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Comparative Molecular Field Analysis and QSAR on Substrates Binding to Cytochrome P450 2D6

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Abstract—In this study, we utilized comparative molecular field analysis (CoMFA) to gain a better understanding of the steric and electrostatic features of the cytochrome P450 2D6 (CYP2D6) active site. The training set consists of 24 substrates with reported K_M values from liver microsomal CYP2D6 spanning an activity range of almost three log units. The low energy conformers were fit by root mean square (RMS) to minaprine at the site of metabolism and to the protonated nitrogen. In this manner, we constructed two CoMFA models, one model with a distance constraint and another without. The model with the distance parameter (non-cross-validated $R^2 = 0.99$) was approximately equal to the CoMFA without a distance parameter (non-cross-validated $R^2 = 0.98$). Validation of our CoMFA was accomplished by predicting the K_M values of 15 diverse CYP2D6 substrates not in the original training set resulting in a predictive $R^2 = 0.62$. Finally, we also pursued correlations of pK_a and log P with CYP2D6 substrate K_M in an effort to investigate other physicochemical properties.

Introduction

Cytochrome P450's (P450) are a multigene family of hemoproteins which are involved in the oxidative metabolism of a variety of foreign and endogenous compounds.[1] Of the more than 30 human isoenzymes identified thus far, the major P450's responsible for metabolism are CYP3A4, CYP2D6, CYP1A2 and the CYP2C subfamilies. P450′s are primarily expressed in the liver,[2] although significant amounts can be found in other tissue types.

Several mechanisms by which P450's catalyze the introduction of oxygen into appropriate substrates have been reported.[3–9] The mechanism includes the addition of two electrons and molecular O_2 to an iron atom with a consequent oxidation state of IV or V. The coordination sphere for the Fe (IV or V) includes four porphyrin nitrogens, one sulfur atom (for 2D6 the Cys443 in the Cys pocket) and a sixth position occupied by water.[10]

CYP2D6 is of particular interest because it was one of the first P450 polymorphisms to be discovered[11] and has been subsequently correlated with human disease.[12–15] It has been shown that genetic polymorphisms of enzymes can result in different biotransformation rates[16,17] which can seriously impact the development of new drugs.[18] Thus, the ability to interpret structural and physicochemical properties of compounds as a means for identifying a compound as a potential CYP2D6 substrate is an important goal in predicting potential drug interactions, new drug discovery and drug development.

There are several properties that control the metabolism of any substrate by CYP2D6. These include: (1) topography of the active site, (2) steric interactions between the ligand and the active site pocket, and (3) key electronic interactions. Steric hinderance can impede the access of the iron-oxygen complex to the possible site(s) of metabolism on CYP2D6 substrates. Computer modeling efforts have demonstrated that CYP2D6 hydroxylates its substrates at a site either 5 or 7 Å and a distance greater than 7 Å[19] from a protonated nitrogen. Recently, a 10 Å distance has also been proposed.[20] The specific interaction of the protonated nitrogen has been suggested to interact with Asp301,[21] aligning the substrate above the heme group for further catalysis.[22] These differences in structural distances have led to the

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classification of compounds which are metabolized by CYP2D6 as 5, 7 and 10 Å substrates (Fig. 1).

The important role that the protonated nitrogen of CYP2D6 substrates play in their metabolism has led to the suggestion that tight ionic binding, and consequently pK_a, may play a major role in imparting lower K_M's for compounds which bind to CYP2D6 in comparison to other P450's. Further, this property has also been suggested to impart the highly regioselective biotransformation ability of these enzymes.[23] However, since all substrates contain this moiety, other properties must be important for good substrate–enzyme interaction such as: lipophilicity or log P,[24] π–π interactions,[25] stereochemistry,[26,27] and H-bonding ability.[28]

Many pharmacophore models,[29–37] substrate models[38–50] and supporting spectroscopic studies such as CD[51,52] and NMR[53–55] on CYP2D6 have been reported. To date, models that have globally attempted to predict CYP2D6 metabolism have focused on the incorporation of known CYP2D6 substrates into one effective model with modest success.[19,56,57]

Comparative Molecular Field Analysis (CoMFA) is a useful technique for investigating important 3-dimentional (3-D) quantitative structure activity relationships (QSAR) between molecular steric and electrostatic differences and biological responses. Previously CoMFA has been used to predict the metabolism of compounds by CYP3A4,[58] CYP2B6[59] and CYP2C9.[60] Recently a 3-D/4D predictive model for CYP2D6 inhibitors has been published[61] but as yet, no CoMFA model has been reported for CYP2D6 substrates. With this in mind, we have used the CoMFA module within SYBYL (Tripos Inc.) to develop a *predictive* 3-D model for CYP2D6 substrates. In this study we further validated the CoMFA by predicting K_M values of CYP2D6 substrates not in the original dataset. Further we correlated the pK_a and log P of CYP2D6 substrates with their K_M values in an effort to investigate the proposed importance of these to physicochemical properties in CYP2D6 metabolism.

Methods

Kinetic values

K_M's were obtained from the literature for the training set, which includes Bupranolol,[62] Carteolol,[63] Clemastine,[64] Clozapine,[65] Debrisoquine,[66] Dextromethorphan,[66] Diphenhydramine,[64] Iloperidine,[67] Imipramine,[68] MDMA,[69,70] Metamphetamine,[69] Methoxyphenamine,[71] *S*-Metropolol,[72] *R*-Metoprolol,[72] Minaprine,[72] MPTP,[73] *S*-Propranolol,[74] *R*-Propranolol,[74] Ritonavir,[75] Tolterodine,[76] *S*-Venlafaxine,[77] and *R*-Venlafaxine.[77] In the same manner, literature K_M's were obtained for the test set which includes Bufuralol[78] *m*-Tyramine,[79] Mexiletine,[80] Nortriptyline,[81] *p*-Tyramine,[79] Hydroxyzine,[64] Promethazine,[64,82] *R*-Propafenone,[83] *S*-Propafenone,[83] Terfenadine,[84] Desipramine,[85] *R*-Mianserin,[86] S-Mianserin[86] and Biperiden.[63] These data are summarized in Tables 1 and 3.

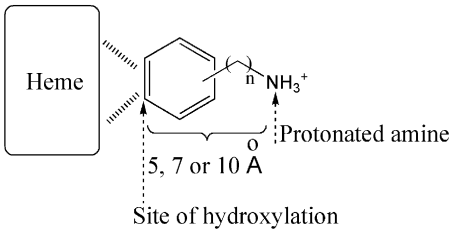

Figure 1. 5, 7 and 10 Å hydroxylation model.

Log P values

All log P values for compounds in this study were taken from reported literature values. A summary of these values are shown in Table 1.

Conformational analysis

The structures of **1**,[88] **2**,[89] **3**,[90] **4**,[91] **5**,[92] **7**,[93] **8**,[94] **10**,,[95] **11**–**13**,[96] **14**,[97] **17**–**20**,[98] **18**[99] **19**,[100] **20**,[100] **22**,[101] **23**,[102] **24**,[102]

Table 1. Predicted and actual K_M values for analogues forming the training set

Compd	Cambridge ref. code	Type of metabolism	Molecular volume (Å^3)[a]	Log P[b]	PK_a	K_M (μM)
1	BUPROL	HY	294.0	2.80	nd	0.27±0.02
2	BAMWER	HY	306.0	−0.46	nd	183
3	CLEMAS	HY	356.1	5.05	nd	32.8±8.7
4	NDNHCL01	NDEM	313.0	2.99	8.0	25±0.7
5	ACICOD	ODEM	301.4	1.07	8.1	149
6	CEGSAI[c]	HY	196.4	0.75	12.9	15
7	DEXORP		275.5	3.99	8.3	22.9
8	FIMTAW	HY	289.6	3.27	9.0	18.6±5.2
9	KUHNUW[d] FALQAK	HY	439.7	nd	nd	39.7±10.8
10	CIMPRA	HY	295.6	4.80	9.5	12.9
11	NEDMIS	DEM	216.2	nd	nd	1.4±0.2
12	NEDMIS	DEM	216.5	nd	nd	3.2±0.2
13	NEDMIS[e]	HY	181.1	2.32	nd	32
14	COWTEN	DEM	204.4	1.66	10.4	59.2±22.4
15	BUPROL[f]	HY DEM	312.3	1.88[c]	9.6	41
16	BUPROL	HY DEM	315.5	1.88[c]	9.6	41
17	AFJEX10	HY	309.3	3.13	7.4	5.26
18	DOWNAE01	HY	208.2	2.71	nd	19
19	BEMBOK	HY	295.1	3.56	9.6	6
20	BEMBOK	HY	286.0	3.56	9.6	6
21	SIGBOZ[g] AHPBUT	HY		nd	nd	10
22	IPRAMI	HY	354.4	nd	nd	7
23	KIDGUZ	DEM NDEM	298.4	2.11	nd	52
24	KIDGUZ	DEM NDEM	302.5	2.11	nd	22

[a]Molecular volumes calculated in the SHADED SURFACES module of SYBYL using default parameters and a probe radius of 1.4.
[b]Ref. 87.
[c]Modified CEGSAI to debrisoquine.
[d]Modified and joined KUHNUW and FALQAK to form Iloperidine.
[e]Modified NEDMIS to Metamphetamine.
[f]Modified BUPROL to Metoprolol.
[g]Modified and joined SIGBOZ and AHPBUT to form Ritonavir.

26,[103] **27**,[104] **28**,[105] **29**,[107] **30**,[106] **31**,[107] **32**,[107] **33**,[108] **34**,[108] **36**,[95] **37**,[109] and **38**[109] (Fig. 2) were obtained from the Cambridge Structural database (CSD). Compounds **6**,[110] **9**,[111] **15**,[88] **16**,[88] **21**,[112,113] **25**,[114] **35**,[115] and **39**[116] were built within SYBYL using or by joining starting X-ray structures. A hydrogen was added to an amine in each substrate to make a protonated amine in all cases. These structures were energy minimized with the Tripos force field,[117] without solvent, using default bond angles and distances while neglecting electrostatics. Side chains when added for compounds within the same structural class (i.e., bufurolol, metoprolol, carteolol), and modeled after the fully extended conformation of the X-ray structure observed for minaprine. To obtain low energy conformers, we utilized GRIDSEARCH to rotate bonds described for each compound in Tables 2 and 3. Conformations of our substrates which were similar to the X-ray structure of minaprine were selected for alignment.

Molecular alignment

All conformations for each compound in the training set (Table 2) were fit within the FIT routine of SYBYL without changing the low energy conformation previously obtained. Our dataset which has large structural diversity, was fit to minaprine by overlapping two points, (1) their protonated amines and (2) the carbon or heteroatom of each molecule at the site of hydroxylation and the atom para or opposite to the site of metabolism. In the same manner, substrates in the test set were aligned (Table 3)

CoMFA calculations

CoMFA was calculated for all conformers as listed in Table 4, (using default parameters except where noted), in the QSAR option of SYBYL 6.5 on a silicon graphics Octane computer. The CoMFA grid spacing was 2.0 Å

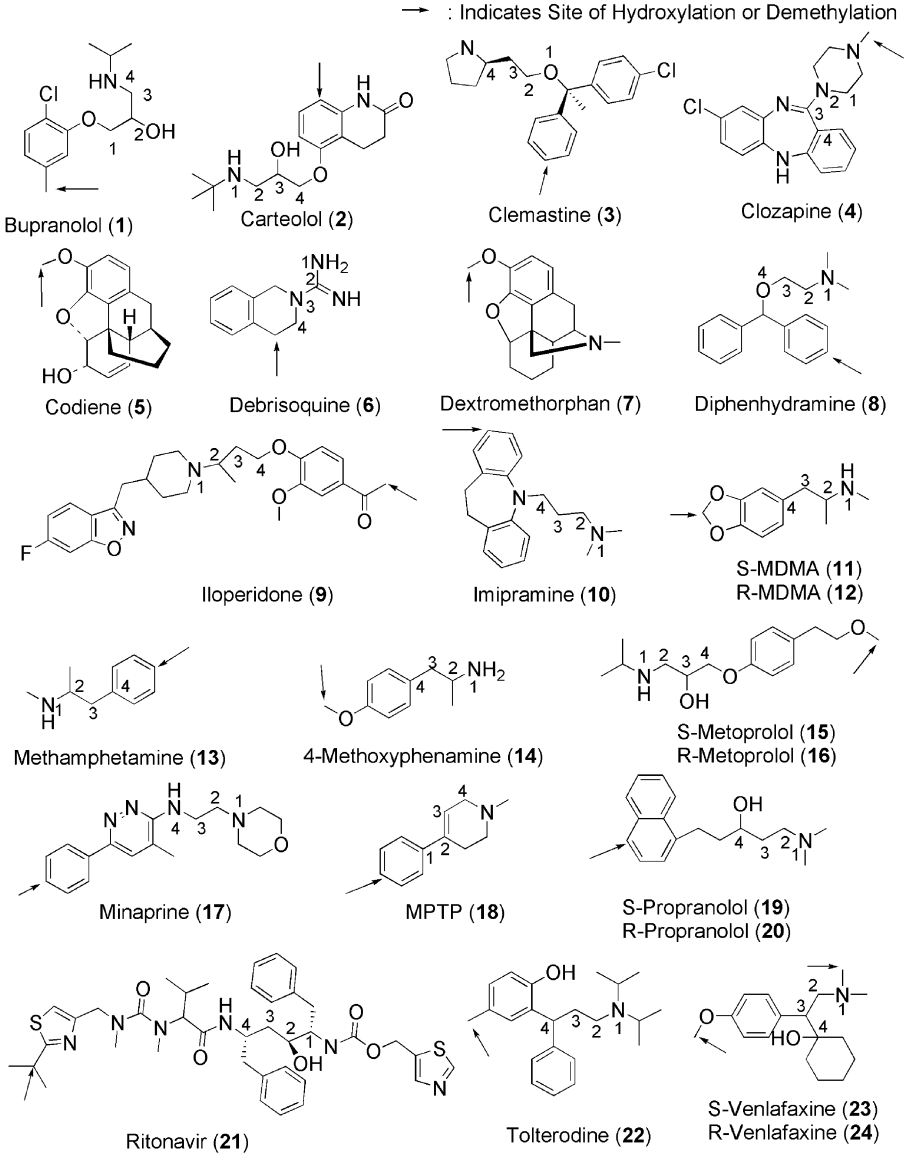

Figure 2. Substrates used in the training set.

Table 2. Conformational analysis and physical properties for compounds in this study

Compd	Distance (Å)	Energy (kcal/mol)	Torsion angle atoms (torsion angle °)
1	9.2	5.40	C1C2C3N4 (177.9)
2	8.4	8.72	N1C2C3C4 (−175.4)
3a	8.1	13.67	C1C2C3O1 (62)
3b	9.6	15.02	C1C2C3O1 (179.4)
4	8.1	15.9	C1N2C3N4 (5.3)
5	8.0	23.75	
6	4.4	3.01	N1C2N3C4 (−118.9)
7	8.1	10.8	
8	7.0	4.48	N1C2C3O4 (−47.8)
9a	10.7	30.97	N1C2C3C4 (−160.5)
9b	9.5	31.93	N1C18C20C21 (91.8)
9c	8.2	30.78	N1C18C20C21 (−155.4)
9d	5.4	30.37	N1C18C20C21 (−145.9)
10a	6.7	16.88	N1C2C3C4 (171.7)
10b	7.0	17.02	N1C2C3C4 (57.0)
11a	8.2	17.17	N1C2C3C4 (175.5)
11b	6.8	17.12	N1C2C3C4 (59.7)
12	8.0	17.18	N1C2C3C4 (175.1)
13	6.6	3.52	N1C2C3C4 (−175.3)
14	8.3	3.15	N1C2C3C4 (−177.4)
15	7.9	5.4	N1C2C3C4 (−177.4)
16	7.9	5.5	N1C2C3C4 (−179.7)
17	6.2	11.7	N1C2C3N4 (−169.5)
18	7.2	7.4	C1C2C3C4 (−142.3)
19a	8.6	7.4	N1C2C3C4 (−179.5)
19b	6.2	6.3	N1C2C3C4 (−179.7)
20a	6.2	6.3	N1C2C3C4 (−179.7)
20b	8.6	7.4	N1C2C3C4 (60.8)
21	6.7	37.57	C1C2C3C4 (−165.2)
22	8.9	16.28	N1C2C3C4 (−179.3)
23	7.5	11.3	N1C2C3C4 (−64)
24	8.5	14.3	N1C2C3C4 (138.2)

in the x, y, and z directions, and the grid region was automatically generated by the CoMFA routine to encompass all molecules with an extension of 4.0 Å in each direction. An sp^3 carbon (sterics) and a charge of +1.0 (electrostatics) were used as probes to generate the interaction energies at each lattice point. The default value of 30 kcal/mol was used as the maximum electrostatic and steric energy cutoff.

Partial least squares (PLS) analysis

Cross-validated (preliminary and final models) and non-cross-validated PLS analyses (final models I and II) were performed within the SYBYL/QSAR routine. Cross-validation of the dependent column (log $1/K_M$) and the CoMFA column was performed with 2.0 kcal/mol column filtering. Scaled by the CoMFA standard deviation, the final cross-validated analysis generated an optimum number of components (Table 5). For each model the cross-validated R^2, standard error of the estimate for the complete model, the F value, and the optimum number of components is reported in Table 5.

The relative steric and electrostatic contributions to the final model were contoured as the standard deviation multiplied by the coefficient at 80% for favored steric (contoured in green) and favored positive electrostatic (contoured in blue) effects and at 20% for disfavored steric (contoured in yellow) and favored negative electrostatic (contoured in red) effects, as shown in Figures 4 and 5. On the basis of this analysis, the K_M values for the test set substrates **24**–**39** (Table 3) were predicted and the observed versus predicted values correlated and plotted in Figure 7.

Results and Discussion

The substrates that compose this study had readily available literature reported K_M values and represent a diverse class of structures which investigates various

Table 3. Physico-chemical properties for substrates in the test set

Compd	Cambridge refcode	Met[a]	Distance[b]	Energy[c]	Volume[b]	Log P	pK_a	K_M	Test set log ($1/K_M$)		
									Obs	Pred	Res
25	SOYDEP[c]	HY	7.9	17.6	321.0	3.5	9.0	18	−1.26	−0.89	−0.46
26	KIVJEE[d]	HY	6.5	2.6	172.2	0.62	9.3	58.2	−1.76	−1.79	0.03
27	JIZJEH	HY	6.8	2.8	218.4	2.57	9.0	16	−1.20	−1.21	−0.01
28	JINGIW	HY	7.1	14.0	289.8	nd	nd	1.35	0.13	−0.70	−0.83
29	KIVJEE	HY	6.0	2.6	172.2	0.62	9.3	190±19.5	−2.28	−1.35	−0.93
30	PROP1C10	HY SM	6.2	14.7	280.2	4.55	9.1	37.1±7.8	−1.57	−1.62	0.05
31	FAHHAX	HY	7.5	5.2	395.0	4.16	7.1	36±7.4	−1.56	−1.56	0.00
32	FAHHAX	HY	7.5	5.2	395.0	4.16	7.1	36±7.4	−1.56	−1.45	−0.11
33	CEWWAC01	HY	6.9	6.3	350.4	33.21	9.0	3.0	−0.48	−0.63	0.15
34	CEWWAC01	HY	6.8	6.4	350.4	3.21	9.0	5.3	−0.72	−1.11	0.39
35	BOWGUP	HY	10.7	17.8	535.2	3.22	nd	13	−1.11	−1.28	0.17
36	CIMPRA[e]	HY	6.7	17.4	298.2	4.90[b]	10.4	11.7	−1.07	−1.08	0.01
37	BUCVAW	HY	7.1	7.0	270.8	nd	nd	4.77±4.93	−0.67	−1.09	0.42
38	BUCVAW	HY	7.1	14.4	272.7	nd	nd	4.77±4.93	−0.67	−1.11	0.44
39	FAYBIQ	HY	6.3	37.7	320.1	nd	nd	37	−1.57	−1.62	0.05

nd, no data; SM, sulfur metabolism.
[a]Type of metabolism.
[b]MV, molecular volume. Molecular volumes were calculated as a Connolly surface using the SHADED SURFACES module within SYBYL with default conditions and a probe radius of 1.4 Å.
[c]Modified to SOYDEP into bufurolol.
[d]Modified KIVJEE into *m*-tyramine.
[e]Modified CIMPRA into desipramine.

Table 4. Final cross-validated and non-cross-validated PLS

Compd	K_M	Cross-validated log ($1/K_M$)			Non-cross-validated log ($1/K_M$)			Non-cross-validated with distance log ($1/K_M$)		
		Obs	Pred	Res	Obs	Pred	Res	Obs	Pred	Res
1	0.27	0.57	0.68	−0.12	0.57	0.57	0.00	0.57	0.57	0.00
2	183	−2.26	−1.96	−0.30	−2.26	−2.20	−0.06	−2.26	−2.20	−0.06
3a	32.8	−1.52	−1.54	0.03						
3b		−1.52	−1.56	0.05						
3c		−1.52	−1.54	0.03	−1.52	−1.54	0.02	−1.52	−1.54	0.02
3d		−1.52	−1.69	0.17	−1.52	−1.58	0.06	−1.52	−1.58	0.06
4a	25	−1.40	−1.54	0.14						
4b		−1.40	−1.34	−0.05	−1.40	−1.37	−0.03	−1.40	−1.37	−0.03
5a	149	−2.17	−2.07	−0.10						
5b		−2.17	−2.07	−0.10	−2.17	−2.21	0.04	−2.17	−2.21	0.04
6	15	−1.18	−1.13	−0.05	−1.18	−1.23	0.05	−1.18	−1.23	0.05
7a	22.9	−1.36	−1.34	−0.02	−1.36	−1.29	−0.07	−1.36	−1.29	−0.07
7b		−1.36	−1.25	−0.11						
8a	18.6	−1.27	−1.18	−0.09						
8b		−1.27	−1.18	−0.09	−1.27	−1.16	−0.11	−1.27	−1.16	−0.11
9a	39.7	−1.60	−1.64	0.04	−1.60	−1.64	0.04	−1.60	−1.64	0.04
9b		−1.60	−1.40	−0.19						
9c		−1.60	−1.66	0.06	−1.60	−1.58	−0.02	−1.60	−1.58	−0.02
9d		−1.60	−1.63	0.03	−1.60	−1.56	−0.04	−1.60	−1.56	−0.04
9e		−1.60	−1.63	0.03	−1.60	−1.63	0.03	−1.60	−1.63	0.03
9f		−1.60	−1.66	0.06						
10a	12.9	−1.11	−0.93	−0.18						
10b		−1.11	−1.13	0.02	−1.11	−1.07	−0.04	−1.11	−1.07	−0.04
10c		−1.11	−1.07	−0.04	−1.11	−1.03	−0.08	−1.11	−1.03	−0.08
11a	2.0	−0.30	−0.41	0.11						
11b		−0.30	−0.20	−0.10	−0.30	−0.23	−0.07	−0.30	−0.23	−0.07
11c		−0.30	−0.46	0.16	−0.30	−0.34	0.04	−0.30	−0.34	0.04
11d		−0.30	−0.52	0.22						
12a	2.0	−0.30	−0.49	0.19	−0.30	−0.45	0.14	−0.30	−0.45	0.14
12b		−0.30	−0.59	0.29						
12c		−0.30	−0.33	0.03						
12d		−0.30	−0.33	0.03	−0.30	−0.34	0.04	−0.30	−0.34	0.04
13	32.0	−1.51	−1.06	−0.45						
14a	59.2	−1.77	−1.67	−0.10	−1.77	−1.72	−0.05	−1.77	−1.72	−0.05
14b		−1.77	−1.95	0.18						
15	69.0	−1.84	−1.94	0.10	−1.84	−1.79	−0.05	−1.84	−1.79	−0.05
16	41.0	−1.61	−1.83	0.22	−1.61	−1.80	0.18	−1.61	−1.80	0.18
17	5.26	−0.72	−0.75	0.03	−0.72	−0.78	0.06	−0.72	−0.78	0.06
18	19.0	−1.28	−1.11	−0.17	−1.28	−1.05	−0.23	−1.28	−1.05	−0.23
19a	6.0	−0.78	−0.90	0.12						
19b		−0.78	−0.63	−0.14						
19c		−0.78	−0.64	−0.14	−0.78	−0.69	−0.09	−0.78	−0.69	−0.09
19d		−0.78	−0.87	0.09	−0.78	−0.88	0.10	−0.78	−0.88	0.10
20a	6.0	−0.78	−0.88	0.10						
20b		−0.78	−0.66	−0.12	−0.78	−0.74	−0.03	−0.78	−0.74	−0.03
20c		−0.78	−0.88	0.10	−0.78	−0.84	0.06	−0.78	−0.84	0.06
21	10.0	−1.00	−1.09	0.09	−1.00	−1.00	0.00	−1.00	−1.00	0.00
22a	7.0	−0.85	−0.88	0.03						
22b		−0.85	−0.85	0.01	−0.85	−0.91	0.06	−0.85	−0.91	0.06
23a	22.0	−1.34	−1.37	0.02						
23b		−1.34	−1.35	0.01	−1.34	−1.31	−0.03	−1.34	−1.31	−0.03
23c		−1.34	−1.22	−0.12						
23d		−1.34	−1.43	0.08						
24a	52.0	−1.72	−1.63	−0.09	−1.72	−1.78	0.06	−1.72	−1.78	0.06
24b		−1.72	−1.78	0.07						
24c		−1.72	−1.75	0.03	−1.72	−1.70	−0.02	−1.72	−1.70	−0.02

areas of the enzyme active site. The non-cross-validated plot for the training set reveals a model which spans almost 3 log units (Fig. 3) and is predictive of metabolism for the standard 2D6 substrates debrisoquine (**6**) and dextromethorphan (**7**). The CoMFA model correlates remarkably well (over almost three log units of activity) the K_M's for the entire dataset consisting of 5, 7 and 10 Å substrates. The data reveals that the best predictive model can be achieved by placing for each substrate a representative conformer from each class (5, 7 or 10 Å) for all substrates. Thus a summation of conformers appears to contribute to the overall predictive power of the model. We also examined the generation of a model for each distance, for which we were unsuccessful. We rationalize this by considering that all distances likely contribute to the overall K_M.

In an effort to improve the model we investigated a CoMFA which included a distance parameter. This resulted in only a slightly improved correlation over the model without a distance parameter (Table 5) revealing

Table 5. Cross-validated PLS analysis

Component	Preliminary model I[a,b]		Preliminary model II[c,d]		Preliminary model III[e]		Final model		Final model with distance parameter	
	s	R^2	s	R^2	s	R^2	s	R^2	s	R^2
1	−0.096	0.743	0.145	0.522	0.023	0.584	−0.017	0.625	−0.311	0.721
2	−0.002	0.695	0.314	0.472	0.202	0.535	0.190	0.567	0.040	0.627
3	0.134	0.676	0.377	0.453	0.243	0.529	0.289	0.541	0.238	0.569
4	0.186	0.673	0.361	0.463	0.273	0.526	0.294	0.548	0.353	0.533
5[a]	0.203	0.665	0.387	0.458	0.298	0.525	0.339	0.540	0.357	0.542
6	0.228	0.685	0.366	0.470	0.320	0.525	0.328	0.555	0.360	0.551
7					0.294	0.544			0.371	0.558
8									0.357	0.577
Optimum # of components[a]	6		5		6		5		7	
SEE[b]	0.21		0.14		0.09		0.09		0.05	
Non-cross-validated R^2	0.92		0.95		0.98		0.98		0.99	
F value	204.1		187.2		256.5		319.5		581.0	
Prob. of $R^2=0$	0.00		0.00		0.00		0.00		0.00	
SC[c]							0.499		0.495	
EC[d]							0.501		0.492	
DC[e]									0.013	

[a]Optimum number of components.
[b]Standard error of estimate (SEE).
[c]Steric component (SC).
[d]Electrostatic component (EC).
[e]Distance component (DC).

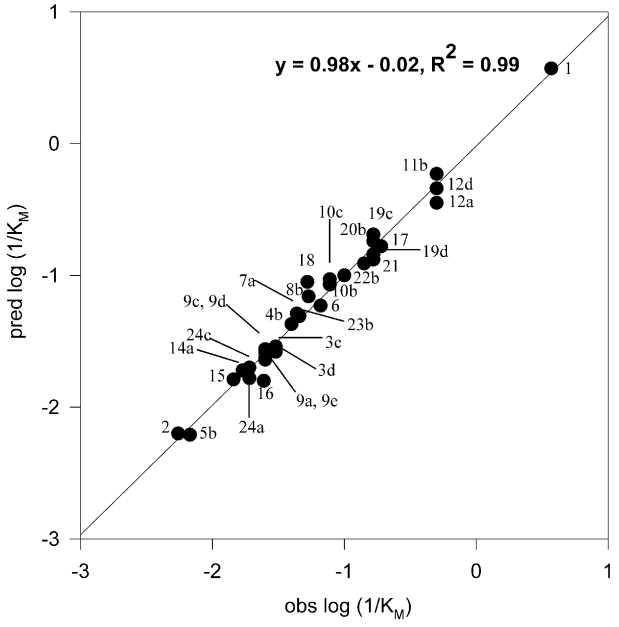

Figure 3. Plot of predicted log K_M versus observed log K_M values for the non-cross-validated CoMFA model.

that the assigned overlap and the steric component adequately describe this important parameter.

Within the steric CoMFA contour map (Fig. 5) we have place minaprine (10 Å) (shown in yellow and red) and debrisoquine (5 Å) (pictured in white) as extreme examples. The steric field is represented by a lipophilic pocket surrounding the active site between the heme and the protonated amine contained in the substrates. This observation is consistent with a NMR study support the presence of a lipophilic enzyme cavity at least 15×7.5 Å and 990 Å^3 in volume in the CYP2D6 active site.[118] Consistent with this report, all of the substrates in our study, fit within the molecular volume constraints previously defined. Our model further reveals two other sterically restricted regions, one which corresponds to the heme plane and a second area which can be accessed by only the 10 Å substrates (Fig. 5).

Examination of the electrostatic fields reveal two areas (Fig. 4) where increased positive charge (blue field) on the substrate should increase CYP2D6 substrate metabolism (smaller K_M). One area is contained near the site of metabolism and a second major area corresponds to 10 Å substrates. Between these two regions is contained an area where more negative charge (red field) on substrates should increase CYP2D6 metabolism (smaller K_M).

Despite the dominance of ion-pair interactions, lipophilicity or log P has been suggested as an important physicochemical property for good substrate binding in a number of systems.[119] For example, log P has been correlated to the inhibition constant (K_i or log K_i) for a series of β-blockers[120] and shown to have an inverse relationship with inhibition for quinidine metabolites.[121] Unfortunately, we were unable to obtain a correlation of log P and K_M for the training set. We were equally unsuccessful in correlating pK_a with K_M. Because our model already correlates greater than 98%, the effect of incorporating pK_a or log P as an additional independent variable in the CoMFA model was negligible.

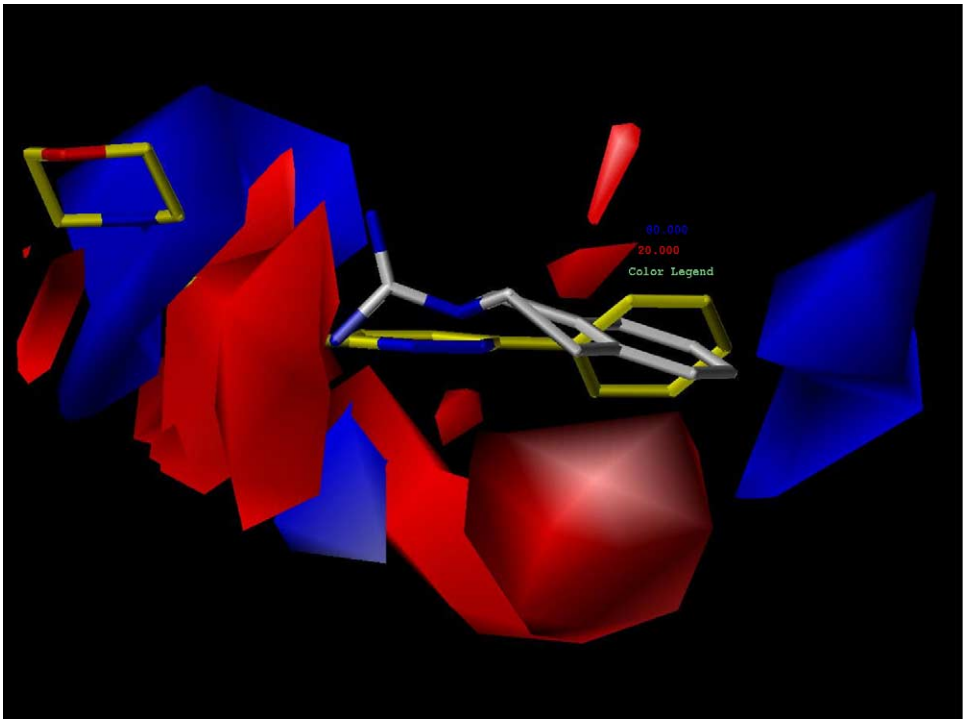

Figure 4. Electrostatic CoMFA fields with distances.

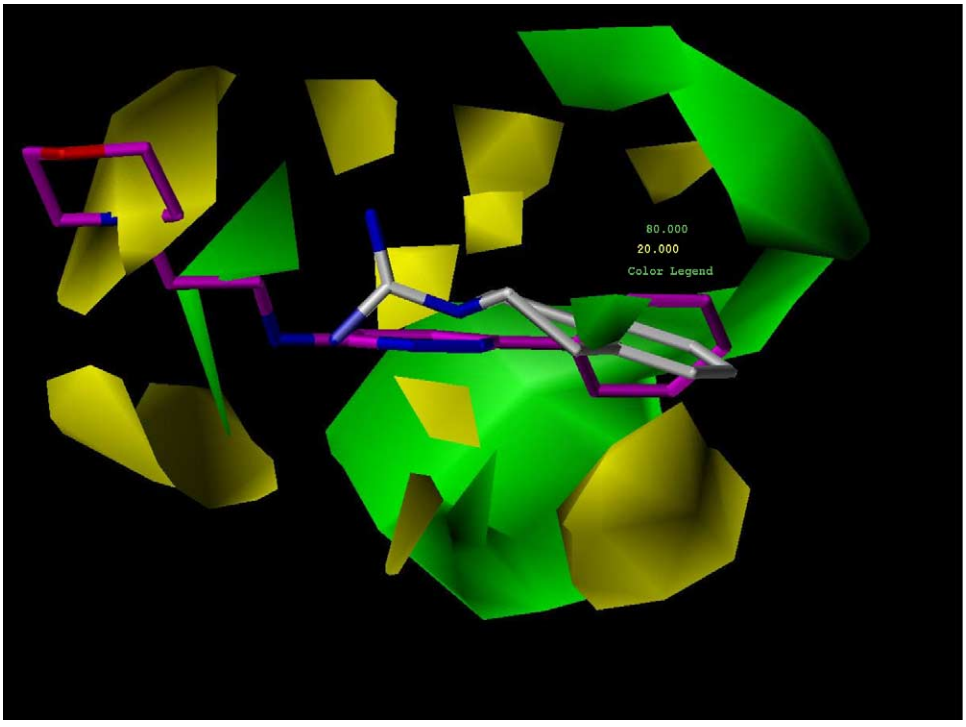

Figure 5. Steric CoMFA fields with distances.

In an effort to validate the CoMFA model, we used 15 substrates in the test set (Fig. 6 and Table 3). A correlation of the predicted K_M versus observed K_M values (Table 5 and Fig. 7) demonstrates the predictive ability ($R^2 = 0.62$) of our model over a range of 2.5 log units.

Further, our model adequately predicted differences between enantiomeric substrates (i.e., Table 3, **33** and **34**), but showed less accuracy in regioselective prediction (Table 3, **26** vs **29**). Overall, the CoMFA demonstrated modest accuracy considering the reported values were obtained from multiple laboratories and were not normalized for consistency. With this in mind, we are encouraged that these results confirm the validity and the predictive utility of this CoMFA model.

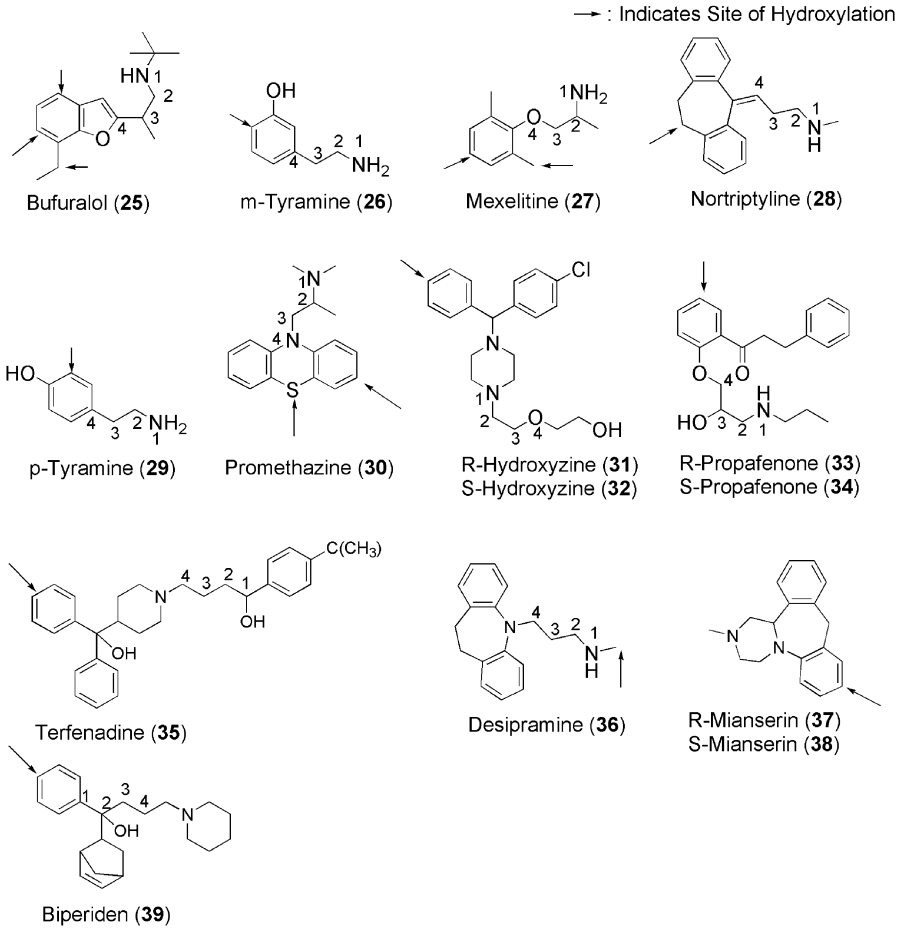

Figure 6. Substrates in the test set.

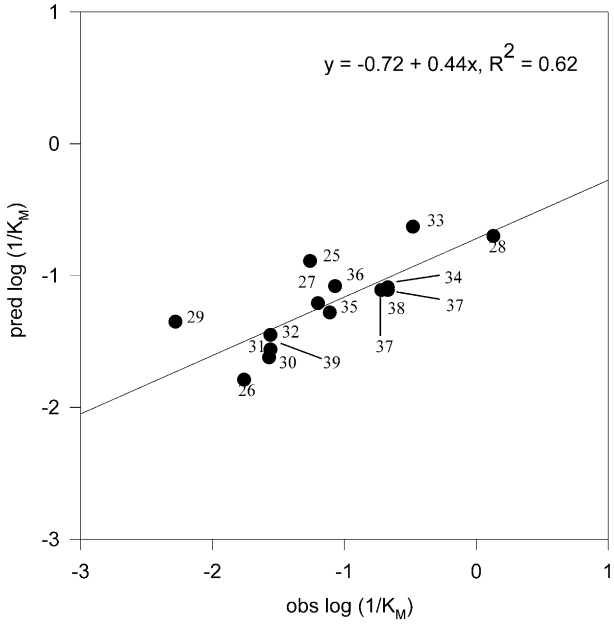

Figure 7. Plot of predicted log K_M versus observed log K_M for the test set.

Conclusions

In this study, we were able to correlate electrostatic and steric differences of 24 substrates of CYP2D6 with microsomal liver K_M. Increased accuracy of prediction was accomplished by including a low energy conformer representative of each of the conformational classes accessible by the substrate. Validation of our model by predicting the microsomal liver K_M of 15 substrates not in the training set confirms the predictive ability and utility of the CoMFA. Unfortunately, in this study we were unable to correlate log P or pK_a with K_M.

This model could be used to predict potential sites of metabolism for any amine containing compound that has a phenyl group 5, 7, or 10 Å away. The utility of the model is that it can be used for compounds that have unknown metabolism. This can be facilitated by orientating the compound (including multiple conformations) in the model and predicting the K_M for that orientation and alignment. The lowest K_M value would be suggestive of the potential site and could be validated by in vitro CYP2D6 metabolism studies. Altogether, this represents the first CoMFA for CYP2D6 substrate metabolism and may prove to be important for future drug development.

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References and Notes

1. Michalets, E. L. *Pharmacotherapy* **1998**, *18*, 84.
2. Wiseman, H.; Lewis, D. F. *Carciogenesis* **1996**, *17*, 1357.
3. Guengerich, F. P.; MacDonald, T. L. *FASEB J*. **1990**, *4*, 2453.
4. Modi, S.; Sutcliffe, M. J.; Primrose, W. U.; Lian, L. Y.; Roberts, G. C. *Nat. Struct. Biol*. **1996**, *3*, 414.
5. Besse, P.; Modi, S.; Boyle, J. M.; Roberts, G. C.; Primrose, W. U. *Biochem. Soc. Trans*. **1997**, *25*, S579.
6. Lewis, D. F.; Ioannides, C.; Parke, D. V. *Drug Metab. Rev*. **1998**, *30*, 709.
7. Lewis, D. F.; Pratt, J. M. *Drug Metab. Rev*. **1998**, *30*, 739.
8. Guengerich, F. P.; Shimada, T.; Bondon, A.; Macdonald, T. L. *Adv. Exp. Med. Biol*. **1991**, *283*, 1.
9. Ebner, T.; Meese, C. O.; Eichelbaum, M. *Mol. Pharm*. **1995**, *48*, 1078.
10. Davydov, R. M.; Greshner, Z.; Rukpaul, K. *Mol. Biol*. **1979**, *13*, 1397.
11. Mahgoub, A. A.; Idle, J. R.; Dring, L. G.; Lancaster, R.; Smith, R. L. *Lancet* **1977**, *2*, 584.
12. Kapitany, T.; Meszaros, K.; Lenzinger, E.; Schindler, S. D.; Barnas, C.; Fuchs, K.; Sieghart, W.; Aschauer, H. N.; Kasper, S. *Schizophrenia Res*. **1998**, *32*, 101.
13. Agundez, J. A.; Martinez, C.; Olivera, M.; Gallardo, L.; Ladero, J. M.; Rosado, C.; Prados, J.; Rodriguez-Molina, J.; Resel, L.; Benitez, J. *Brit. J. Cancer* **1998**, *78*, 1361.
14. Bordet, R.; Broly, F.; Destee, A.; Libersa, C. *Clin. Neuropharm*. **1994**, *17*, 484.
15. McCann, S. J.; Pond, S. M.; James, K. M.; Le Couteur, D. G. *J. Neurol. Sci*. **1997**, *153*, 50.
16. Darmansjah, I.; Muchtar, A. *Clin. Pharmacol. Ther*. **1992**, *52*, 449.
17. Kroemer, H. K.; Eichelbaum, M. *Life Sci*. **1995**, *56*, 2285.
18. Belpaire, F. M.; Bogaert, M. G. *Acta Clin. Belg*. **1996**, *51*, 254.
19. De Groot, M. J.; Ackland, M. J.; Horne, V. A.; Alex, A. A.; Jones, B. C. *J. Med. Chem*. **1999**, *42*, 1515.
20. De Groot, M. J.; Bijloo, G. J.; Hansen, K. T.; Vermeulen, N. P. *Drug Metab. Dispos*. **1995**, *23*, 667.
21. Ellis, S. W.; Hayhurst, G. P.; Smith, G.; Lightfoot, T.; Wong, M. M.; Simula, A. P.; Ackland, M. J.; Sternberg, M. J.; Lennard, M. S.; Tucker, G. T. *J. Biol. Chem*. **1995**, *270*, 29055.
22. Koyman, L. M. H. *J. Comp. Aid. Mol. Des*. **1993**, *7*, 281.
23. Smith, D. A. *Drug Metab. Rev*. **1991**, *23*, 355.
24. Ferrari, S.; Leemann, T.; Dayer, P. *Life Sci*. **1991**, *48*, 2259.
25. Haining, R. L.; Jones, J. P.; Henne, K. R.; Fisher, M. B.; Koop, D. R.; Trager, W. F.; Rettie, A. E. *Biochemistry* **1999**, *38*, 3285.
26. Narimatsu, S.; Kato, R.; Horie, T.; Ono, S.; Tsutsui, M.; Yabusaki, Y.; Ohmori, S.; Kitada, M.; Ichioka, T.; Shimada, N.; Kato, R.; Ishikawa, T. *Chirality* **1999**, *11*, 1–9.
27. Ellis, S. W.; Rowland, K.; Ackland, M. J.; Rekka, E.; Simula, A. P.; Lennard, M. S.; Wolf, C. R.; Tucker, G. T. *Biochem. J*. **1996**, *316*, 647.
28. Williams, M. A.; Goofellow, J. M.; Thornton, J. M. *Protein Sci*. **1994**, *3*, 1224.
29. Zvelebil, M. J.; Wolf, C. R.; Sternberg, M. J. *Protein Engin*. **1991**, *4*, 271.
30. Helms, V.; Deprez, E.; Gill, E.; Barret, C.; Hui, B.; Hoa, G.; Wade, R. C. *Biochemistry* **1996**, *35*, 1485.
31. Smith, D. A.; Jones, B. C. *Biochem. Pharmacol*. **1992**, *44*, 2089.
32. Mackman, R.; Tschirret-Guth, R. A.; Smith, G.; Hayhurst, G. P.; Ellis, S. W.; Lennard, M. S.; Tucker, G. T.; Wolf, C.R; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys*. **1996**, *331*, 134.
33. Lewis, D. F.; Eddershaw, P. J.; Dickins, M.; Tarbit, M. H.; Goldfarb, P. S. *Chem. Biol. Interact*. **1998**, *115*, 175.
34. De Groot, M. J.; Vermeulen, N. P.; Kramer, J. D.; Van Acker, F. A.; Donne-Op, D.; Den Kelder, G. M. *Chem. Res. Toxicol*. **1996**, *9*, 1079.
35. De Groot, M. J.; Bijloo, G. J.; van Acker, F. A.; Fonseca Guerra, C.; Snijders, J. G.; Vermeulen, N. P. *Xenobiotica* **1997**, *27*, 357.
36. Islam, S. A.; Wolf, C. R.; Lennard, M. S.; Sternberg, M. J. *Carcinogenesis* **1991**, *12*, 2211.
37. De Groot, M. J.; Ackland, M. J.; Horne, V. A.; Alex, A. A.; Jones, B. C. *J. Med. Chem*. **1999**, *42*, 4062.
38. Lewis, D. F.; Eddershaw, P. J.; Goldfarb, P. S.; Tarbit, M. H. *Xenobiotica* **1997**, *27*, 319.
39. Wiseman, H.; Lewis, D. F. *Carcinogenesis* **1996**, *17*, 1357.
40. Lewis, D. F.; Lake, B. G.; Parke, D. V. *Xenobiotica* **1995**, *25*, 1355.
41. Koymans, L. M.; Vermeulen, N. P.; Baarslag, A.; Donne-Op, D.; Kelder, G. M. *J. Comput. Aided Mol. Des*. **1993**, *7*, 281.
42. Lewis, D. F.; Lee-Robichaud, P. *J. Steroid Biochem. Mol. Biol*. **1998**, *66*, 217.
43. De Groot, M. J.; Vermeulen, N. P. *Drug Metab. Rev*. **1997**, *29*, 747.
44. De Groot, M. J.; Bijloo, G. J.; Martens, B. J.; Van Acker, F. A.; Vermeulen, N. P. *Chem. Res. Toxicol*. **1997**, *10*, 41.
45. De Groot, M. J.; Vermeulen, N. P.; Kramer, J. D.; Van Acker, F. A.; Donne-Op, D.; Kelder, G. M. *Chem. Res. Toxicol*. **1996**, *9*, 1079.
46. De Groot, M. J.; Bijloo, G. J.; Hansen, K. T.; Vermeulen, N. P. *Drug Metab. Dispos*. **1995**, *23*, 667.
47. Segall, M. D.; Payne, M. C.; Ellis, S. W.; Tucker, G. T.; Boyes, R. N. *Xenobiotica* **1998**, *28*, 15.
48. Segall, M. D.; Payne, M. C.; Ellis, S. W.; Tucker, G. T.; Boyes, R. N. *Eur. J. Drug Metabol. Pharm*. **1997**, *22*, 283.
49. Koymans, L. M. *Pharm. World Sci*. **1993**, *15*, 86.
50. De Groot, M. J.; Havenith, R. W.; Vinkers, H. M.; Zwaans, R.; Vermeulen, N. P.; Van Lenthe, J. H. *J. Comput.-Aided Mol. Des* **1998**, *12*, 183.
51. Andersson, L. A.; Johnson, A. K.; Peterson, J. A. *Arch. Biochem. Biophys*. **1997**, *345*, 79.
52. Andersson, L. A.; Peterson, J. A. *Biochem. Biophys. Res. Commun*. **1995**, *211*, 389.
53. Smith, G.; Modi, S.; Pillai, I.; Lian, L. Y.; Sutcliffe, M. J.; Pritchard, M. P.; Friedberg, T.; Roberts, G. C.; Wolf, C. R. *Biochem. J*. **1998**, *331*, 783.
54. Modi, S.; Paine, M. J.; Sutcliffe, M. J.; Lian, L. Y.; Primrose, W. U.; Wolf, C. R.; Roberts, G. C. *Biochemistry* **1996**, *35*, 4540.
55. Modi, S.; Primrose, W. U.; Boyle, J. M.; Gibson, C. F.; Lian, L. Y.; Roberts, G. C. *Biochemistry* **1995**, *34*, 8982.
56. De Groot, M. J.; Bijloo, G. J.; Martens, B. J.; van Acker, F. A.; Vermeulen, N. P. *Chem. Res. Toxicol*. **1997**, *10*, 41.
57. Islam, S. *Carcinogenesis* **1991**, *12*, 2211.
58. Ekins, S.; Bravi, G.; Wikel, J. H.; Wrighton, S. A. *J. Pharmacol. Exp. Ther*. **1999**, *291*, 424.
59. Ekins, S.; Bravi, G.; Ring, B. J.; Gillespie, T. A.; Gillespie, J. S.; Vandenbranden, M.; Wrighton, S. A.; Wikel, J. H. *J. Pharmacol. Exp. Ther*. **1999**, *288*, 21.
60. Jones, J. P.; He, M.; Trager, W. F. *Drug Metab. Dispos*. **1995**, *24*, 1.
61. Ekins, S.; Bravi, G.; Binkley, S.; Gillespie, J. S.; Ring, B. J.; Wikel, J. H.; Wrighton, S. A. *Pharmacogenetics* **1999**, *9*, 477.

62. Appanna, G.; Tang, B. K.; Miller, R.; Kalow, W. *Drug Metab. Dispos.* **1996**, *24*, 303.
63. Kudo, S.; Uchida, M.; Odomi, M. *Eur. J. Clin. Pharmacol.* **1997**, *52*, 479.
64. Hamelin, B. A.; Bouayad, A.; Drolet, B.; Gravel, A.; Turgeon, J. *Drug Metab. Dispos.* **1998**, *26*, 536.
65. Linnet, K.; Olesen, O. V. *Drug Metab. Dispos.* **1997**, *25*, 1379.
66. Kronbach, T.; Mathys, D.; Gut, J.; Catin, T.; Meyer, U. A. *Anal. Biochem.* **1987**, *162*, 24.
67. Mutlib, A. E.; Klein, J. T. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 1285.
68. Ball, S. E.; Ahern, D.; Scatina, J.; Kao, J. *Brit. J. Clin. Pharmacol.* **1997**, *43*, 619.
69. Lin, L. Y.; Di Stefano, E. W.; Schmitz, D. A.; Hsu, L.; Ellis, S. W.; Lennard, M. S.; Tucker, G. T.; Cho, A. K. *Drug Metab. Dispos.* **1997**, *25*, 1059.
70. Tucker, G. T.; Lennard, M. S.; Ellis, S. W.; Woods, H. F.; Cho, A. K.; Lin, L. Y.; Hiratsuka, A.; Schmitz, D. A.; Chu, T. Y. *Biochem. Pharmacol.* **1994**, *47*, 1151.
71. Roy, S. D. *Xenobiotica* **1990**, *20*, 55.
72. Belpaire, F. M.; Wijnant, P.; Temmerman, A.; Rasmussen, B. B.; Brosen, K. *Eur. J. Clin. Pharmacol.* **1998**, *54*, 261.
73. Coleman, T.; Ellis, S. W.; Martin, I. J.; Lennard, M. S.; Tucker, G. T. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 685.
74. Rowland, K.; Ellis, S. W.; Lennard, M. S.; Tucker, G. T. *Brit. J. Clin. Pharmacol.* **1996**, *42*, 390.
75. Kumar, G. N.; Rodrigues, A. D.; Buko, A. M.; Denissen, J. F. *J. Pharmacol. Exp. Ther.* **1997**, *281*, 1506.
76. Postlind, H.; Danielson, A.; Lindgren, A.; Andersson, S. H. *Drug Metab. Dispos.* **1998**, *26*, 289.
77. Otton, S. V.; Ball, S. E.; Cheung, S. W.; Inaba, T.; Rudolph, R. L.; Sellers, E. M. *Brit. J. Clin. Pharmacol.* **1996**, *41*, 149.
78. Yamazaki, H.; Guo, Z.; Persmark, M.; Mimura, M.; Inoue, K.; Guengerich, F. P.; Shimada, T. *Mol. Pharm.* **1994**, *46*, 568.
79. Hiroi, T.; Imaoka, S.; Funae, Y. *Biochem. Biophys. Res. Commun.* **1998**, *249*, 838.
80. Konishi, H.; Morita, K.; Minouchi, T.; Yamaji, A. *Eur. J. Drug Metab. Pharm.* **1999**, *24*, 149.
81. Olesen, O. V.; Linnet, K. *Drug Metab. Dispos.* **1997**, *25*, 740.
82. Nakamura, K.; Yokoi, T.; Inoue, K.; Shimada, N.; Ohashi, N.; Kume, T.; Kamataki, T. *Pharmacogenetics* **1996**, *6*, 449.
83. Kroemer, H. K.; Botsch, S.; Heinkele, G.; Schick, M. *Methods Enzymol.* **1996**, *272*, 99.
84. Jones, B. C.; Hyland, R.; Ackland, M.; Tyman, C. A.; Smith, D. A. *Drug Metab. Dispos.* **1998**, *26*, 875.
85. Von Moltke, L. L.; Greenblatt, D. J.; Duan, S. X.; Daily, J. P.; Harmatz, J. S.; Shader, R. I. *J. Pharm. Sci.* **1998**, *87*, 1184.
86. Koyama, E.; Chiba, K.; Tani, M.; Ishizaki, T. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 21.
87. Craig, P. N.; Newton, J. *Comprehensive Medicinal Chemistry*; Drayton, C., Ed.; Pergamon: Oxford, 1990; Vol. 6, p. 242.
88. Gadret, M.; Goursolle, M.; Leger, J. M.; Colleter, J. C. *Acta Crystallogr., Sect. B* **1975**, *31*, 2780.
89. Kido, M.; Nakagawa, K.; Fujiwara, T.; Tomita, K. I. *Chem. Pharm. Bull.* **1981**, *29*, 2109.
90. Terzis, A. *Cryst. Struct. Commun.* **1978**, *7*, 91.
91. Fillers, J. P.; Hawkinson, S. W. *Acta Crystallogr., Sect. B* **1982**, *38*, 1750.
92. Liebman, A. A.; Malarek, D. H.; Blount, J. F.; Nelson, N. R.; Delaney, C. M. *J. Org. Chem.* **1978**, *43*, 737.
93. Gylbert, L.; Carlstrom, D. *Acta Crystallogr., Sect. B* **1977**, *33*, 2833.
94. Wiedenfeld, H.; Knoch, F. *Acta Crystallogr., Sect. C (Cr. Str. Commum.)* **1987**, *43*, 1359.
95. Post, M. L.; Horn, A. S. *Acta Crystallogr., Sect. B* **1977**, *33*, 2590.
96. Morimoto, B. H.; Lovell, S.; Kahr, B. *Acta Crystallogr., Sect. C (Cr. Str. Commun.)* **1998**, *54*, 229.
97. Okabe, N.; Takimoto, E.; Fujiwara, T.; Yamagata, Y.; Tomita, K. I. *Acta Crystallogr., Sect. C (Cr. Str. Comm.)* **1985**, *41*, 87.
98. Michel, A.; Gustin, R.; Evrard, G.; Durant, F. *Bull. Soc. Chim. Belg.* **1982**, *91*, 49.
99. Flippen-Anderson, J. L.; Gilardi, R.; George, C. *Acta Crystallogr., Sect. C (Cr. Str. Commun.)* **1986**, *42*, 1185.
100. Laguerre, M.; Leger, J. M.; Carpy, A.; Seances, C. R. *Acad. Sci., Ser. 2* **1981**, *293*, 145.
101. Chawdhury, S. A.; Koch, M. H. *Acta Crystallogr., Sect. B* **1976**, *32*, 1286.
102. Yardley, J. P.; Husbands, G. E.; Stack, G.; Butch, J.; Bicksler, J.; Moyer, J. A.; Muth, E. A.; Andre, T.; Fletcher, III, E. H.; James, M. N.; Sielecki, A. R. *J. Med. Chem.* **1990**, *33*, 2899.
103. Okabe, N.; Mori, S.; Sasaki, Y. *Acta Crystallogr., Sect. C (Cr. Str. Commun.)* **1991**, *47*, 1448.
104. Sivy, J.; Kettmann, V.; Fresova, E. *Acta Crystallogr., Sect. C (Cr. Str. Commun.)* **1991**, *47*, 2695.
105. Klein, C. L.; Banks, T. A.; Rouselle, D. *Acta Crystallogr., Sect. C (Cr. Str. Commun.)* **1991**, *47*, 1478.
106. Shastry, C. I.; Seshadri, T. P.; Prasad, J. S.; Achar, B. N. *Crystallography*. **1987**, *178*, 283.
107. Gongdu, Z.; Sheng, J.; Huaxue, J. *J. Struct. Chem.* **1986**, *5*, 49.
108. Shin, W. *Bull. Korean Chem. Soc.* **1985**, *6*, 7.
109. Van Meerssche, M.; Declercq, J. P. *Bull. Soc. Chim. Belg.* **1983**, *92*, 307.
110. Maryanoff, B. E.; Molinari, A. J.; McComsey, D. F.; Maryanoff, C. A.; Wooden, G. P.; Olofson, R. A. *J. Org. Chem.* **1983**, *48*, 5074.
111. Jottier, W. I.; De Winter, H. L.; Peters, O. M.; Blaton, N. M.; De Ranter, C. J. *Acta Crystallogr., Sect. C (Cr. Str. Commun.)* **1992**, *48*, 1827.
112. Zhao, X.; Chang, Y.; Fowler, F. W.; Lauher, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 6627.
113. Nakamura, N.; Suda, H.; Takita, T.; Aoyagi, T.; Umezawa, H. *J. Antibiot.* **1976**, *29*, 102.
114. Berthelot, P.; Vaccher, C.; Flouquet, N.; Luyckx, M.; Brunet, C.; Boulanger, T.; Frippiat, J. P.; Vercauteren, D. P.; Debaert, M.; Evrard, G.; Durant, F. *Eur. J. Med. Chem.* **1991**, *26*, 395.
115. Van Roey, P.; Smith, G. D.; Duax, W. L.; Umen, M. J.; Maryanoff, B. E. *J. Am. Chem. Soc.* **1982**, *104*, 5661.
116. Codding, P. W. *Acta Crystallogr., Sect. B (Str. Sci.)* **1986**, *42*, 632.
117. Clark, M. D.; Cramer, III, R. D.; Opdenbosch, N. V. *J. Comp. Chem.* **1989**, *10*, 982.
118. Ellis, S. W.; Rowland, K.; Ackland, M. J.; Rekka, E.; Simula, A. P.; Lennard, M. S.; Wolf, C. R.; Tucker, G. T. *Biochem. J.* **1996**, *316*, 647.
119. Smith, D. A. *Drug Discov. Today* **1997**, *2*, 406.
120. Ferrari, S.; Leemann, T.; Dayer, P. *Life Sci.* **1991**, *48*, 2259.
121. Ching, M. S.; Blake, C. L.; Ghabrial, H.; Ellis, S. W.; Lennard, M. S.; Tucker, G. T.; Smallwood, R. A. *Biochem. Pharm.* **1995**, *50*, 833.