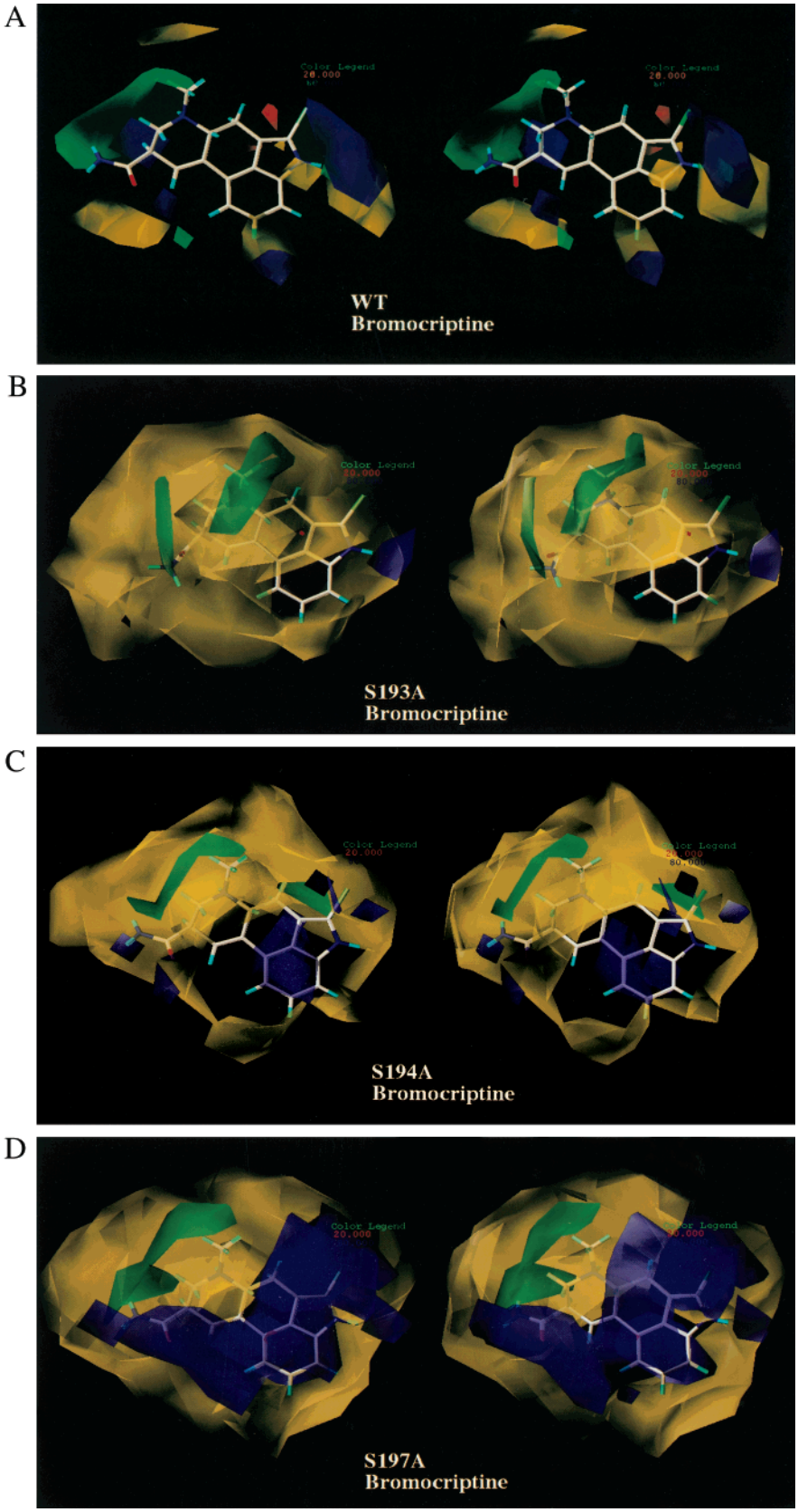


Figure 4. Contour map of steric and electrostatic fields (standard deviation times coefficient) from WT and mutant D2 DA receptor CoMFA models. Figure shown is reproduced at the following URL: http://www.utexas.edu/pharmacy/divisions/pharmtox/faculty/wilcox.html. Color coding is as follows: Blue = positive charge favors high affinity; red = positive charge does not favor high affinity. Green = steric bulk favors high affinity; yellow = steric bulk does not favor high affinity. Panel A: WT, bromocriptine template. Panel B: S193A, bromocriptine template. Panel C: S194A, bromocriptine template. Panel D: S197A, bromocriptine template.

The contour maps showed several potentially important characteristics. First, all maps exhibited little in the way of electrostatic features in the vicinity of the cationic nitrogen (Figure 4, panels A−D). This suggests

that *differences* among the training set compounds in this region of the molecule are not of major importance in high-affinity binding at WT or mutant D2 DA receptors. This is consistent with the importance of the electrostatic bond between the N+ and the Asp of transmembrane region III in binding of all D2 ligands. Steric features near the cationic nitrogen are of some importance in differentiating agonist binding affinity, since sterically unfavored regions predominated only in the mutant receptors.

A second salient characteristic is that, whereas hydrogen-bonding regions are highly significant for favoring high affinity in all variants of the D2 receptor, these regions are not the same in all receptor variants. For the WT and S193A receptor models, the regions in which positive charge is favored are mostly restricted to the vicinity of the hydrogen-bonding nitrogen, although electrostatically favored regions figure more prominently in the WT than in the S193A model. In contrast, for the S194A and S197A CoMFA models there are extensive regions near the hydroxylated aromatic ring in which positive electrostatic charge favors high affinity. In fact, there is something of a progression of increasing importance of a larger electrostatically favored region for positive charge from WT to S193A to S194A and S197A receptors. Only in the S197A model do the positive electrostatically favorable regions extend dramatically between the aromatic ring, and the ring containing the hydrogen-bonding hydroxyl. However, all models show that regions of positive charge near the hydrogen-bonding regions of the molecule favor high-affinity binding.

Steric features, especially regions in which steric bulk is unfavored for high affinity, appear considerably more prominent in each of the mutant receptor models than in the WT model. All models have in common a sterically favored region that lies close to the cationic nitrogen. The similarities between S194A and S197A models are fairly striking. Here, sterically favored regions are found near the alkyl moieties of the high-affinity ergoline compounds. Overall, there are large regions in both S194A and S197A models where positive charge favors high-affinity agonist binding.

Contour Maps of the WT Receptor with Bromocriptine vs Pergolide Templates. Figure 5 (panels A−B) compares the contour maps for the WT receptor using bromocriptine vs pergolide templates. This comparison was made to validate the proposal to use as template for the S193A receptor that drug which showed the biggest increase in affinity (see below). First, both maps exhibited little in the way of electrostatic features in the vicinity of the cationic nitrogen. This suggests that differences among the training set compounds in this region of the molecule are not of major importance in high-affinity binding at WT receptors for models based on either template. Second, the regions in which positive charge is favored include a large region in the vicinity of the hydrogen-bonding regions and additional regions where the compound may interact with stabilizing aromatic residues of the receptor. Highly similar electrostatically favored regions are apparent for both the bromocriptine- and pergolide-based contour maps. Third, steric features, especially regions in which steric bulk is unfavored for high affinity, are not especially prominent for the WT models (compared with the other mutant models, Figures 4 and 6). Both bromocriptine- and pergolide-based models have in common a sterically favored region that lies close to the cationic nitrogen. Essentially the only difference between the bromocriptine- and pergolide-based models for the WT D2 receptor is the small region in which positive charge is electrostatically unfavored for high-affinity agonist binding in the bromocriptine-based but not the pergolide-based model. Together, these results strongly suggest that the differences between CoMFA models for the WT vs the various mutant D2 receptors cannot be attributed to choice of template compound. However, the results are also consistent with the idea that use of a biologically/chemically optimal template can lead to model differences which are subtle but of potential utility.

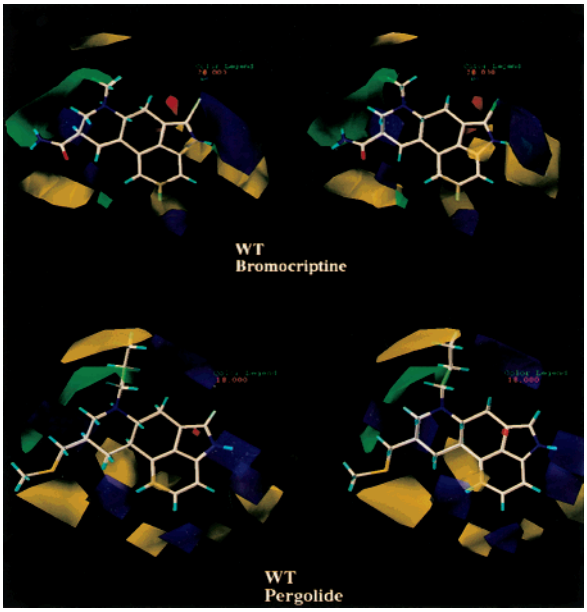


Figure 5. Contour map of steric and electrostatic fields (standard deviation times coefficient) from the WT D2 DA receptor CoMFA models (stereoview). Figure shown is reproduced at the following URL: http://www.utexas.edu/pharmacy/divisions/pharmtox/faculty/wilcox.html. Color coding is as follows: Blue = positive charge favors high affinity; red = positive charge does not favor high affinity. Green = steric bulk favors high affinity; yellow = steric bulk does not favor high affinity. Panel A: WT, bromocriptine template. Panel B: WT, pergolide template.

Contour Maps of the S193A Receptor with Bromocriptine vs Pergolide Templates. Figure 6 (panels A−B) compares the contour maps for the S193A receptor using bromocriptine vs pergolide templates. Noteworthy is the fact that the key features identified above for the S193A receptor using a bromocriptine-based template are virtually identical to those derived using pergolide as the template. While the dominant features are highly similar between the two models, there are some subtle and perhaps significant differences. First, while the regions in which positive charge favors high-affinity are identical between the models, the regions in which positive charge are not favorable for high-affinity are distinct. In the bromocriptine-based model, this region (red) is distant from the electrostatically favored region (blue), whereas in the pergolide-based model the unfavored region is larger and slightly

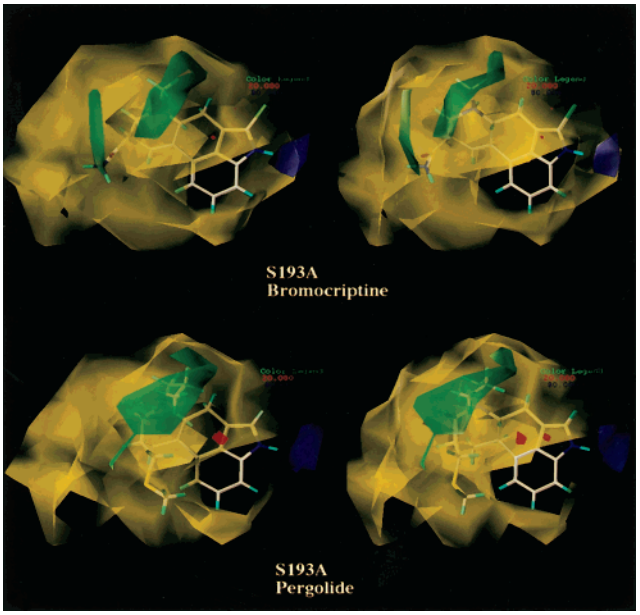


Figure 6. Contour map of steric and electrostatic fields (standard deviation times coefficient) from the S193A D2 DA receptor CoMFA models (stereoview). Figure shown is reproduced at the following URL: http://www.utexas.edu/pharmacy/divisions/pharmtox/faculty/wilcox.html. Color coding is as follows: Blue = positive charge favors high affinity; red = positive charge does not favor high affinity. Green = steric bulk favors high affinity; yellow = steric bulk does not favor high affinity. Panel A: S193A, bromocriptine template. Panel B: S193A, pergolide template.

closer to the favored region. Second, whereas the bromocriptine-based model has a sterically favored region near the cationic nitrogen and in the region where the highest-affinity ergolines project alkyl substituents, there is a second sterically favored region in this model toward the other end. In contrast, the pergolide-based model shows a single major electrostatically favored region around the cationic nitrogen and alkyl substituent region. As indicated below (protein homology modeling) this observation is consistent with the role played by the alkyl substituents in facilitating a stable binding of the highest affinity ergolines to the D2 receptor. This validates the use of an alternate template compound.

Predictive Abilities of the CoMFA Models. Affinities were obtained for WT and mutant D2 DA receptors for three test compounds not in the training set (Table 4). The affinity of the receptors for the test compounds, 4-MNP-DHX, α-ergocriptine, and 6-Cl−PB, exhibited a considerable range, as did the structures of these compounds (Figure 1). Table 4 shows the results of predicting the affinity of these compounds at the WT and mutant D2 receptors, and this is depicted graphically in Figure 7.

As shown in Table 4, predictions of agonist affinity from each of the four bromocriptine-based CoMFA models was quite good. Residual values for measured − predicted affinity ranged from 0 to 0.03 μM for 4-MNP-DHX, from 0 to 2.8 nM for α-ergocriptine, and from 0 to 3 μM for 6-Cl-PB. Interestingly, the predictions for drug affinities at the S193A receptor were improved when pergolide was used as the CoMFA template rather than bromocriptine (smaller residual values, Table 4).

A further important aspect of the four standard

Table 4. Prediction of Affinity of Test Compounds at WT and Mutant D2 Receptors[a]

test compound	wild type	wild type (perg)	S193a	S193a (perg)	S194a	S197a
4MNP-DHX						
measured	0.3	0.3	1.3	1.3	0.4	0.7
predicted	0.27	0.31	1.26	1.31	0.37	0.7
residual	0.03	−0.01	0.04	−0.01	0.03	0
α-ergocriptine						
measured	0.00062	0.00062	0.0018	0.0028	0.0028	0.001
predicted	0.0035	0.0028	0.0036	0.0028	0.0044	0.001
residual	−0.00288	−0.00218	−0.0018	−0.001	−0.0014	0
6-CI-PB						
measured	3	3	13	13	13	3
predicted	3	2.83	9.87	14.15	12.43	2.92
residual	0	0.17	3.13	−1.15	0.57	0.08

[a] K_d values shown are in μM.

CoMFA models is that they predicted different affinities for the test compounds. Predicted affinities for 4-MNP-DHX ranged from 0.3 to 1.3 μM; those for 6-Cl-PB ranged from 3 to 13 μM; and those for α-ergocriptine ranged from 0.6 to 4 nM. Thus, the four major CoMFA models clearly differentiate among compounds with diverse structures in terms of predicted affinity, and the measured and predicted affinities were highly correlated ($R^2 = 0.99$; Figure 7).

Protein Homology Modeling of Agonist Binding to D2 Receptors. Overall, the dominant effect of the mutations on docking is to shift the drugs *toward* the mutated residue and allow more room for the bulky groups. For the three mutants (S193A, S194A, S197A), the CoMFA results indicate that two features primarily determine differential binding. These are the drug's bulky, hydrophobic groups that face nonpolar residues at one edge of the binding pocket (F110, F361) and the ancillary pocket (Y380) and those that lie toward W358 (Figure 8). Two of these residues, W358 and F361, are in the fingerprint region of helix VI (CWLPFF) that is highly conserved in all G protein coupled receptors. In these experiments, docking was performed with DA and (S)-PPHT as catecholamines and bromocriptine and pergolide as ergolines.

When Ser-193 was mutated to Ala, pergolide binding was most enhanced while the binding of PPHT was decreased. The most important steric feature for better binding according to the CoMFA contour maps (Figure 4, panel B) is the propyl group attached to the positively charged nitrogen of pergolide, which points toward W358. PPHT also has a propyl group on the nitrogen, which points in the same direction. But PPHT has a phenyl group that points toward the binding and ancillary pockets (see Methods: "Docking of agonists into D2 receptor models"). The CoMFA map suggests that bulk in this location is unfavorable for better binding. This implies that the phenyl ring is sterically hindered in interaction with these pockets. This explains the lack of enhancement of the binding of PPHT vs the binding of pergolide with the S193A mutation.

Mutation of Ser-194 to Ala decreases binding of lisuride and (S)-PPHT. One (S)-PPHT phenyl group that is in an unfavorable area of the steric surface points toward the binding pocket, close to F361. In lisuride, one ethyl group on the secondary amine of the peptide group also is directed toward this sterically unfavorable

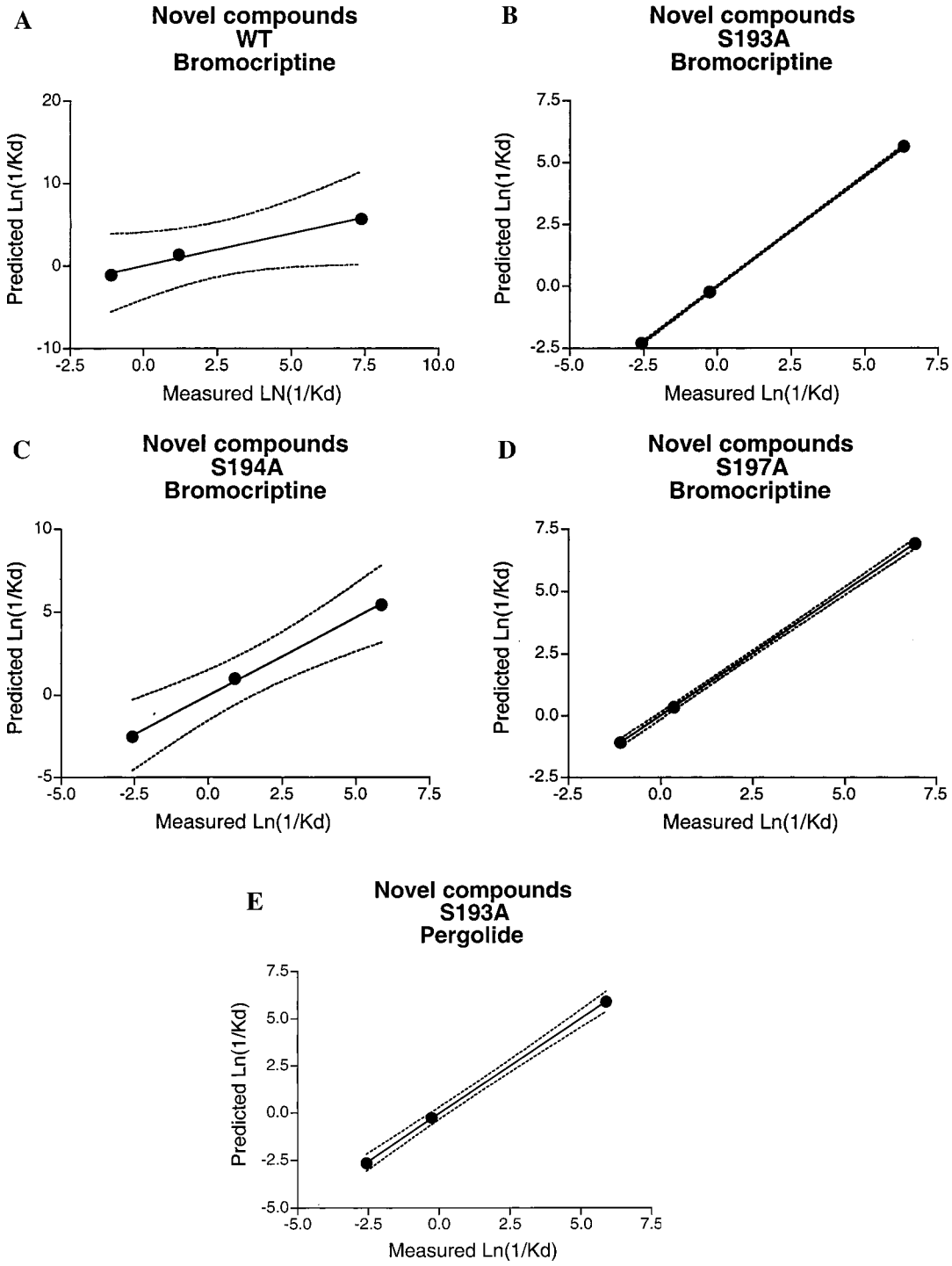


Figure 7. Correlation (non-cross-validated R^2) between measured and predicted affinities for compounds not in the training set at WT and mutant D2 receptors. Panels A−D used bromocriptine as the template. Panel E used pergolide as the template. Panel A: WT, bromocriptine template. Panel B: S193A, bromocriptine template. Panel C: S194A, bromocriptine template. Panel D: S197A, bromocriptine template. Panel E: S193A, pergolide template.

surface. Close contacts with F361 may thus account for their less tight binding for this mutant. In Ser-197 changed to Ala, the favorable steric surface is toward F361, and binding of these ligands is restored to WT values.

In the current homology model, the reduced binding affinity of S193A for dopamine can be explained indirectly, that is, from the hydrophobic interactions outlined above. Since S193 is buried in helix contacts and inaccessible to drugs in the current model, we cannot account for the binding affinity changes in terms of hydrogen bonding of dopamine or any of the drugs to the receptor. In contrast, S194 and S197 hydroxyl groups are accessible to the binding site, and decreased hydrogen bonding can directly effect the dopamine binding for S194A and S197A mutations.

Of all the test agonists docked by the serine mutations, bromocriptine was among the least affected. This may be because bromocriptine contains a bulky group not included in the present model that is much more important for binding affinity than the steric features important for compounds such as PPHT and pergolide.

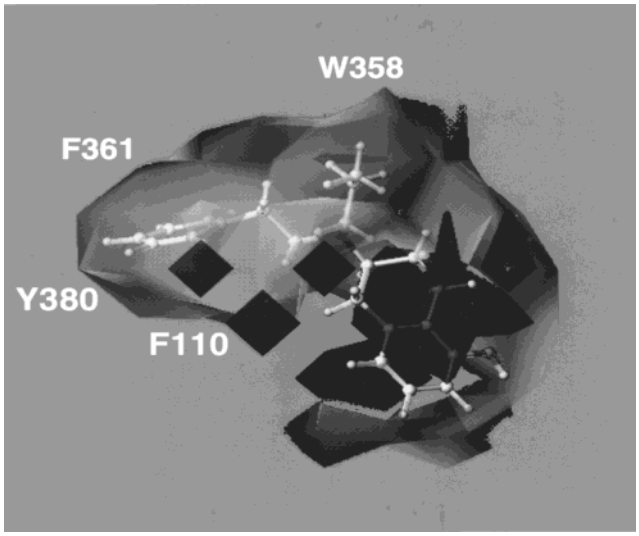


Figure 8. Relationship between aligned compounds of the training set and key amino acid residues of the S194A D2 receptor.

For the WT D2 receptor, the CoMFA steric map (Figure 4, panel A) shows that the most favorable determinants for binding lie in the direction of the peptide unit in bromocriptine, where this bulky group is found. This stabilizing bulky ring may contribute substantially to binding. This is consistent with the 10-fold higher binding affinity of bromocriptine for the WT dopamine receptor as compared with pergolide.

Discussion

Overall Quality of the Models. The present results add to the information on agonist interactions with WT and mutant D2 dopamine receptors obtained from classical studies with drug enantiomers[29] and protein modeling.[27,28,30,31] Here, we determined the extent to which CoMFA-derived models for WT and mutant D2 DA receptors differed for the same training set of DA agonists and using the same template (bromocriptine). The predictive utility of the CoMFA models for the three DA agonists not in the training set (4-MNP-DHX, Cl-PB, α-ergocriptine) was generally excellent; the differences between the measured and predicted affinities were quite small. Across CoMFA models there was a range of predicted affinities for each test compound that was highly correlated with the differences in measured affinities among the WT and the three mutant D2 receptors. Differences were also observed for contour maps derived from the CoMFA models. It is of special note that the use of the same training set of structurally diverse agonists to predict affinities measured at WT and mutant D2 DA receptors allows a more direct comparison of the computational chemistry models than has previously been possible. These results suggest that the CoMFA-derived WT and mutant D2 DA receptor models may have utility for predicting affinities of novel agonists. Thus, a major finding of the present report is that high-quality CoMFA models that accurately predict agonist affinity across D2 receptor variants and differ among themselves are obtainable using the differential QSAR method.

A second major result of the present work is the direct comparison among CoMFA-derived contour maps for agonist binding to WT and three Ser-to-Ala mutant D2 receptors. Significantly, there were striking differences in the distribution of favored positively charged regions and of regions in which steric bulk was favored among the models. That these differences occurred using the same training set of compounds and the same template compound (bromocriptine) argues that the differences are the result of the mutations per se. These results support the contention that a comparison among contour maps may be useful in rationalizing the results of in vitro mutagenesis experiments and may enable the prediction of new lead compounds that would show greater selectivity at one or another receptor variant. If such selectivity could be achieved among a set of receptors differing in a single amino acid residue among 415, then the approach could be readily adapted to a comparison of endogenous receptor subtypes. In turn, the differential QSAR method described in the present and in another recent paper[12] could yield development of subtype selective agonists of therapeutic utility in Parkinson's disease, schizophrenia, and addiction.

The third major result of the present report was the "proof of principle" whereby an alternative template compound, pergolide, was utilized to derive a CoMFA model for the S193A receptor. Prior to performing this comparison it was necessary to establish that choice of a particular template compound for the drug alignments was not itself responsible for the differences in the resulting CoMFA models. This was accomplished with the WT receptor using bromocriptine and pergolide as the templates (Figure 5). The two models were similar in almost all regards. Overall, it appeared that choice of template did not result in significant differences in CoMFA models for the WT receptor. This validation was extended via a comparison between bromocriptine- and pergolide-based models for the S193A receptor (Figure 6). Pergolide was unique among the compounds studied in that it demonstrated a substantial increase in affinity with the point mutation. The two S193A CoMFA models, one each with bromocriptine and pergolide as templates for the same test set of drugs, were consistent. However, the differences between them were potentially important in two regards. First, the predictions of affinity for several test compounds were even closer to measured values when using the pergolide- than the bromocriptine-based model. This argues for an improved quantitative prediction using the alternate template. Second, the contour maps differed slightly between the two models in ways which both chemical intuition and the drug docking studies discussed below suggested might be real. Together, the novel differential QSAR/dual CoMFA template approach to evaluation of a single receptor variant offers exciting possibilities for determining the structural bases for agonist affinity.

A further goal of the present study was to compare docking of CoMFA-derived agonist structures to the WT and mutant D2 homology-based receptor models. This approach was inspired by our previous success in docking of the CoMFA-derived agonist structures to key residues of the D2 receptor model.[12] Interpretation of the contour maps according to the protein homology models of the docked DA agonists revealed important correspondences among binding affinity, the agonist substitutions at the cationic nitrogen, and the receptor

active sites for agonists. Thus, homology modeling-based docking was able to identify and refine the physical factors responsible for the CoMFA and extend the interpretation of the CoMFA in a more realistic manner than is obtainable using CoMFA alone. This is an exceptionally important and unique aspect of the present studies since the concept of a differential QSAR/dual CoMFA template approach implies a more serious extrapolation of meaning from contour maps than would be appropriate when one is studying a single receptor.

Homology modeling could account for steric features that determined binding for the mutant D2 receptors. Interestingly, these features were *distant* from the site of hydrogen bonding thought to interact directly with the Ser residues of transmembrane helix V. This suggests that hydrogen bonding features are relatively promiscuous in changing partners and/or that the binding pocket is unusually accessible to water. This concept is consistent with the experiments of Javitch.[32] Water in this pocket between the drug and the receptor could replace the lost hydrogen bonding capacity of the serine-mutant receptors. A similar effect has been seen in the X-ray structure of a Thr to Ala mutation of T4 phage lysozyme. In that work, a water molecule replaced the lost −OH from Thr on the enzyme.[33] Javitch[34] also reported the water accessibility of 10 contiguous residues including Ser193 to Ser197 in transmembrane helix V of the D2 dopamine receptor. Our homology model of the D2 receptor has Ser193 between helices IV and V rather than accessible to the binding pocket. This may reflect the fact that the model is of the high-affinity agonist binding conformation of the receptor, whereas the affinities used here as dependent variables are of the low-affinity agonist binding conformation, consistent with the conformation of the receptor "seen" by agonist upon initial binding. However, it may also be the case that, as Javitch hypothesizes, part of helix V is nonhelical and could be exposed to DA agonists.[34] Updated models and further experimental data are needed to distinguish these possibilities.

In conclusion, we have demonstrated the utility of differential QSAR in CoMFA investigations and the benefit of homology modeling in interpreting the results of CoMFA contour maps. This work suggests that the power of such combined approaches (point mutation, CoMFA, plus homology modeling) can be applied to other systems. The results show the particular utility of comparing WT and multiple mutant receptors rather than studying a single receptor variant. The results also demonstrate that a differential QSAR/dual CoMFA template variant of differential QSAR may be of even greater utility in the search for selective agonists and an understanding of their modes of binding.

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