

Marketed Drugs Can Inhibit Cytochrome P450 27A1, a Potential New Target for Breast Cancer Adjuvant Therapy[§]

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ABSTRACT

Cytochrome P450 CYP27A1 is the only enzyme in humans converting cholesterol to 27-hydroxycholesterol, an oxysterol of multiple functions, including tissue-specific modulation of estrogen and liver X receptors. Both receptors seem to mediate adverse effects of 27-hydroxycholesterol in breast cancer when the levels of this oxysterol are elevated. The present work assessed druggability of CYP27A1 as a potential antibreast cancer target. We selected 26 anticancer and noncancer medications, most approved by the Food and Drug Administration, and evaluated them first in vitro for inhibition of purified recombinant CYP27A1 and binding to the enzyme active site. Six strong CYP27A1 inhibitors/binders were identified. These were the two antibreast cancer pharmaceuticals anastrozole and fadrozole, antiplatelet drug bicalutamide, sedative dexmedetomidine, and two

antifungals ravuconazole and posaconazole. Anastrozole was then tested in vivo on mice, which received subcutaneous drug injections for 1 week. Mouse plasma and hepatic 27-hydroxycholesterol levels were decreased 2.6- and 1.6-fold, respectively, whereas plasma and hepatic cholesterol content remained unchanged. Thus, pharmacologic CYP27A1 inhibition is possible in the whole body and individual organs, but does not negatively affect cholesterol elimination. Our results enhance the potential of CYP27A1 as an antibreast cancer target, could be of importance for the interpretation of Femara versus Anastrozole Clinical Evaluation Trial, and bring attention to posaconazole as a potential complementary anti-breast cancer medication. More medications on the US market may have unanticipated off-target inhibition of CYP27A1, and we propose strategies for their identification.

Introduction

Cytochrome P450 27A1 (CYP27A1) is a ubiquitous sterol 27-hydroxylase acting on cholesterol in extrahepatic tissues, bile acid intermediates in the liver, and vitamin D₃ in the kidneys (Supplemental Fig. 1) (Wikvall, 1984; Masumoto et al., 1988). Despite broad sterol specificity, CYP27A1 is a highly regio- and stereo-specific enzyme producing all 27-hydroxycholesterol (27HC) in humans. Cholesterol 27-hydroxylation serves two main physiologic purposes: cholesterol elimination and cellular regulation. 27HC is a means of cholesterol transport from extrahepatic tissues to the liver (Meaney et al., 2002). 27HC is also a bioactive molecule interacting with different regulatory proteins, including liver X receptors (Kalaany and Mangelsdorf, 2006). Complete deficiency of CYP27A1 activity leads to cerebrotendinous xanthomatosis (CTX), an autosomal recessive and slowly progressive disease characterized by deposition of cholesterol and cholestanol (reduced form of cholesterol) in the brain and other tissues, neurologic dysfunction, and ocular

abnormalities. Patients with CTX usually have normal or below normal levels of plasma cholesterol; nevertheless, they frequently develop premature atherosclerosis and osteoporosis (Bjorkhem, 2013). Yet a partial lack of CYP27A1 activity in *CYP27A1*^{+/-} heterozygous individuals does not lead to CTX (Bjorkhem, 2013).

Recent studies in cell culture and animals revealed a new role of 27HC when the oxysterol levels were elevated either through pharmacologic treatments, diet-induced hypercholesterolemia, or genetic manipulations (Umetani et al., 2007; DuSell et al., 2008). 27HC was discovered to bind to estrogen receptors (ER) and act as a selective ER modulator (SERM) eliciting system-specific adverse effects. In the vascular wall, 27HC functioned as an ER antagonist and inhibited estrogen-related cardioprotection (Umetani et al., 2007, 2014; Umetani and Shaul, 2011). Conversely, in breast tumors, 27HC served as a partial ER agonist and stimulated tumor growth in several mouse models of breast cancer (Nelson et al., 2013; Wu et al., 2013). Through action on liver X receptors, 27HC also increased breast tumor metastasis (Nelson et al., 2013). In bone, 27HC attenuated estrogen action and had negative effects on bone mineralization (DuSell et al., 2010). Epidemiologic and other studies support the role of 27HC as a SERM. Indeed, in women, menopause was found to be accompanied by elevations of plasma 27HC (Burkard et al., 2007) and is known to dramatically increase the risk of coronary heart disease and ER-positive breast cancer (Patel et al., 2007; Lloyd-Jones et al., 2009). However, it is unclear whether

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ABBREVIATIONS: CTX, cerebrotendinous xanthomatosis; ER, estrogen receptor; 27HC, 27-hydroxycholesterol; HPCD, 2-hydroxypropyl- β -cyclodextrin; KP_i, potassium phosphate buffer; SERM, selective ER modulator.

27HC is deleterious in women of reproductive age, that is, when plasma levels of 27HC are low and estrogen levels are physiologic. A model is proposed, according to which effects of 27HC on ER function are determined by the levels of both 27HC and estrogen, with the ER function being affected when the levels of 27HC increase and estrogen levels decrease (Umetani et al., 2007).

CYP27A1 is highly abundant in human breast cancer specimens with enzyme expression correlating with the tumor grade (Nelson et al., 2013). This finding along with the new role of 27HC as a SERM (Umetani et al., 2007; DuSell et al., 2008) suggests that CYP27A1 inhibition could be a new therapeutic option for the treatment of ER-positive breast cancer in postmenopausal women. We envision that CYP27A1 inhibitors could be used in combination with CYP19A1 (aromatase) inhibitors, the first-line adjuvant treatment of this type of breast cancer (Dutta and Pant, 2008). Alternatively, CYP19A1 and CYP27A1 could be inhibited simultaneously by the same drug. The caveat is that the whole-body complete inhibition of CYP27A1 is not desirable, as it may lead to a pharmacologically induced form of CTX. Accordingly, the inhibition should only be partial—CYP27A1^{+/−} individuals do not develop CTX (Bjorkhem, 2013)—or targeted to specific organs. The mammary gland is a particularly suitable organ for tissue-specific drug delivery with efforts currently underway to administer some of the anticancer medication through the skin via transdermal patches (Li et al., 2010; Xi et al., 2010). This advantage of the mammary gland and body's tolerability to a partial lack of CYP27A1 activity supports enzyme potential as a pharmacologic target. In this work, we undertook the first step in assessing CYP27A1 as a therapeutic target: we investigated the druggability of the enzyme. We used mostly marketed medications and identified several pharmaceuticals that have the potential to inhibit CYP27A1 in humans. Our results justify further investigation of CYP27A1 as a therapeutic target.

Materials and Methods

CYMAL-7 was from Anatrace (Maumee, OH). R-Bicalutamide was from Toronto Research Chemicals (Toronto, Canada). Cholesterol and [³H]cholesterol were from Steraloids (Newport, RI) and American Radiolabeled Chemicals (St. Louis, MO), respectively. All other chemicals were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Recombinant CYP27A1, adrenodoxin reductase, and adrenodoxin were expressed and purified, as described (Murtazina et al., 2004).

Animals. All animal studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health and were approved by Case Western Reserve University's Animal Care and Use Committee. Four-week-old C57BL/6J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Animal Resource Center at Case Western Reserve University until they reached the age of 4–5 months. Animals were maintained in a standard 12-hour light (~10 lux)/12-hour dark cycle environment with water and food provided ad libitum.

Screening Enzyme Assay. Drugs were screened for the effect on CYP27A1 activity with cholesterol, which was reconstituted in vitro, as described (Heo et al., 2012), except that phospholipid vesicles were replaced with detergent CYMAL-7. Briefly, incubations were carried out in 1 ml 50 mM potassium phosphate buffer (KP_i), pH 7.2, containing 1 mM EDTA and 0.01% CYMAL-7. Drug (46 μM) or vehicle was added to the buffer first, followed by the addition of purified recombinant CYP27A1 (0.07 μM), adrenodoxin (3.5 μM), adrenodoxin

reductase (0.35 μM), [³H]cholesterol (13 nM or ~250,000 cpm), and unlabeled cholesterol (2.3 μM, a concentration equal to 0.5 *K_m* CYP27A1 for cholesterol). Enzymatic reaction was initiated by addition of NADPH (1 mM) and was carried out for 4 minutes at 37°C. The reaction was terminated by addition of 5 ml CH₂Cl₂. Organic phase was isolated, evaporated, and analyzed by high-performance liquid chromatography, as described (Mast et al., 2004). Cholesterol was added from 10 mM stock in 45% aqueous 2-hydroxypropyl-β-cyclodextrin (HPCD). Drugs were added from 5 mM stocks in water (cisplatin, fluvoxamine, temozolomide, aminolevulinic acid, and thioperamide), 4.5% aqueous HPCD (exemestane and imexon), methanol (aminoglutethimide, anastrozole, aprepitant, axitinib, azacitidine, bexarotene, clofarabine, crizotinib, dacarbazine, dexmedetomidine, fadrozole, hexaconazole, letrozole, pamidronate, posaconazole, procarbazine, ravuconazole, tranlycypromine, and vorinostat), or dimethyl sulfoxide (bicalutamide). The solvent for drug stocks was determined by drug solubility: if a 5 mM drug stock could not be prepared in water, 4.5% aqueous HPCD, methanol, and dimethyl sulfoxide were then sequentially tested for the ability to dissolve the drug at the desired concentration.

Spectral (Drug-Binding) Assay. This assay is based on the perturbations in the P450 spectrum caused by changes in the coordination chemistry of the P450 heme iron and the identity the sixth heme iron ligand, usually a water molecule in a resting substrate-free P450 enzyme (Dawson et al., 1982; Poulos et al., 1986; Isin and Guengerich, 2008). If this water molecule is displaced and the sixth coordinate position is left unoccupied (typical for substrate binding), the Soret peak in the P450 absolute spectrum shifts from 416–418 nm toward the shorter wavelength (blue shift), and the difference spectrum develops a peak at 380–393 nm and a trough at 415–420 nm P450, the so-called type I spectral response (Schenkman et al., 1967). If the sixth coordinate position becomes occupied by a nitrogen-containing functionality (often the case with drug binding), the shift of the P450 Soret peak is toward the longer wavelength (red shift), and the difference spectrum has a trough at 390–410 nm and a peak at 421–435 nm, the so-called type II spectral response (Schenkman et al., 1967). There may be a third type of response, reverse type I response (a mirror reflection of a type I response), believed to reflect the movement of a water molecule back to the sixth coordinate position (Chun et al., 2001). Sometimes, substrate or drug binding does not elicit P450 spectral response. This does not necessarily mean a lack of binding, but could be a case of compound positioning in the enzyme active site at a distance to the heme iron (Isin and Guengerich, 2008). Bicalutamide, the strongest CYP27A1 inhibitor in the screening enzyme assay, was used for optimization of the conditions of the binding assay, which was carried out as described (Mast et al., 2006) at 30°C in a 1-ml solution of 50 mM KP_i (pH 7.2) containing 0.4 μM CYP27A1, 1 mM EDTA, 0.01% CYMAL-7, 10% glycerol, and 0.1 M NaCl. Drugs were added from 1–5 mM stocks in the same vehicle as used for studies of drug effects on CYP27A1 activity. These conditions produce the lowest *K_d* for bicalutamide, maximal P450 spectral response, and a clear isosbestic point in the CYP27A1 difference spectrum, indicating unchanged stoichiometry of the spectral conversion during the titration (<http://goldbook.iupac.org/I03310.html>). Apparent *K_d* values were calculated using the $\Delta A = (\Delta A_{\max}[L]) / (K_d + [L])$ equation, in which ΔA is the spectral response at different ligand (drug) concentrations [L], and ΔA_{\max} is the maximum amplitude of drug-induced spectral response. Drugs that elicited spectral response in CYP27A1 were then tested for CYP27A1 binding in 50 mM KP_i (pH 7.2) containing 1 mM EDTA and 0.01% CYMAL-7, but lacking glycerol and NaCl, as these conditions were similar to those of enzyme assay.

Determination of IC₅₀ and K_i. These parameters of CYP27A1 inhibitions were determined in the reconstituted system in vitro using the same conditions as in the screening enzyme assay, except the drug concentrations varied from 0.002 to 120 μM, and the cholesterol concentration was 4.6 μM. The data were fit to the following equation by Graph-Pad Prism software:

$$\% \text{ Control Activity} = B + \frac{A - B}{1 + 10^{([\text{I}] - \text{logIC}_{50})}}$$

in which [I] is the inhibitor concentration, IC_{50} is the inflection point, A is the CYP27A1 activity (100%) in the absence of inhibitor, and B is the residual CYP27A1 activity (%) at maximum inhibitor concentrations. Because the determination of IC_{50} was carried out at a cholesterol concentration equal to the K_m , $K_i = \text{IC}_{50}/2$, assuming a model of competitive inhibition.

Mouse Treatments with Anastrozole. All animal-handling procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and conformed to the standards of the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Drug administration was as described (Macedo et al., 2008) using a 1 mg/ml solution of anastrozole in 0.3% hydroxypropylcellulose and 0.9% NaCl. Mice in the treatment group ($n = 8$) received a daily subcutaneous injection of 200 μl (200 μg) anastrozole, whereas animals in the control group ($n = 6$) received a daily injection (200 μl) of the vehicle. The treatment time was 7 days. Animals were then sacrificed, and blood was collected in the EDTA-coated tubes for plasma isolation by 1000g centrifugation for 10 minutes at 4°C. The liver was isolated as well as described (Zheng et al., 2015) and used along with plasma for the determination of cholesterol and 27HC content by gas chromatography–mass spectrometry, as described (Zheng et al., 2015).

Statistical Analysis. All data represent mean \pm S.D. All in vitro assays were carried out in triplicate. All quantifications in mice represent the average of the individual measurements in at least six animals. Statistical significance was determined by a two-tailed, unpaired Student's *t* test and defined as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Selection of Drugs for Testing on CYP27A1. Drug selection was based on approaches developed in our prior studies of CYP46A1 and CYP11A1 (Mast et al., 2012, 2013a), which like CYP27A1 act on cholesterol as a substrate, but generate different products, 24-hydroxycholesterol and pregnenolone, respectively. We used intuitive predictions and were guided by the knowledge of the CYP27A1 substrate specificities, shape of the active sites of CYP46A1 and CYP11A1, and the previously generated CYP27A1 model based on crystal structure of CYP11A1 (Mast et al., 2006, 2008, 2011; Charvet et al., 2013). Cumulatively, these data suggested that the CYP27A1 active site should represent a banana-shaped tunnel connecting the protein surface and buried heme group, the site of catalysis in P450 enzymes.

The website of the National Cancer Institute lists 116 drugs approved by the Food and Drug Administration for treatment of different types of cancers (Supplemental Table 1). We found chemical structures of all of these drugs and visually inspected these structures. We excluded 38 bulky compounds and 22 peptide-based pharmaceuticals, as they are unlikely to enter the CYP27A1 active site because of spatial constraints. We also excluded seven drugs that were cost prohibitive, and two compounds that absorb in the 370- to 470-nm region and would be difficult to evaluate in the spectral binding assay. We next excluded 21 compounds whose structures have hindered nitrogens, as a hindered nitrogen is unlikely to coordinate the heme iron and form the N-Fe bond that often inhibits P450s (Gigon et al., 1968). The remaining compounds were categorized into four groups, as follows: nucleotide derivatives; platinum-containing, antibreast cancer drugs; aromatase inhibitors; and others. We selected one representative

drug from nucleotide derivatives and platinum-containing compounds, all aromatase inhibitors, and other drugs on the list. We also included seven noncancer pharmaceuticals identified as strong inhibitors of cholesterol-metabolizing CYP46A1 and CYP11A1 in our previous studies (Mast et al., 2010, 2012, 2013a,b). Thus, a total of 26 compounds were selected for evaluations in the present work. Chemical structures of these compounds are shown in Fig. 1.

CYP27A1 Inhibition in the Screening Enzyme Assay. Eight compounds were found to inhibit CYP27A1 activity by $\geq 40\%$ (an arbitrary cutoff limit) (Fig. 2). Of them, four (bicalutamide, anastrozole, dexmedetomidine, and fadrozole) inhibited CYP27A1 activity by $\geq 75\%$ (strong inhibitors), and four (procarbazine, thioperamide, ravuconazole, and posaconazole) inhibited the P450 by $\geq 43\%$ (moderate inhibitors).

CYP27A1 Response to Drugs in the Binding Assay. All drugs tested in the screening assay were then evaluated in the spectral binding assay. Of them, seven induced spectral response in CYP27A1, and these were seven of the eight drugs that inhibited the enzyme by $\geq 40\%$ in the screening assay (Fig. 3; Table 1); only procarbazine was spectrally silent. Five of these seven spectral inducers (dexmedetomidine, fadrozole, thioperamide, anastrozole, and ravuconazole) elicited a classic type II spectral response in the CYP27A1 difference spectrum (a trough at 390–410 nm and peak at 421–435 nm) (Dawson et al., 1982), but had differential effects on the P450 absolute spectrum. The typical type II red shift of the Soret peak was elicited only by dexmedetomidine, fadrozole, and thioperamide, but not by anastrozole and ravuconazole. The red shift was larger in the case of dexmedetomidine (7 nm) than fadrozole (4 nm) and thioperamide (2 nm), suggesting that the latter two induce only partial shifts. The CYP27A1 spectral response to addition of bicalutamide and posaconazole was different and of a rare reverse type I in the difference spectrum (a trough at 380–390 nm and peak 415–420 nm) (Schenkman et al., 1967) with no changes in the absolute spectrum. Thus, seven of eight top CYP27A1 inhibitors in the screening assay (except procarbazine) elicited perturbations around the P450 heme iron, suggesting that they enter the enzyme active site and act as competitive inhibitors.

Drug-induced spectral changes enabled the determination of the apparent K_d values (Table 1), which were relatively low, within a 1–20 μM range. These values, however, did not always correlate with the extent of CYP27A1 inhibition in the screening assay. Bicalutamide and posaconazole, which had the lowest apparent K_d s, inhibited CYP27A1 by 92 and 43%, respectively, whereas fadrozole and thioperamide, whose apparent K_d s were the highest, inhibited the P450 by 75 and 47%, respectively. Therefore, the conditions of the spectral assay were changed to make them more similar to those of the enzyme assay by excluding 10% glycerol and 0.1 M NaCl from the titration buffer. The new assay conditions decreased the K_d s for three drugs (dexmedetomidine, fadrozole, and ravuconazole) from 2.6-fold (ravuconazole) to 5.7-fold (fadrozole), but still did not always correlate with the inhibition of enzyme activity. Therefore, the IC_{50} plots were next generated to characterize the effectiveness of drug inhibition.

CYP27A1 Inhibition as Assessed by In Vitro IC_{50} and K_i . Four drugs were selected for these quantitative characterizations. These were bicalutamide, anastrozole, and fadrozole, the three strongest CYP27A1 inhibitors in the screening

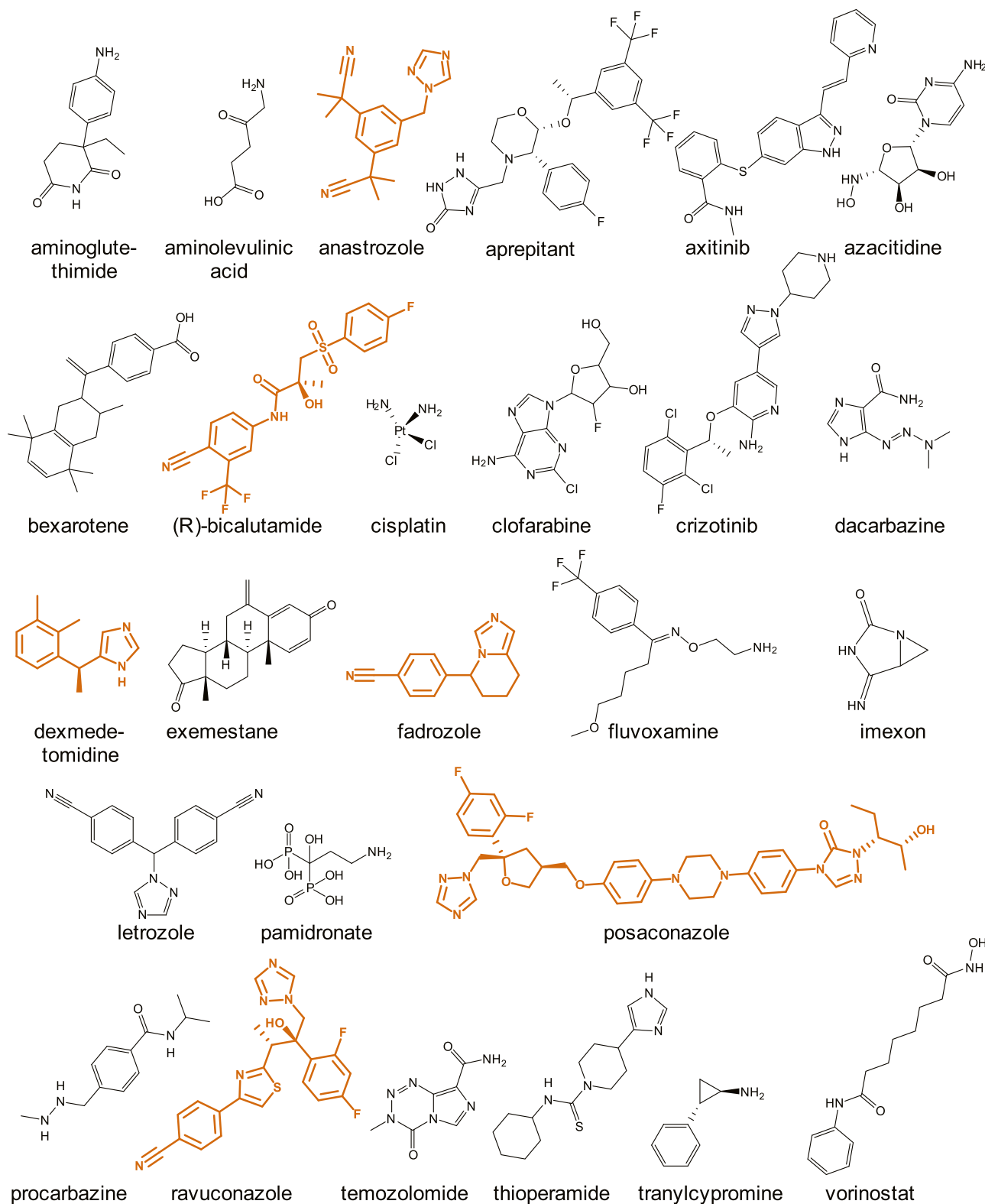


Fig. 1. Chemical structures of the drugs evaluated in the present work. The structures in orange are of the strong CYP27A1 inhibitors or binders.

assay, plus posaconazole, whose moderate CYP27A1 inhibition in the screening assay did not correlate with a low apparent K_d . Dexmedetomidine was excluded because it is an intravenous sedative, impractical for a daily outpatient use. Ravuconazole and thioperamide were excluded because they

are still experimental drugs and thioperamide had a relatively high $20 \mu\text{M}$ K_d . The IC_{50} plots were generated (Fig. 4) and K_i values were determined ($\text{IC}_{50} = 2K_i$) because spectral studies suggested a competitive mode of inhibition. Almost a complete inhibition of CYP27A1 activity was observed in the IC_{50} plots

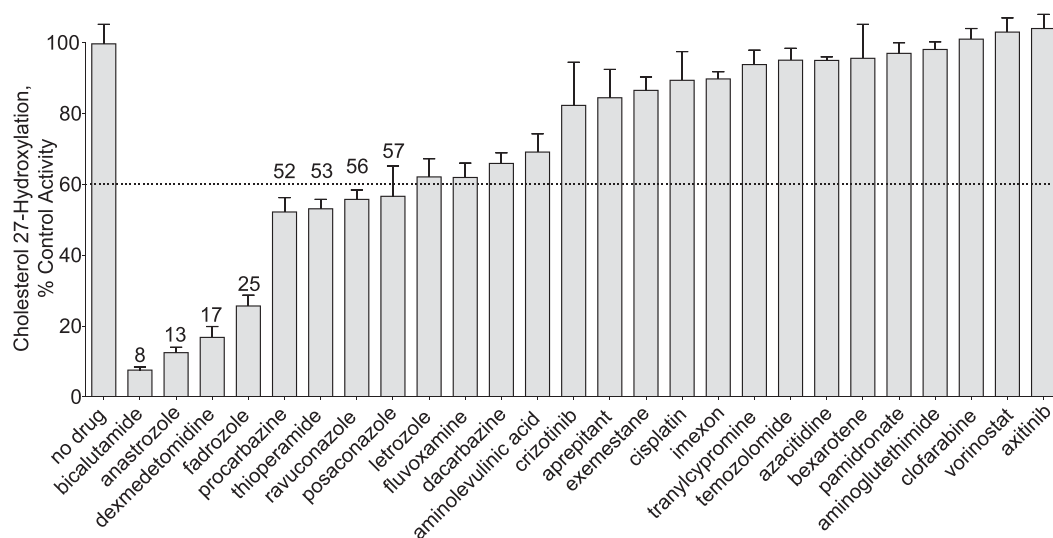


Fig. 2. Effect of drugs on CYP27A1 cholesterol 27-hydroxylase activity in the screening assay. Assay conditions are described in *Materials and Methods*. The results represent mean \pm S.D. of three independent measurements. Numbers above the bars indicate percentage of CYP27A1 activity relative to control incubations containing no drug.

of bicalutamide, anastrozole, and fadrozole, yielding K_i s of 1.1, 1.2, and 4.6 μM , respectively. In contrast, posaconazole inhibited CYP27A1 only by $\sim 50\%$ with apparent K_i of 1.9 μM . This partial CYP27A1 inhibition was likely due to the limited posaconazole solubility in aqueous solutions, as this compound has the highest $\log P$ value among the tested drugs (Table 1). Limited solubility of posaconazole also provides an explanation for the relatively low CYP27A1 inhibition in the screening assay, as the effective drug concentration was probably much lower than of the other seven top inhibitors. By contrast, posaconazole's K_d was likely affected to a lesser extent because much lower (≥ 3 -fold) posaconazole concentrations were used in the spectral binding assay as compared with the screening and IC_{50} assays.

CYP27A1 Inhibition In Vivo in Mice. Mice were treated with anastrozole at a drug concentration (200 $\mu\text{g}/\text{mouse}$ per day) established to be maximally effective in reducing tumor growth in the mouse intratumoral aromatase xenograph model (Macedo et al., 2008). The effect on CYP27A1 was assessed by the measurements of plasma 27HC as the indicator of the whole-body CYP27A1 inhibition. We also measured hepatic levels of 27HC to determine the liver contribution to the whole-body CYP27A1 inhibition. A 7-day treatment of mice with anastrozole reduced the plasma and hepatic concentrations of 27HC by 2.6- and 1.6-fold, respectively (Fig. 5), suggesting that CYP27A1 inhibition in the liver was smaller than in extrahepatic organs. In contrast, plasma and hepatic levels of cholesterol were not altered in anastrozole-treated mice (Fig. 5) consistent with only minor, under normal conditions, contribution of cholesterol 27-hydroxylation to a total-body cholesterol elimination (Lund et al., 1996).

Discussion

The major findings of the present work are that CYP27A1 is a druggable target and that six pharmaceuticals—bicalutamide, anastrozole, dexmedetomidine, fadrozole, ravuconazole, and posaconazole—bind to purified recombinant

enzyme (Fig. 3) and have low micromolar values of either in vitro K_d s (bicalutamide, dexmedetomidine, fadrozole, ravuconazole, and posaconazole) (Table 1) or K_i s (bicalutamide, anastrozole, and fadrozole) (Fig. 4). The K_d – K_i comparison for the three drugs that completely inhibited CYP27A1 in the IC_{50} assay (bicalutamide, anastrozole, and fadrozole) reveals that the apparent drugs' K_d s are either higher or similar to their K_i s. Consequently, the K_i s of dexmedetomidine and ravuconazole, which were not determined in the present study, are also likely in the low micromolar range. The same is perhaps true for posaconazole, whose apparent K_d s were in the low micromolar range and whose determination of the K_i was affected by the drug's solubility. The pharmaceutical industry usually uses 1 μM value of in vitro K_i as a cutoff limit for prediction of in vivo inhibition, and considers values of ≥ 10 –30 μM as typically not associated with significant in vivo inhibition (Obach et al., 2005). Accordingly, the K_d and K_i values determined in the present work indicate that bicalutamide, anastrozole, dexmedetomidine, fadrozole, ravuconazole, and posaconazole have the potential to inhibit CYP27A1 in humans. This prediction is supported by a proof-of-concept treatment of mice with anastrozole (Fig. 5), demonstrating the CYP27A1 inhibition is possible in vivo. Animal studies also revealed that the observed partial inhibition of CYP27A1 does not lead to changes of plasma and hepatic cholesterol, an important finding because plasma and hepatic cholesterol are altered in *Cyp27a1*^{−/−} mice (decreased 1.2-fold and increased >3 -fold, respectively) lacking cholesterol 27-hydroxylase activity (Dubrac et al., 2005). Thus, it is possible to partially inhibit CYP27A1 in vivo, but not affect metabolism of cholesterol.

Practical and clinical significance of the identification of potential CYP27A1 inhibitors is drug-specific, as these compounds have different medical indications. Of immediate importance could be the data on CYP27A1 inhibition by the inhibitors of aromatase (or CYP19A1), the major target in the ER-positive breast cancer. In this group of drugs, the strongest CYP27A1 inhibition was by anastrozole (87%), followed by fadrozole (75%), letrozole (38%), and exemestane

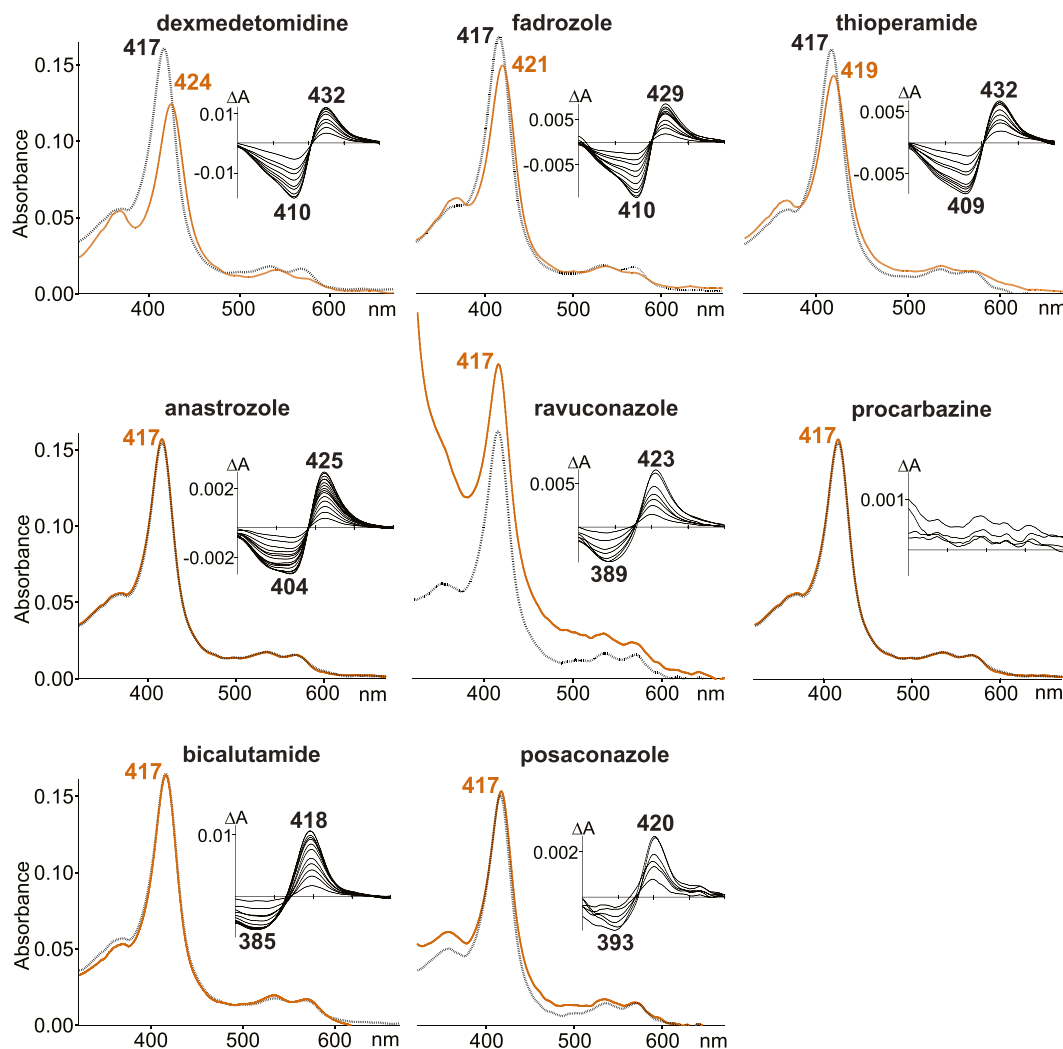


Fig. 3. Absolute and difference (right side insets) spectra of CYP27A1 in the absence (black dashed line) and presence (orange line) of different drugs. Numbers above and below the spectra indicate the wavelengths of absorption maxima or minima. Absolute spectra were recorded using $1.2 \mu\text{M}$ CYP27A1 and $50 \mu\text{M}$ drug. Difference spectra were recorded using $0.4 \mu\text{M}$ CYP27A1 and a varying drug concentration against increasing amounts of drug vehicle. The buffer was 50 mM KP_i (pH 7.2) containing 1 mM EDTA, 0.01% CYMAL-7, 10% glycerol, and 0.1 M NaCl.

(13%) (Table 1). This pattern is different from the known inhibition of CYP19A1 led by letrozole (>98.9–99.1%), exemestane (97.9%), anastrozole (96.7–97.3%), and fadrozole (82.4–92.6%) (Geisler, 2011). Thus, letrozole and exemestane, the two strongest inhibitors of CYP19A1, are weak inhibitors of CYP27A1, whereas anastrozole and fadrozole are strong inhibitors for both CYP19A1 and CYP27A1. Anastrozole represents the third generation of aromatase inhibitors, which are more potent than the second generation inhibitors (e.g., fadrozole) and are also more selective toward CYP19A1 (Dutta and Pant, 2008). We demonstrate in this work that, despite improved selectivity, anastrozole has the $1.2 \mu\text{M}$ *in vitro* K_i for inhibition of CYP27A1-mediated cholesterol 27-hydroxylation (Fig. 4) and inhibits CYP27A1 in mice (Fig. 5). This finding may be of key significance for the interpretation of the results of the ongoing Femara (letrozole) versus Anastrozole Clinical Evaluation Trial conducted on postmenopausal women with ER-positive breast cancer (O'Shaughnessy, 2007; Monnier, 2010). This trial investigates whether different CYP19A1 inhibition by letrozole and anastrozole translates into differences in their clinical efficacy (O'Shaughnessy, 2007). Our data may help to

understand the results of this trial, especially if letrozole does not offer a greater clinical benefit as compared with anastrozole because a smaller inhibition of CYP19A1 by anastrozole could be compensated by the off-target inhibition of CYP27A1, an effect beneficial for the breast cancer treatment (Nelson et al., 2013).

Of practical significance could be CYP27A1 inhibition by ravuconazole and posaconazole despite that the two drugs were unable to significantly inhibit CYP27A1 in our *in vitro* studies (Fig. 2). This low inhibition is probably due to high drug lipophilicity, a reason for low bioavailability of oral posaconazole, and enhancement of drug bioavailability to up to 47% by high-fat food (Lipp, 2010). Drug lipophilicity and low apparent K_d values for CYP27A1 (Table 1) make, however, ravuconazole and especially posaconazole, an approved Food and Drug Administration drug, good candidates for breast-specific drug delivery via transdermal patches, a route of administration that is currently under investigation (Li et al., 2010; Xi et al., 2010). Alternatively, posaconazole could be administered orally in combination with other antibreast cancer medications.

TABLE 1
A comparison of the screening and spectral assay data

Drug	Screening Assay CYP27A1 Activity ^a	Spectral Assay				log <i>P</i> ^e
		Apparent <i>K</i> _d ^a		$\Delta A_{\max}^b \times 10^{-3}$	$\lambda_{\min}/\lambda_{\max}$ in Difference Spectrum	
	%	μM			nm	
No drug	100					
Bicalutamide	8 ± 1	3.4 ± 0.9		43 ± 4		385/418
Anastrozole	13 ± 2	9.1 ± 2.1		16 ± 1		404/425
Dexmedetomidine	17 ± 3	5.8 ± 0.1	(1.5 ± 0.1)	90 ± 1	(42 ± 7)	410/432
Fadrozole	25 ± 3	20.4 ± 2.5	(3.6 ± 0.1)	55 ± 3	(28 ± 2)	410/429
Procabazine	52 ± 4			NSR ^d		
Thioperamide	53 ± 3	18.1 ± 3.8		46 ± 3		409/432
Ravuconazole	56 ± 3	3.9 ± 0.4	(1.5 ± 0.2)	32 ± 1	(28 ± 2)	389/423
Posaconazole	57 ± 9	2.8 ± 0.1		13 ± 1		393/420
Letrozole	62 ± 5			NSR		
Fluvoxamine	62 ± 4			NSR		
Dacarbazine	66 ± 3			NSR		
Aminolevulinic acid	69 ± 5			NSR		
Crizotinib	82 ± 12			NSR		
Aprepitant	84 ± 8			NSR		
Exemestane	87 ± 4			NSR		
Cisplatin	89 ± 8			NSR		
Imexon	90 ± 2			NSR		
Tranylcypromine	94 ± 4			NSR		
Temozolomide	95 ± 3			NSR		
Azacitidine	95 ± 1			NSR		
Bexarotene	96 ± 10			NSR		
Pamidronate	97 ± 3			NSR		
Aminoglutethimide	98 ± 2			NSR		
Clofarabine	101 ± 3			NSR		
Vorinostat	103 ± 4			NSR		
Axitinib	104 ± 4			NSR		

^aCholesterol 27-hydroxylation; all results represent mean ± S.D. of three independent measurements. Numbers in parentheses indicate binding parameters in the buffer lacking glycerol and NaCl.

^bNormalized to micromoles of CYP27A1.

^clog*P*, Octanol-water partition coefficient.

^dNSR, no spectral response; the response was <0.009 absorbance units when 0.4 μM CYP27A1 was titrated up to an 18 μM drug.

^eExperimental values.

^fTheoretical values.

Only strong CYP27A1 inhibitors bicalutamide and dexmedetomidine are of unclear relevance for use in breast cancer. Bicalutamide is the anti prostate cancer drug contraindicated for women (Cockshott, 2004). Dexmedetomidine is not gender-specific, but is used for a short time only to sedate patients in intensive care units. Both drugs and other CYP27A1

inhibitors are of value for analysis of chemical structures that interact with CYP27A1 (shown in orange in Fig. 1). These structures share at least two common features. One is conformational flexibility, a feature indicated by crystal structures of bicalutamide cocomplexes with the androgen receptor and CYP46A1 (Bohl et al., 2005; Mast et al., 2013c),

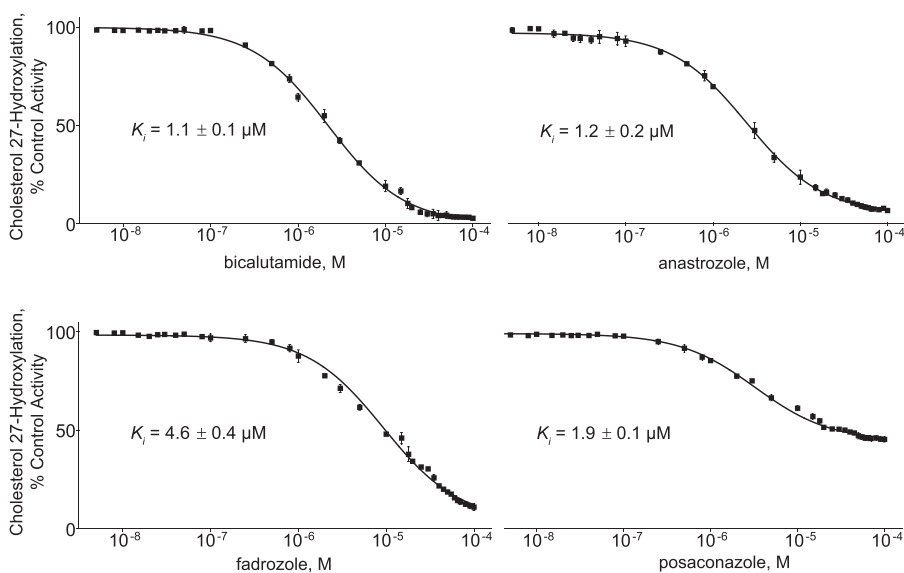


Fig. 4. The IC₅₀ plots for the inhibition of cholesterol 27-hydroxylation by bicalutamide, anastrozole, fadrozole, and posaconazole. Assay conditions are described in *Materials and Methods*. The results represent mean ± S.D. of three independent measurements.

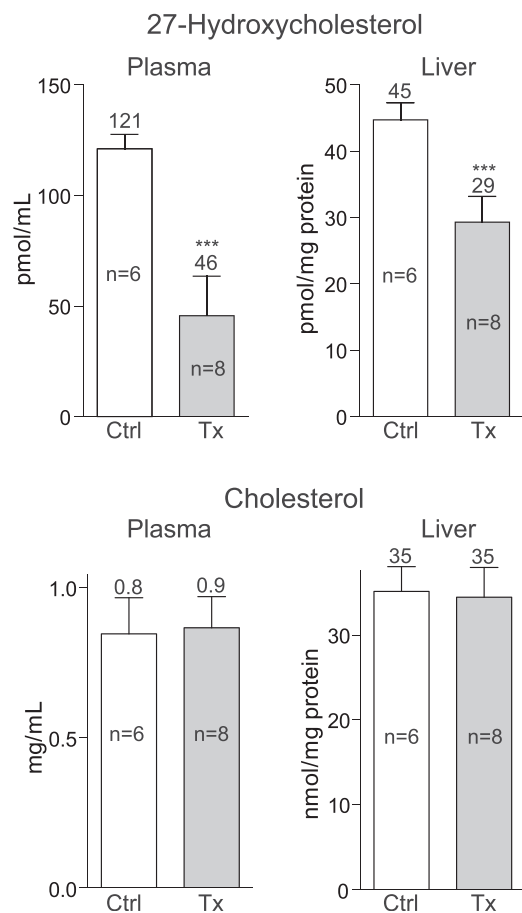


Fig. 5. Effect of anastrozole treatment on the levels of 27HC and cholesterol in mouse plasma and liver. Anastrozole treatment is described in *Materials and Methods*. The results represent mean \pm S.D. of the measurements in individual animals. Ctrl, control (vehicle-treated animals); Tx, anastrozole-treated mice. *** $P < 0.001$.

and posaconazole cocomplexes with CYP46A1 and CYP51s from different pathogenic species (Chen et al., 2010; Lepsheva et al., 2010; Mast et al., 2013b). These crystal structures reveal protein-specific drug conformations and demonstrate how bicalutamide and posaconazole can adopt different conformations and fit the shape of the active site of very different proteins. The same likely is true for anastrozole, dexmedetomidine, and ravuconazole, whose primary targets are CYP19A1, α -adrenergic receptors, and CYP51s from invading pathogens, respectively. Only fadrozole has a rigid structure, but this drug is small and should be easily accommodated in the active site of CYP27A1, which binds such large substrates as cholesterol.

Another feature that likely underlies unanticipated drug interactions with CYP27A1 is the presence of unhindered functionalities that enable the formation of a coordinate bond with the P450 heme iron. All top eight CYP27A1 inhibitors have these functionalities (Fig. 3), but they seemed to interact differently with the P450 heme iron, as indicated by spectral studies (Fig. 3). Only dexmedetomidine elicited a classic type II response in both absolute and difference CYP27A1 spectra, suggesting direct coordination of the P450 heme iron with one of its imidazole nitrogens. Fadrozole and thioperamide induced only partial Soret peak shifts, suggesting that their nitrogens coordinate the heme iron indirectly, probably via

the bridging water molecule, as was found in the P450, whose absolute spectrum underwent a partial drug-induced red shift (Seward et al., 2006). A lack of any Soret peak shifts in the absolute spectra of anastrozole- and ravuconazole-bound CYP27A1, but a type II response in their difference spectra, is an intriguing new finding that has not yet been reported. Both drugs have the cyano group(s), which we hypothesize could coordinate the heme iron. Crystallographic studies are required (underway in this laboratory) to establish unambiguously the molecular basis of this type of spectral response. Similarly, crystallographic studies are required to understand the reason of a rare reverse type I spectral response elicited in CYP27A1 by bicalutamide and posaconazole. We envision that a fluorine atom in the terminal phenyl ring of bicalutamide or posaconazole (Fig. 1) comes into close proximity to the aqua heme ligand and, by affecting the properties of this water molecule, elicits the spectral shift in CYP27A1. A combination of conformational flexibility and unhindered functionalities is not uncommon among marketed drugs. Hence, more medications likely have unanticipated off-target inhibition of CYP27A1, and a strategy for their identification could include a search of the common structural features found in the strong CYP27A1 inhibitors.

Another strategy for the identification of CYP27A1 inhibitors among marketed pharmaceuticals could be screening of drugs designed to bind to the primary targets of the identified CYP27A1 inhibitors (the androgen and α_2 -adrenergic receptors as well as CYP19A1 and CYP51). In this study, we tested CYP19A1 (aromatase) inhibitors and found common inhibiting drugs—anastrozole and fadrozole. We also found common inhibiting drugs with CYP51—ravuconazole and posaconazole. The next step is to screen modulators of the androgen and α_2 -adrenergic receptors that can be administered long-term to women. Thus, if CYP27A1 is determined to be a new therapeutic target for breast cancer, medications are already on the market that inhibit CYP27A1, with more medications likely to be found, if additional screenings are performed.

In summary, we screened 26 marketed drugs and identified 8 with substantial in vitro inhibition of CYP27A1 in the enzyme assay. We conducted additional in vitro and in vivo drug characterizations that suggested that 6 of the investigated drugs have potential to inhibit CYP27A1 in humans. Our data demonstrate that CYP27A1 is a druggable target and that the aromatase inhibitor anastrozole may have an unanticipated inhibition of CYP27A1. The present work encourages evaluations of posaconazole as an antibreast cancer agent and proposes strategies for identification of additional CYP27A1 inhibitors among marketed drugs.

Authorship Contributions

Participated in research design: Mast, Pikuleva.

Conducted experiments: Mast, Lin.

Performed data analysis: Mast, Lin, Pikuleva.

Wrote or contributed to the writing of the manuscript: Mast, Lin, Pikuleva.

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