Three-Dimensional Quantitative Structure-Activity Relationship Analysis of Human CYP51 Inhibitors

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ABSTRACT:

CYP51 fulfills an essential requirement for all cells, by catalyzing three sequential mono-oxidations within the cholesterol biosynthesis cascade. Inhibition of fungal CYP51 is used as a therapy for treating fungal infections, whereas inhibition of human CYP51 has been considered as a pharmacological approach to treat dyslipidemia and some forms of cancer. To predict the interaction of inhibitors with the active site of human CYP51, a three-dimensional quantitative structure-activity relationship model was constructed. This pharmacophore model of the common structural features of CYP51 inhibitors was built using the program Catalyst from multiple inhibitors (n = 26) of recombinant human CYP51-mediated lanosterol 14α-demethylation. The pharmacophore, which consisted of one hydrophobe, one hydrogen bond acceptor, and two ring aromatic features, demonstrated a high correlation between observed and predicted IC₅₀ values (r = 0.92). Validation of this pharmacophore was performed by predicting the IC₅₀ of a test set of commercially available (n = 19) and CP-320626-related (n = 48) CYP51 inhibitors. Using predictions below 10 μM as a cutoff indicative of active inhibitors, 16 of 19 commercially available inhibitors (84%) and 38 of 48 CP-320626-related inhibitors (79.2%) were predicted correctly. To better understand how inhibitors fit into the enzyme, potent CYP51 inhibitors were used to build a Cerius² receptor surface model representing the volume of the active site. This study has demonstrated the potential for ligand-based computational pharmacophore modeling of human CYP51 and enables a high-throughput screening system for drug discovery and data base mining.

Cytochrome P450 enzymes (P450s) are a widely studied, large superfamily of heme-thiolate proteins involved in the metabolism of endobiotics and xenobiotics across eukaryotes and prokaryotes (Nelson et al., 1996). The clinical relevance of P450s is their central role in drug metabolism, which can occur in all human tissues and may be inhibited by the coadministration of competing xenobiotics for the same enzyme (Wrighton et al., 1995). Much less is known about the endogenous functions of P450, although their role in steroid metabolism is an exception. It has been postulated that other endogenous signaling pathways (Chan et al., 1998) and substrates for the same enzymes (Strolin Benedetti and Harwood, 1999). This pharmacophore model of the common structural features of CYP51 inhibitors was built using the program Catalyst from multiple inhibitors (n = 26) of recombinant human CYP51-mediated lanosterol 14α-demethylation. The pharmacophore, which consisted of one hydrophobe, one hydrogen bond acceptor, and two ring aromatic features, demonstrated a high correlation between observed and predicted IC₅₀ values (r = 0.92). Validation of this pharmacophore was performed by predicting the IC₅₀ of a test set of commercially available (n = 19) and CP-320626-related (n = 48) CYP51 inhibitors. Using predictions below 10 μM as a cutoff indicative of active inhibitors, 16 of 19 commercially available inhibitors (84%) and 38 of 48 CP-320626-related inhibitors (79.2%) were predicted correctly. To better understand how inhibitors fit into the enzyme, potent CYP51 inhibitors were used to build a Cerius² receptor surface model representing the volume of the active site. This study has demonstrated the potential for ligand-based computational pharmacophore modeling of human CYP51 and enables a high-throughput screening system for drug discovery and data base mining.

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In conclusion, our study demonstrates the potential of computational pharmacophore modeling of human CYP51 and enables a high-throughput screening system for drug discovery and data base mining.
There have been many studies usingazole, oxysterol, and mecha-
nism-based inactivator-type inhibitors of fungal or mammalian CYP51 (Trzaskos et al., 1986, 1993, 1995; Frye et al., 1993, 1994). In addition, the purification (Sonoda et al., 1993) and expression of human CYP51 have allowed the determination of its regulation by oxysterols (Stromstedt et al., 1996). However, because of the mem-
brane-bound nature of mammalian P450s, there is as yet no crystal structure for human CYP51. Therefore, our understanding of the struc-
tural requirements of the human CYP51 active site are limited to homol-
geny models constructed using various bacterial P450s (Ishida et al., 1988; Tuck et al., 1991, 1992; Tafi et al., 1996; Talele et al., 1997; Holte and Fattorusso, 1998; Lewis et al., 1999), including Mycobacterium tuberculo-
sis CYP51 (Matsura et al., 2005; Rupp et al., 2005) and the crystal structure of M. tuberculosis CYP51 (Podust et al., 2001).

There have also been few quantitative structure–activity relationship
(QSAR) models of CYP51 to date and they have dealt exclu-
sively with the fungal CYP51s (Tafi et al., 1996; Fujita, 1997). The utility of more classic QSAR models has also been reviewed for the production of azole-type agricultural fungicides (Fujita, 1997). How-
ever, approaches to modeling the common features of inhibitors of
other human P450s have been widely shown using a Catalyst phar-
macophore approach (Ekins et al., 2001), comparative molecular field
analysis (Jones et al., 1996), and other QSAR methods (Ekins et al.,
2003). These techniques have also been used to predict a test set of
molecules excluded from the training set (Ekins et al., 1999).

Recently a series of dual-acting hypoglycemic and hypcholester-
olemic agents were found to inhibit glycogen phosphorylase and
human CYP51 (Harwood et al., 2005). The prototype of this series,
CP-320626, inhibited glycogen phosphorylase and human CYP51
with IC_{50} values of 155 nM and 4 \mu M, respectively, reduced glyco-
genolysis and cholesterologenesis in cultured cells, and lowered plasma glucose and plasma cholesterol in experimental animals (Har-
wood et al., 2005). To understand the characteristics of these CP-
320626-related CYP51 inhibitors and other inhibitors of this human
CYP51 enzyme, we have used a computational approach to produce a
predictive Catalyst pharmacophore. This model was constructed using a training set of structurally diverse commercially available
molecules and CP-320626-related CYP51 inhibitors with varying IC_{50}
values. The validity of the predictions made by this model was then
validated using test sets of molecules not in the training set.
These test sets contained both commercially available molecules and structurally diverse CP-320626-related CYP51 inhibitors for which in
vitro data were subsequently generated.

Materials and Methods

Materials. Human cytochrome P450 reductase was obtained from Panvera
(now In vitro, Madison, WI). Rat microsomal lipid was prepared as de-
scribed by Sundin et al. (1987). Triarimol, CP-320626, and all other related
CYP51 inhibitors denoted as A-P and 1–48 (see supplemental Table for
structures) in the tables were synthesized as described previously (Hoover et
al., 1999, 2001). The pharmacophore method described below represents the
interactions of the ligands used and cannot be related easily to features in the
protein binding site. This method has also been applied to other proteins of
relevance to absorption, distribution, metabolism, excretion, and toxicity
(Ekins and Swaan, 2004). The receptor surface modeling method also de-
scribed below attempts to provide information relating to the volume require-
ments of the enzyme active site for the heme and nonheme binding compounds with
highest affinity.

Molecular Modeling. The computational molecular modeling studies were
3 cells was partially purified using the method of Stromstedt et al. (1996).
Protein concentrations were measured using a BCA Protein Assay kit (Fierce
Chemical, Rockford, IL) with bovine serum albumin as a standard as described by the manufacturer. Cytochrome P450 content was measured by the method
of Omura and Sato (1964). Initial rate conditions for the formation of 4,4-
dimethylcholest-8,14,24-trien-3β-ol (triene) were determined in preliminary
studies with the CYP51 reconstitution system. In brief, 25 \mu M of lanosterol (1
mM, suspended in a mixture of 50:50 tyloxapol-acetone, dioleoyl phosphatidyl
choline micelles (5 mg/ml), methanol, and 100 mM potassium phosphate
buffer, pH 7.4) was added to tubes to provide a final lanosterol concentration of
50 \mu M (approximate K_m). Inhibitors (ranging from 0.01 to 200 \mu M in
methanol) or methanol (control) were then added to the tubes, and the lanos-
sterol-inhibitor mixture was allowed to dry under nitrogen for 10 min. To dried
substrate and inhibitor tubes, 20 \mu l of CYP51, 125 \mu l of human reduct-
ase, and 50 \mu l of rat lipid were added and allowed to sit at room temperature
for 10 to 15 min for enzyme reconstitution. Buffer (100 mM potassium
phosphate, pH 7.4, with 20% glycerol, 0.1 mM diithiothreitol, 0.1 mM EDTA,
and 0.5 mM potassium cyanide) was added to the tubes after the reconstituti-
ation period for a final incubation volume of 0.5 ml. Tubes were preincubated for 2
min at 37°C, and the reaction was initiated by the addition of 50 \mu l of a
NADPH generating system (final incubation concentration; 10 mM MgCl_2,
0.54 mM NADP, 6.2 mM tri-isocitric acid, and 0.5 U/ml isocitrate dehydro-
genase). Reactions were terminated at 60 min with the addition of 25 \mu l of
ergosterol (1 mg/ml in ethyl acetate), followed by extraction with 5 ml of ethyl
acetate. Tubes were then vortexed before centrifugation followed by removal
of 3 to 4 ml of supernatant and transfer to fresh tubes and evaporation under
nitrogen at 50°C. The evaporated samples were reconstituted in a 150-\mu l mobile phase, and 25 to 50 \mu l was injected onto the high-performance liquid chromatography system (see below).

Triene formation was determined using a modification of the method of
Harwood et al. (1996). In brief, triene metabolite and ergosterol were sepa-
rated on a Waters Nova-Pak C_18 column (4.0 \mu M; 150 mm \times 3.9 mm; Waters,
Bedford, MA) with a mobile phase consisting of methanol-acetonitrile-
high-performance liquid chromatography grade water (45:45:10), at a flow rate of
1.5 ml/min. The triene and ergosterol were monitored by UV detection at 248
nm using a SpectroMonitor variable wavelength detector (LDC Analytical,
Riviera Beach, FL) and the Multichrom data acquisition system (version. 2.11;
Fisons Instruments, Beverly, MA) was used for data collection, analysis, and
reporting. Approximate retention times for triene metabolite and ergosterol
were 25 and 30 min, respectively.

Interpretation of CYP51 Lanosterol Demethylase Assay Results. The
percent inhibition of triene formation with methanol controls was determined with
each inhibitor at three to six concentrations in triplicate and the average
value was used as described previously (Harwood et al., 2005). The IC_{50}
was calculated from a graph of percent CYP51 activity remaining versus inhibitor
concentration using DeltaGraph (version 4.0; Monterey, CA).

Molecular Modeling. The computational molecular modeling studies were
carried out using a Silicon Graphics Octane workstation and based on a
pharmaphore methodology described previously for other P450s (Ekins et
al., 1999, 2001). The pharmacophore method described below represents the
features on the ligands used and cannot be related easily to features in the
protein binding site. This method has also been applied to other proteins of
relevance to absorption, distribution, metabolism, excretion, and toxicity
(Ekins and Swaan, 2004). The receptor surface modeling method also de-
scribed below attempts to provide information relating to the volume require-
ments of the enzyme active site for the heme and nonheme binding compounds
with highest affinity.

Modeling with Catalyst. The three-dimensional structures of CYP51 in-
hbitors from the present study or literature data sets were built interactively
using Catalyst (version 4.0; Accelrys Software, Inc., San Diego, CA). The
number of conformers generated for each inhibitor was limited to a maximum
of 255 with an energy range of 0 to 20 kcal/mol. A training set of 26 molecules
was selected and used to build the pharmacophore (Table 1). Ten hypothoses
were generated using these conformers for each of the molecules, and IC_{50}
values were generated after selection of the following features for the inhibi-
tors: hydrogen bond donor, hydrogen bond acceptor, hydrophobic, positive
ionizable, and ring aromatic. After assessment of all 10 hypotheses generated,
the lowest energy cost hypothesis was considered the best.
The goodness of the structure activity correlation was estimated by means of
the correlation coefficient r value. Catalyst also calculates the total energy
cost of the generated pharmacophores from the deviation between the esti-
mated activity and the observed activity, combined with the complexity of the
<table>
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<th>Inhibitor</th>
<th>Structure</th>
<th>Observed CYP51 IC₅₀/μM</th>
<th>Estimated CYP51 IC₅₀/μM</th>
<th>Inhibitor</th>
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**TABLE 1**
Commercial and CP-320626 analog CYP51 inhibitors used as a catalyst training set.
TABLE 1—Continued

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<th>Inhibitor</th>
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<th>Estimated CYP51 IC50</th>
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*Experimental data are the average of triplicate determinations.

An energy minimization on the conformers of the molecule (Catalyst tutorials release 3.0; Accelrys Software, Inc.). The computational predictions derived using the fit to the CYP51 Catalyst pharmacophore were compared with the observed in vitro values. We selected an acceptable arbitrary cutoff for potency of inhibition as accepted of \( \leq 10 \) \( \mu M \).

Catalyst CYP51 Pharmacophore Validation Using Permuting of Activity Data. The statistical significance of the pharmacophore hypothesis used was tested by permuting (randomizing) the structures and the activities and then repeating the Catalyst hypothesis generation procedure 10 times.

Modeling with Cerius2. The three-dimensional structures of CYP51 inhibitors generated using Catalyst as described previously were imported into Cerius2 (Accelrys Software, Inc.). Inhibitors with an IC50 \( \leq 10 \) \( \mu M \) were selected, and a receptor surface model (Hahn and Rogers, 1995) was generated. Additional molecules were then fitted to this model to discover which features were outside of the volume occupied by the active molecules.

Results

CYP51 in Vitro Inhibition Data. Partially purified CYP51 was characterized for protein and carbon monoxide difference spectra (data not shown). Total P450 was determined as 0.22 nmol/mg of protein. The assay conditions were also developed to provide optimal lanosterol turnover using a final ratio of P450/reductase of 1:6. The \( K_m \) determined for lanosterol with the reconstituted human CYP51 was 30 \( \mu M \) (data not shown), in agreement with previously determined values (Lamb et al., 1998). Using a standard concentration of 30 \( \mu M \) lanosterol, 93 commercially available molecules including the competitive inhibitor CP-320626 (Harwood et al., 2005) and related CYP51 inhibitors were screened in the IC50 assay (Tables 1–3).

Catalyst CYP51 Pharmacophore Model. Catalyst uses a collection of molecules with inhibitory activity over multiple orders of magnitude for the enzyme of interest to construct a model of the structural features (pharmacophore) necessary for interaction of the molecules with the active site of the enzyme. The resultant pharmacophore hypotheses explain the variability of the potency of inhibition with respect to the geometric localization of these features of the molecules. The IC50 values for structurally diverse inhibitors of CYP51 generated in this study cover \( >3 \) orders of magnitude (0.13–>200 \( \mu M \)) (Table 1). These values were used to build a pharmacophore, with the lowest energy model yielding four structural features (Fig. 1) necessary for inhibitor binding to the active site of CYP51: one hydrophobe, one hydrogen bond...
TABLE 3

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<tr>
<th>CP-320626-Related Molecules</th>
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*Experimental data are the average of triplicate determinations.*

acceptor, and two ring aromatic features (additional hydrophobes). The Catalyst pharmacophore demonstrated a good correlation of observed versus estimated IC_{50} values (r = 0.92) (Fig. 2).

The total energy cost of the hypothesis (117.3) and the null hypothesis (154.1) suggest that the model was significant as the difference was sizeable. Permuting the training set molecule structures with activities can be used to provide further evidence that the model is statistically reliable (Ekins et al., 1999). After permuting the hypothesis 10 times the mean r value decreased considerably to 0.32, and the mean energy cost difference increased to 148.7, almost identical to the null hypothesis value.

Catalyst CYP51 Pharmacophore Validation Using Test Sets of IC_{50} Values. After constructing the Catalyst 3D-QSAR model for a training set of 26 IC_{50} values generated by this laboratory in vitro (Table 1), the model was used to predict IC_{50} values of a test set of 19 commercially available molecules for which in vitro IC_{50} values were also generated (Table 2) and not included in the initial Catalyst model. In 16 cases, the IC_{50} values were correctly predicted to be either active (IC_{50} <10 µM) or inactive molecules (IC_{50} >10 µM). The poorly predicted molecules were made up of 5.3% false negatives (n = 1, bifonazole) and 10.5% false positives (n = 2, triarimol and azacompazole).

A second test set of IC_{50} values was composed of 48 CP-320626-related CYP51 inhibitors of which 38 were correctly predicted using the Catalyst 3D-QSAR model following the criteria as described above (Table 3). The poorly predicted molecules were made up of 4.2% false negatives (n = 2, molecules 1 and 37) and 16.6% false positives (n = 8, molecules 7, 12, 38, 39, 44, 45, 46, and 47).

Modeling with Cerius². Fifteen molecules with in vitro IC_{50} values <10 µM produced a volume using the receptor surface model software present in Cerius² (Fig. 3A). When fluconazole, an inhibitor with a low affinity for human CYP51 was fitted to this model, part of the molecule lies outside of the volume (Fig. 3B).

Discussion

In humans, beyond its obvious role in cholesterol and steroid hormone biosynthesis (Trzaskos et al., 1986), the physiological roles
of CYP51 in, for example, spermatogenesis and oogenesis (Stromstedt et al., 1998) are poorly defined. In addition, inhibition of cholesterol biosynthesis through inhibition of mammalian CYP51 results in an increase in cellular lanosterol concentrations (Miettinen, 1988), the consequences of which remain poorly defined. Furthermore, the poor specificity of most current mammalian CYP51 inhibitors for CYP51 versus steriodogenic or drug-metabolizing P450s (Hartman and Sanged, 1979; Strolin Benedetti and Bani, 1999) implies that molecules aimed at other therapeutic targets should preferably avoid interaction with this enzyme.

Because there is no crystal structure for the human membrane-bound CYP51, alternative techniques must be considered to understand whether a novel molecule will bind to it. It is possible, however, to begin to understand the specificity of an enzyme without possessing information regarding its crystal structure. The most widely used computational method for modeling in the cytochrome P450 field is homology modeling based on crystallized bacterial P450s. The initial models of the active site for CYP51 from Saccharomyces cerevisiae and an inactive mutant (Ishida et al., 1988) were constructed using alignment with P450cam. By using these, novel substrate analogs were developed as competitive inhibitors for this enzyme (Tuck et al., 1991). The formation of a fungal CYP51 bipheny1-iron complex also yielded information as to the approximate height of the cavity (10 Å) above the porphyrin ring in the active site (Tuck et al., 1992). Further work studying the try helix of the fungal CYP51 with lanosterol has shown that Gly-310 is an important residue, although interactions at His-317 and Met-313 may also occur (Vanden Bossche et al., 1995). It has been suggested that whereas ketoconazole and itraconazole have multiple interactions with the CYP51 I helix, fluconazole has fewer and may result in its lower antifungal activity (Vanden Bossche and Koymans, 1998). Comparative homology models have identified differences in the active sites of Candida albicans and S. cerevisiae (Boschott and Grant, 1994), in agreement with earlier work (Yoshida and Aoyama, 1987). When the human CYP51 I helix sequence is aligned with those from C. albicans and S. cerevisiae (Vanden Bossche and Koymans, 1998), there are six amino acids that are different between the fungal and the human P450s. It is therefore likely that the surface volume of inhibitors for fungi and human CYP51 could be markedly different due to the alteration in the active site caused by these amino acid substitutions. Numerous studies have used homology modeling and docking for CYP51 to show lanosterol hydrogen bonding interactions (Holtje and Fattorussu, 1998), triazole andazole inhibitors interacting with the hydrophobic active site and the heme iron (Talele et al., 1997), and functional group modifications that alter enzyme-inhibitor interactions (Tsukuda et al., 1998). CYP51 homology models have been used in de novo design (Ji et al., 2000) and in understandingazole binding to human and crystallized M. tuberculosis CYP51 enzymes (Matsuura et al., 2005; Rupp et al., 2005). The latter study indicated that the human enzyme may have a larger and differently shaped active site cavity due to the replacement of Phe-255 with Leu-310 (Matsuura et al., 2005; Rupp et al., 2005).

Although there are data sets in the literature describing the fungicidal activity of CYP51 inhibitors (Stehmann and de Waard, 1995), there is little information relating to human disease. The present study has described, to our knowledge, the first 3D-QSAR relationship using in vitro data for inhibitors of human CYP51. By using 26 structurally diverse molecules with a range of inhibitory activity, we constructed a predictive pharmacophore model that was used to score the activity of test molecules and predict whether new chemical entities might interact with this enzyme. The model for the data set generated in this study suggested a pharmacophore with four features, indicating the predominance of hydrophobic features (Fig. 1) in agreement with previous reports (Matsuura et al., 2005; Rupp et al., 2005) and a high correlation (r = 0.92) (Fig. 2) between predicted and observed IC50 values.

One of the best ways to test the validity of a 3D-QSAR is to predict the activity of molecules excluded from the training set and then compare these to observed values. In addition to prospectively assessing many proprietary compounds, several commercially available molecules were evaluated in vitro as they had either important endogenous roles (cholesterol, arachidonic acid, progesterone, pregnenolone, and anandamide), were known P450 substrates (erythromycin, tolbutamidine, lovastatin, chloropropamide, and propafenone), or were known P450 inhibitors (sulfaphenazole, fluconazole, azaconazole, econazole, bifonazole, terconazole, itraconazole, triamcinol, and sulfynpyrazone). It was thought that these molecules would provide a structurally diverse test set to validate the pharmacophore constructed from a similarly structurally diverse training set including heme-binding azoles and non-heme-binding CP-320626-related compounds (Harwood et al., 2005). In vitro IC50 values were generated for CYP51 inhibition for 19 commercially available molecules. Sixteen of the 19 (84%) molecules fulfilled the criterion (Table 2). Non-heme-binding CP-320626-related CYP51 inhibitors were also successfully predicted using the same pharmacophore model with an ~80% prediction rate (Table 3). These results suggest that the CYP51 pharmacophore has utility for both classification of novel molecules that are either known heme binders or nonheme binders with an acceptable level of accuracy for early screening.

By aligning the most potent CYP51 inhibitors (azoles and CP-320626-related compounds) with the pharmacophore, it was possible...
to generate a receptor surface model of their volume. The receptor surface model of these active inhibitors forms an elongated shape (Fig. 3A), not too dissimilar from that of lanosterol and the volume generated by Rupp et al. (2005). This receptor surface model can also be used to explain the apparent lack of inhibitory activity toward human CYP51 by inactive molecules, by first mapping them to the pharmacophore and then placing them inside this receptor surface model. Features outside of the volume could suggest poor steric interactions within the active site of the enzyme that would be unfavorable for binding and therefore inhibition. As an example, we have found a fluconazole to the pharmacophore and shown that a triazole ring is outside of the surface volume generated by the active compounds (Fig. 3B). This may explain the lack of potency of fluconazole toward the human CYP51 but subsequent activity toward the fungal P450.

Indeed this is in agreement with a recent study that has also shown the human CYP51 but subsequent activity toward the fungal P450. This may explain the lack of potency of fluconazole toward the active site of this P450 and visually describe the selectivity of these studies.

References


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