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A Suite of Activity-Based Probes for Human Cytochrome P450 Enzymes

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Abstract

Cytochrome P450 (P450) enzymes regulate a variety of endogenous signaling molecules and play central roles in the metabolism of xenobiotics and drugs. We recently showed that an aryl alkyne serves as an effective activity-based probe for profiling mouse liver microsomal P450s in vitro and in vivo. However, individual P450s display distinct substrate and inhibitor specificities, indicating that multiple probe structures may be required to achieve comprehensive coverage of this large and diverse enzyme family. Here, we have synthesized a suite of P450-directed, activity-based protein profiling (ABPP) probes that contain: 1) varied chemical architectures validated as mechanism-based inhibitors of the P450 enzyme family, and 2) terminal alkyne groups for click chemistry conjugation of reporter tags. This set of probes was screened against a wide cross-section of human P450s, leading to the discovery of an optimal set of probes that provide broad coverage of this enzyme family. We used these probes to profile the effects on P450 activity of aromatase inhibitors in current clinical use for the treatment of breast cancer. We describe the surprising discovery that one of these aromatase inhibitors, anastrozole, significantly increases probe-labeling of P450 1A2, indicative of a heterotypic cooperativity effect on a central P450 isozyme involved in metabolizing numerous drugs and xenobiotics. The results presented herein greatly expand the suite of ABPP probes for profiling P450s and illuminate new applications for these tools to understand P450-drug interactions.

Introduction

The human genome encodes 57 putatively functional cytochrome P450 (P450) enzymes,[1] which are membrane-bound monooxygenases that catalyze the metabolism of a large and diverse number of xenobiotics, drugs, and endogenous signaling molecules.[2] Despite sharing a conserved structural core with an active site heme group, these enzymes are remarkably diverse, exhibiting as little as 16% sequence homology.[3] P450s catalyze a multitude of reactions, including C-hydroxylation, heteroatom oxygenation, dealkylation, epoxide formation, group migration, and bond scission.[4] Several P450 subfamilies have dynamic active site architectures that permit broad substrate specificity[5] and account for their participation in catalyzing the metabolism of 90% of all pharmaceuticals. Other P450 subfamilies are involved in the regulation of endogenous molecules such as bioactive sterols, fatty acids, and eicosanoids. The widespread expression and catalytic activity of the P450 family has implicated these enzymes in cellular toxicity and carcinogenicity, as well as certifying them as targets for inhibition and pro-drug activation.[6–9]

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Supporting Information Available. Probe syntheses and characterization. Gel analysis of an azido-variant of **1**, probe labeling gel profiles for the entire P450 panel; gel analysis of probe labeling of insect cell control proteomes, charts showing quantitative intensity of P450 probe labeling; and concentration dependence of P450 probe labeling. This material is available free of charge via the Internet at http://pubs.acs.org.

The expression, and over-expression, of P450s in tumor cells is well-recognized.[10–13] Cytochrome P450s are key enzymes in cancer formation and cancer treatment, mediating the metabolic activation of numerous precarcinogens and participating in the activation and inactivation of anticancer drugs. P450 1B1, a xenobiotic metabolizing enzyme, is over-expressed in a number of tumor types, and therapeutic strategies have been implemented to harness the catalytic reactivity of P450 1B1 to activate pro-drugs, and to determine the extent of precarcinogen activation.[14–15] Multiple successful strategies to inhibit P450s, including P450 19A1 (aromatase) to treat breast cancer and P450 17A1 to combat androgen-dependent prostate cancer, have also been implemented.[9,16–17] The catalytic activity of a number of human P450s remains unannotated, but several are expressed in tumors, suggesting a potentially broad role for this enzyme family in cancer.[18]

The study of P450 drug metabolism is a critical element of the pharmaceutical development process. *In vitro* systems, such as liver microsomes, hepatocyte cultures, and recombinant P450s are used to evaluate the effects of new pharmaceutical entities on P450 activity and potential drug-drug interactions. These efforts rely largely on the co-administration of drugs with P450 substrates and thus are only applicable to relatively well-characterized members of the P450 family. Moreover, *in vitro* assays may fail to account for myriad post-translational mechanisms that are known to regulate P450 activity in cells. Toward the goal of developing a general strategy to profile P450 activities in native biological systems, we generated a P450-directed activity-based protein profiling (ABPP) probe, **1** (referred to as 2EN-ABP in previous reference).[19] This probe covalently labels P450s in an activity-dependent manner, and these labeling events were detected by appending a fluorescent reporter group via “click chemistry” onto probe-P450 adducts.[19,20] Notably, **1** proved capable of profiling P450 activities in both native liver proteomes and living mice.[21–23]

Probe **1** was designed based on the scaffold of 2-ethynylnaphthalene, a mechanism-based P450 inhibitor that acts via P450-catalyzed oxidation of the aryl alkyne to a highly reactive ketene, which subsequently acylates nucleophilic residues within the P450 active site.[24–25] Although **1** labeled several P450 enzymes in mouse liver, it is unlikely that this agent can serve as a universal ABPP probe for all mammalian P450s, given the wide diversity of substrate selectivities displayed by these enzymes. To expand the coverage of P450s addressable by ABPP, we describe herein the synthesis and functional characterization of a suite of activity-based probes (ABPs) based on scaffolds of known mechanism-based inhibitors of P450s (Figure 1) that operate by one or more of three inhibition mechanisms (Figure 2). [24,26,28,29] We show that these probes have complementary reactivity profiles with a large set of human P450s and can be used to reveal unusual forms of drug-drug interactions that affect P450 activity.

Experimental Section

Materials/Characterization

Unless otherwise noted, all chemicals used for probe syntheses were purchased from Acros (Geel, Belgium), Alfa Aesar (Ward Hill, MA), or Sigma-Aldrich (St. Louis, MO). Tris(2-carboxyethyl) phosphine (TCEP) was purchased from Fluka (St. Louis, MO). The click chemistry ligand, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, was purchased from Sigma-Aldrich. Aromatase inhibitor, anastrozole, was purchased from Toronto Research Chemicals, Inc. (North York, Canada), and formestane was purchased from Sigma Aldrich. Probe **1**[19] and rhodamine-azide[29] were prepared as described previously. Solvents used for synthesis were obtained from a dry still system. NMR spectra were obtained on a Varian-Innova 400 MHz instrument. NMR chemical shifts are reported in ppm downfield relative to the internal solvent peak, and *J* values are in Hz. High resolution-mass spectrometry (HR-MS) was obtained at The Scripps Research Institute Mass Spectrometry Core and recorded on an

Agilent mass spectrometer using ESI-TOF (electrospray ionization – time of flight). Insect cell microsomes containing human P450 enzymes were purchased from BD (San Jose, CA) as BD Supersomes. These are recombinant cDNA-expressed P450s prepared from a baculovirus-infected insect cell system. The insect cell microsomes contain the necessary cytochrome P450 reductase. IC_{50} values were calculated using Prism (GraphPad Software, Inc., La Jolla, CA) after obtaining quantitative optical density data from SDS-PAGE gels. Quantitative optical density data was collected from SDS-PAGE gel images using ImageJ (NIH Freeware). Error values are calculated as standard error.

Synthesis and Characterization of P450-Directed ABPs

Details on the synthesis and analytical characterization of P450-directed ABPs is provide in the Supporting Information.

Proteome Labeling and Analysis

Catalytic activity of P450s is NADPH-dependent, therefore control samples were prepared without NADPH. BD Supersome microsomal proteomes (50 μL of 1.0 mg/mL protein in PBS) were treated with a probe from the suite (**1**–**9**) (20 μM; 0.5 μL of a stock solution in DMSO) in the presence or absence of NADPH (1 mM; 0.5 μL of a stock solution in PBS). Samples were incubated at 37 °C for 1 h. Following incubation, probe-labeled proteomes were treated with rhodamine-azide (100 μM; 6 mM stock solution in DMSO) followed by TCEP (0.5 mM; 25 mM stock in water) and ligand (100 μM; 1.7 mM stock in DMSO:t-butanol (1:4)). The samples were vortexed, and cycloaddition was initiated by the addition of $CuSO_4$ (1 mM; 50 mM stock in water). Samples were vortexed and left at rt in the dark for 1 h at which time 2× SDS-PAGE loading buffer (50 μL) was added. The samples were heated at 90 °C for 8 min, loaded onto SDS-PAGE gels (30 μL per well), and visualized by in-gel fluorescent scanning using a Hitachi FMBio Ile flatbed scanner (MiraiBio, Alameda, CA).

Profiling Inhibition of Probe 1 Labeling of P450 19A1 (Aromatase) by Anastrozole and Formestane

BD Supersome P450 19A1 microsomal proteome (50 μL; 1 mg/mL protein in PBS) was treated with an aromatase inhibitor (0–100 μM) and NADPH (1 mM). Samples were incubated at 37 °C for 15 min, after which **1** (20 μM) was added, and incubations proceeded at 37 °C for an additional 1 h. Click chemistry and visualization as described above.

Global P450 Inhibition Profiles of Aromatase Inhibitors

BD Supersome microsomal proteomes (50 μL of 1.0 mg/mL protein in PBS) were treated with an aromatase inhibitor (2×, 10×, 20×, and 40× the IC_{50} concentration for inhibition of P450 19A1) and NADPH (1 mM). Samples were incubated at 37 °C for 15 min, after which the strongest labeling probe for each P450 was added (20 μM), and incubations proceeded at 37 °C for an additional 1 h. Click chemistry and visualization as described above.

Results and Discussion

Probe Design and Synthesis

Click chemistry-compatible ABPs are composed of three general components: 1) a reactive group for targeting enzyme active sites, 2) a binding group for directing the probe to a particular enzyme family, and 3) a latent alkyne handle for click chemistry attachment of a reporter group to visualize binding events.[20,21] Aryl alkynes satisfy the first two criteria for many P450s, with the aromatic groups providing positive binding interactions with hydrophobic P450 active sites and the aryl-conjugated alkyne promoting mechanism-based enzyme inactivation via a highly reactive ketene intermediate (Figure 2A). In addition to probe **1**, we synthesized four new aryl

alkyne probes **2**,**3**,**4**, and **7**. An aliphatic linker group was added to each probe terminated by an alkyne for use as a click chemsitry handle to append reporter groups for protein detection and/or enrichment. Through development of a variant of **1** that possesses a linker group terminating in an azide, we have determined that the aryl-alkyne functional unit is likely the primary reactive moiety for inactivating P450s (see Supporting Figure 1).

Probe **2** (Scheme 1A) was developed to provide a smaller aryl-binding group compared to **1**. Beginning with commercially available methyl-4-iodobenzoate (**10**), the aryl alkyne moiety was installed via a Sonogashira coupling reaction to give **11** in moderate yield.[30] The trimethylsilane protecting group was removed and the methyl ester saponified in a single step with NaOH giving the free acid **12**.[31] The aliphatic alkynyl linker, 6-amino-1-hexyne, was coupled to the probe core using 1-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI) to give probe **2** in good overall yield. We also synthesized an analogue of probe **1**, probe **3**, where the aryl alkyne and linker groups were placed at the C1 and C2 positions on the naphthalene ring system, respectively. Following our prior synthesis of **1**, the methyl ester of 1-bromo-2-naphthoic acid was synthesized with a coupling reaction giving **14** (Scheme 1B).[32] As with all our aryl alkyne probes, the TMS-protected alkyne was installed onto the molecule with a Sonogashira reaction (**15**)[33] followed by one-pot deprotection and saponification giving the free acid **16**.[33] The click chemistry linker was installed with an HOBt/EDCI promoted coupling reaction to 6-amino-1-hexyne providing probe **3**. The biphenyl probe **4** was synthesized (Scheme 1C) in the same manner as **3**, starting with the esterification of **17**[34] to make **18**,[35] Sonogashira coupling to obtain **19**,[34] base saponification and deprotection of TMS to obtain the free acid **20**, and EDCI/HOBt coupling to the linker to obtain **4**. Probe **4** delivers a bi-aryl ring system with degrees of rotational freedom absent in the other multi-ring aryl alkyne ABPs.

Two aliphatic ABPs, **5** and **6**, were also synthesized. A prior report demonstrated that undec-10-ynoic acid is a potent NADPH-dependent inhibitor of P450s involved in ω-hydroxylation and ω-1-hydroxylation of lauric acid.[36] In these cases, inhibition appeared to be primarily through inhibitor-heme adduct formation. In addition to the mechanism of inhibition via a reactive ketene intermediate (Figure 2A), a second propynyl mechanism may also operate (Figure 2B). This inhibition involves the P450-catalyzed dihydroxylation of the carbon alpha to the alkyne group, followed by rearrangement to a Michael acceptor.[37,38] A nucleophilic amino acid residue can add into the Michael acceptor leading to inhibition by covalent adduction to the apo-P450. Probe **5** was synthesized by a facile coupling of undec-10-ynoic acid (**21**) to the click chemistry linker (Scheme 1D). It is possible that P450s could react with either terminal alkyne on the probe molecule. However, as will be shown later, significant P450 labeling differences were observed between ABPs **5** and **6**. Probe **6** was synthesized with a conjugated terminal alkyne to determine the effect on P450 labeling as compared to probe **5** (Scheme 1E). The synthesis commenced with a Claisen-like condensation yielding **23**,[39] followed by aqueous base saponification of the ester groups to give **24**,[39] and copper promoted mono-decarboxylation providing the free acid of the conjugated alkyne, **25**.[39] Probe **6** was obtained by coupling on the click chemistry linker.

Probe **7** was developed from 7-ethynylcoumarin (7EC), a known mechanism-based inhibitor of human P450 2B1.[40] Compound **26** was converted to the 7-trifluoromethylsulfonate coumarin **27**, followed by a Pd promoted coupling reaction to give the TMS protected 7-ethynylcoumarin ester **28** (Scheme 1F). Consistent with the other probe syntheses, the methyl ester was saponified and the TMS group cleaved with aqueous base giving **29**, followed by HOBt/EDCI coupling of the click chemistry handle to give probe **7** in good yield.

The inactivation of human P450s 2B6, 3A4, and 3A5, and rat P450 2B1 has been demonstrated by the oral contraceptive 17-α-ethynylestradiol (**30**, 17EE).[41–43] Reactive intermediates of

17EE were found to inactivate P450s in a NADPH-dependent mechanism-based manner by a combination of heme alkylation and apo-protein modification. We developed probe **8** as a variant of 17EE by performing a bimolecular nucleophilic substitution reaction with **30** and a tosylate variant of the click chemistry linker to give probe **8** (Scheme 1G).

The final probe in the suite, **9**, was synthesized from a furanocoumarin core, 8-hydroxypsoralen. Furanocoumarins, including 8-methoxypsoralen, are components of many foods and inhibit the metabolism of several xenobiotics. 8-methoxypsoralen is a potent NADPH-dependent mechanism-based inactivator of human P450s 2A6, 2A13, and 2B1.[44–46] The mechanism of inactivation is ascribed to the covalent adduction of the inhibitor to the apo-protein. To generate the furanocoumarin reactive intermediate, a P450 oxidizes the furan alkene to an epoxide, followed by subsequent nucleophilic attack to form the dihydrofuranocoumarin product (Figure 2C). The synthesis of probe **9** (Scheme 1H) commenced with nucleophilic substitution of methylbromoacetate by 8-hydroxypsoralen (**31**) yielding **32**.[47] The methyl ester was saponified with aqueous LiOH to give **33**,[48] followed by HOBt/EDCI coupling of the click chemistry linker yielding probe **9**.

Probe Labeling of Human Cytochrome P450s

Our initial studies with **1** had focused on profiling P450 targets in mouse tissues.[19] Although rodents are useful models for ascertaining P450 function, their P450 repertoires are quite distinct from that of humans; mice contain 102 putatively functional P450s compared to only 57 for humans.[40,49,50] These differences create complications in correlating function of P450s in humans and rodents. The evaluation of human P450 activity, particularly on new pharmaceutical entities, is therefore typically conducted with human hepatocytes or recombinantly expressed human P450s. Following this line of reasoning, we screened our suite of ABPs against a panel of 14 human P450s co-expressed with cytochrome P450 reductase in insect cell microsomes in the presence or absence of NADPH. Following a 1 h incubation, the proteomes were treated with rhodamine-azide under click chemistry conditions and resolved by SDS-PAGE. Activity-based labeling of P450 enzymes was defined as fluorescent signals observed in the 48–55 kDa region of the gel in reactions containing NADPH, but not in reactions lacking NADPH. No labeling was observed in control insect proteomes lacking recombinantly expressed human P450s (Supporting Figure 2).

The panel of enzymes contained the following human P450s: 1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 4A11, 4F2, and 19A1 (aromatase). Eight of these enzymes are directly involved in xenobiotic (pharmaceutical) metabolism – 1A1, 1A2, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 – being highly expressed in liver and responsible for the catalytic conversion of ~75% of all pharmaceutical compounds,[51,52] (P450 3A4 alone is involved in the catalytic metabolism of nearly 50% of known drugs[51]). These drug-metabolizing P450s are remarkably flexible and display broad substrate selectivity. Other enzymes analyzed include: P450 1B1, an extrahepatic P450 often over-expressed in cancer that is involved in estradiol and xenobiotic hydroxylation; P450 2A6, a primarily hepatic P450 involved in xenobiotic metabolism, most notably the mediation of nicotine's metabolic inactivation to cotinine;[53] P450 2J2, an epoxygenase predominantly expressed in liver and heart that metabolizes arachidonic acid to biologically active eicosanoids; P450s 4A11 and 4F2, which are expressed primarily in liver and kidney and catalyze fatty acid hydroxylation; and P450 19A1 (aromatase), an extrahepatic P450 required for estrogen biosynthesis (including conversion of testosterone to estradiol and androstenedione to estrone) and the progression of estrogen receptor positive [ER(+)] post-menopausal breast cancer.

The probe labeling profiles of recombinant insect cell-expressed human P450s were diverse, with some enzymes showing strong reactivity with many probes and others very restricted reactivity with a select number of probes (Figure 3). Intriguing structure-activity relationships

were evident from this library-versus-library analysis. For instance, the two aliphatic alkyne probes (probes **5** and **6**) showed very distinct labeling profiles, with the former probe reacting strongly with P450 1B1, but not P450 2D6, and the latter probe displaying the opposite profile. Both probes contain the same click chemistry linker chain, therefore these binding differences likely illustrate a preference for the long aliphatic chain of ABP **5** by P450 1B1, and a preference for the activated conjugated alkyne of **6** by P450 2D6. Similarly profound reactivity differences also occurred within the aryl alkyne subset of ABPs. Indeed, although most P450 enzymes were labeled by multiple probes, each enzyme showed a unique relative reactivity signature (e.g., compare profiles of four P450s shown in Figure 3; complete labeling profiles for all P450s can be found in Supporting Figure 3).

To more quantitatively assess differences in P450 reactivity profiles, we measured the intensity of each P450-probe labeling event by in-gel fluorescence scanning. To ensure that these measurements reflected activity-based labeling of P450s, signals were calculated as differences in fluorescent intensity of P450 bands in reactions with or without NADPH. Results of this analysis are presented in Figure 4 for the probe labeling profiles of the four P450s shown previously in Figure 3. A more complete presentation of labeling profiles for the P450 panel is provided as a heat-map in Figure 5, where the absolute (Figure 5A) and relative (Figure 5B) labeling signals are shown. The relative signals were determined by normalizing data for each P450 to the strongest labeling event for that enzyme. This normalization facilitates identification of the optimal activity-based probe for each P450, even in cases where relatively low overall signals were observed. Absent from this figure are the labeling profiles of four P450s (2A6, 2B6, 2E1, 4A11) that failed to show strong labeling with any members of the probe suite.

Evaluation of the quantitative labeling data revealed clear differences in probe-P450 reactivity profiles, with some enzymes showing strong preferential reactivity with a single probe (e.g., 2D6 with **6**; 2J2 with **1**) and other enzymes displaying broad reactivity with the probe library [e.g., 2C9, 3A4] (Figure 4 and Figure 5). A brief synopsis of key findings is provided below.

The P450 1A subfamily is critical to drug metabolism and the oxidative catabolism of polycylic and heterocyclic hydrocarbons, typically preferring planar substrates. As seen in the normalized heat map, the aryl alkyne ABPs have the most significant labeling of both P450 1A1 and 1A2. Like the P450 1A subfamily, P450 1B1 is able to activate a broad spectrum of chemical carcinogens, including polycyclic hydrocarbons and heterocyclic and aromatic amines. Interestingly, P450 1B1 showed a very broad probe labeling profiling, with every probe reacting to an appreciable degree with this P450. This finding supports the view that P450 1B1 is capable of activating many different types of carcinogenic compounds and may serve as a useful target for pro-drug activation,[54] particularly for tumor types that overexpress this P450.

Cytochrome P450 3A4 is the most abundant of all P450s in the human body. It has been proposed, and crystal structures and in silico studies have found, that P450 3A4 has an open active site and that reactions are influenced largely by the chemical lability of C-H bonds.[55] Additionally, P450 3A4 is known to exhibit non-Michaelis-Menten steady-state kinetics for many substrates, indicating multiple substrates binding simultaneously.[56] Evaluating our raw data shows that P450 3A4 reacts with most all members of the probe suite, supporting the known flexibility of this enzyme's active site.

Cytochrome P450s involved in fatty acid metabolism, 2J2 and 4F2, were labeled by both aryl and aliphatic alkyne ABPs. Interestingly, and somewhat counter intuitively, P450 2J2, an epoxygenase that oxidizes arachidonic acid, was most strongly labeled by **1**. In contrast, P450 4F2 was most strongly labeled by the fatty acid-derived probe **5**. But, **1** labeled P450 4F2 with the second greatest intensity. In both cases the aliphatic conjugated alkyne probe, **6**, displayed

minimal probe labeling, likely reflecting a preference for interacting with extended, hydrophobic small-molecules. Finally, P450 19A1, which is responsible for estrogen biosynthesis, was labeled moderately by **1** and the two aliphatic probes. The probe labeling profiles for P450s 2J2, 4A11, 4F2, and 19A1 were surprising. These are enzymes with rather specific biological substrates, typically regulating steroid or fatty acid metabolism. However, they all react with ABPs of varying chemical composition, quite distinct from their natural substrates. This highlights that the structural composition of endogenous substrates and non-natural inhibitors/probes can differ considerably for P450s.

A handful of P450s (2A6, 2E1, 4A11) failed to show significant labeling with any of the activity-based probes. The most characteristic and specific reaction of P450 2A6 is coumarin 7-hydroxylation. Additionally, 8-methoxypsoralen is a potent inhibitor of human P450 2A6.[45] In the design of **9** we substituted the 8-methoxy group with an ester bond to the click chemistry linker, which may have hindered probe entrance into the active site of P450 2A6. Though little is known about the P450 2E1 active site, several low molecular weight solvents are potent inhibitors. The only reported alkyne-bearing mechanism-based inhibitor is *tert*-butyl acetylene (for rabbit P450 2E1).[57] Other mechanism-based inhibitors include disulfiram and its primary metabolite diethyldithiocarbamate.[58] P450 4A11, which was weakly labeled by **7**, is involved in ω-hydroxylation of lauric, myristic, and palmitic acids. Surprisingly, neither of the aliphatic ABPs, **5** or **6**, labeled P450 4A11. It is possible that, in such instances where probes would be predicted to bind to P450s, labeling occurs predominantly on the heme molecule rather than on the protein, which would preclude detection by ABPP. Alternatively, we cannot exclude the possibility that these enzymatic preparations contained low levels of expression and/or activity of the corresponding P450s.

Collectively, these data indicate that individual P450 enzymes exhibit distinct probe-labeling signatures. These signatures should facilitate the deduction of P450 identities from cell or tissue proteome, especially in instances where insufficient proteomic material is available for mass spectrometry analysis. Preliminary studies where we have tested the concentration-dependence of P450 labeling suggest that labeling signatures can be further refined by adjusting the concentrations of individual probes. For instance, while P450 2B6 strong labeling with multiple probes tested at 20 μM, this enzyme exhibited selective reactivity with probe **2** tested at 2 μM (see Supporting Figure 5).

Evaluation of Aromatase Inhibitors

As demonstrated in our previous report,[19] P450 ABPs can be used to measure alterations in P450 activity due to chemical compounds that act as inhibitors and inducers. For many years estrogen-receptor positive (ER+) post-menopausal breast cancer was treated with tamoxifen, a selective estrogen receptor modulator. However, tamoxifen is an ER agonist in some tissues, which has led to increased incidence of other cancer types, such as endometrial cancer.[59] Addiitonally, resistance to tamoxifen therapy is a problem for many patients.[9] Aromatase inhibitors, which block the biosynthesis of estrogen, have therefore moved to a position of frontline therapy for ER+ breast cancer.[59] Aromatase inhibitors are separated into two classes, type I and II (Figure 6A). Type I inhibitors (formestane) are irreversible steroidal mechanism-based inhibitors; they are androgen substrate analogs that bind competitively but irreversibly to the enzyme active site. Type II inhibitors (anastrozole) are non-steroidal triazoles; they bind reversibly to the enzyme and orient within the aromatase active site such that the azole nitrogens interact with the Fe of the prosthetic heme group.[60]

Our quantitative profiling experiments identified **1** as the most potent probe for P450 19A1 (Figure 5). We therefore asked whether probe **1** could report on the binding of aromatase inhibitors to P450 19A1. Type I (formestane) and type II (anastrozole) aromatase inhibitors were independently added to insect cell expressed human P450 19A1, followed by addition of

1, and subsequent click chemistry to rhodamine-azide (Figure 6B). We determined an IC_{50} = 0.43 μM and 1.0 μM for formestane and anastrozole, respectively (Figure 6B). These values are in-line with those reported previously in the literature.[61] This data demonstrates that **1** can monitor the effects of inhibitory drugs on aromatase activity. We next considered whether we could identify the consequences of aromatase inhibitors on probe labeling of the other P450s in our panel.

Profiling P450 Inhibition by Aromatase (P450 19A1) Inhibitors

A major concern for all pharmaceuticals is their off-target effects. Aromatase inhibitors, both type I and II, are considered highly selective. There are no published reports of off-target changes to P450 activity due to formestane; however inhibition of P450s 1A2, 2C9, and 3A4 has been reported for anastrozole at concentrations of 8, 10, and 10 μM, respectively.[62] These inhibitory effects were determined by co-incubating human liver microsomes with anastrozole and P450-specific substrates. We treated each of the 14 human P450s in our panel with inhibitors at approximately 2×, 10×, 20×, and 40× the IC_{50} concentration of aromatase inhibition for the respective inhibitor, followed by probe labeling with the specific ABP that most strongly labeled each P450 at 20 μM (Figure 5), and visualization with click chemistry to rhodamine-azide.

Each aromatase inhibitor affected the probe labeling of two P450s (in addition to their effects on P450 19A1). Formestane decreased probe **5** labeling of P450 2C19 and **1** labeling of P450 3A4 (Figure 7A), while anastrozole resulted in inhibited labeling of P450 2C9 by probe **5** and, interestingly, increased labeling of P450 1A2 by probe **3** up to 175% of control values (Figure 7B). This last finding indicates that anastrozole and probe **3** may show heterotypic cooperativity with P450 1A2. Heterotypic cooperativity, where the metabolism of one substrate is increased in the presence of a second compound, is known to occur with many P450 isoforms and this form of drug-drug interaction can have a major impact on the rate of in vivo clearance for specific small-molecules.[63] Cooperativity of human P450 1A2 has previously been observed with polycyclic hydrocarbons.[64] The heterotropic cooperative effects associated with human P450 1A2 are ascribed to a more productive rearrangement of the substrate for P450 catalysis because of either steric packing or more specific P450-ligand interactions due to the presence of a second compound.[64] To our knowledge, these data provide the first evidence of heterotypic cooperativity for anastrozole and P450 1A2 (or any other P450) and suggest that this aromatase inhibitor could produce unanticipated drug-drug interactions due to activation of P450 1A2. Since previous studies have also reported an inhibitory effect of anastrozole on P450 1A2, we speculate that the (positive or negative) impact of this aromatase inhibitor on P450 1A2 activity will depend on the specific nature of the test substrate. More generally, our results underscore the versatility of ABPP for characterizing both increases and decreases in P450 activity that occur in response to drug treatment.

Conclusions

Here, we have synthesized and tested a new set of ABPP probes directed toward the P450 family of enzymes. These probes were all synthesized in ‘clickable’ form, empowering them with the potential to profile P450 enzymes in vitro and in vivo.[19] Screening of the probes against a panel of human P450 enzymes identified optimal probes for individual P450s. Promiscuous probes that target many P450s (e.g., probes **1–3** and **5**) could be readily distinguished from more selective probes that showed preferred labeling with individual P450s (e.g., probes **6–8**). Conversely, some P450s (e.g., 2C9, 2D6, and 3A4) exhibited broad reactivity with the suite of ABPP probes, likely reflecting malleable active site structures that can accommodate a range of small-molecules. Other P450s, however, showed highly restricted or, in certain cases, no reactivity with members of our probe set. We further demonstrated that

ABPP probes can detect not only inhibition, but also stimulation of P450 activities by small-molecules, uncovering a previously unappreciated activation of P450 1A2 by the anti-cancer drug anastrozole. Considering the central role that P450s play in metabolizing drugs, xenobiotics, and endogenous bioactive molecules, we anticipate that the ABPP probe suite described herein should prove of value for globally assessing P450 function in a wide range of biological systems. For instance, the probes could be used to profile P450s in human liver biopsies isolated from different disease conditions, as well as to characterize these enzymes and their drug interactions in living cells and animals. On this note, we have previously shown that probe 1 is capable of profiling P450 activities and drug interactions in living mice[19]. Future work may also include the optimization of probe selectivity for individual P450s, such that these enzymes can be independently monitored in primary human specimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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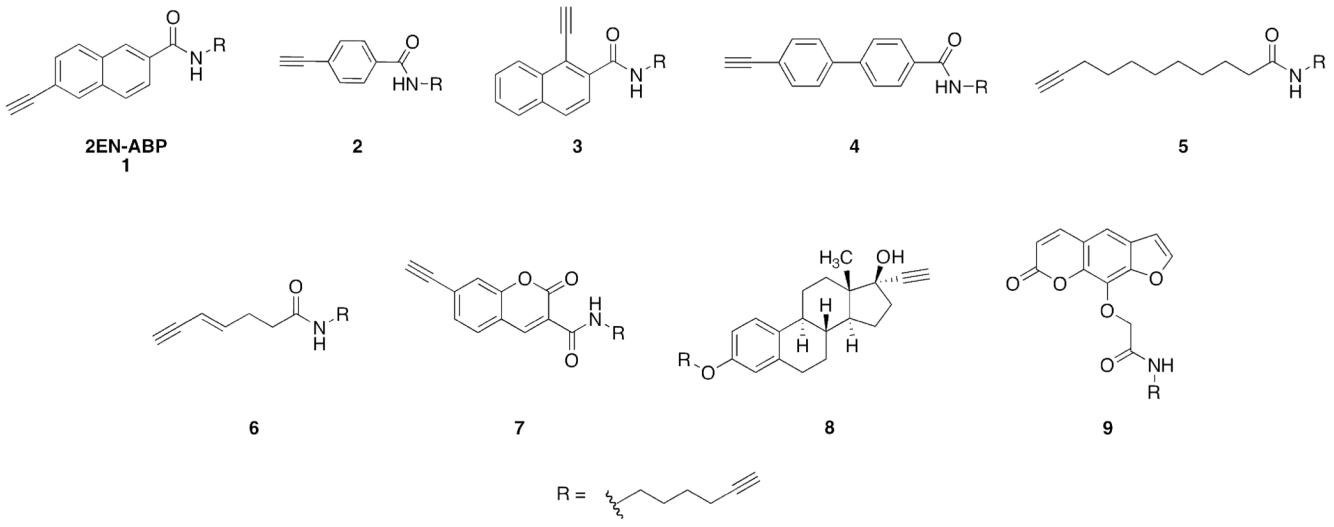


Figure 1.
Structures of P450-directed activity-based probes (ABPs).

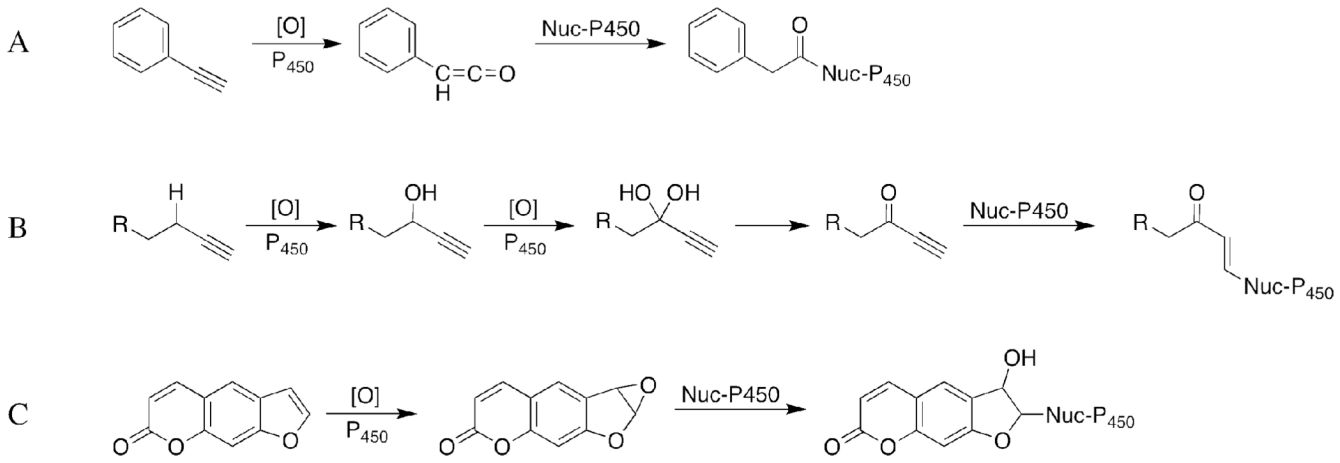


Figure 2.

Modes of mechanism-based inactivation of cytochrome P450 enzymes. (A) Aryl alkynes are oxidized to reactive ketene intermediates that inactivate P450s by covalent adduction to the apo-protein. (B) Propynyl-bearing inhibitors are oxidized to a reactive Michael acceptor and inactivate P450s by covalent adduction to the heme or apo-protein. (C) Furanocoumarin inhibitors are oxidized to the reactive furan epoxide and form covalent adducts to the P450 apo-protein.

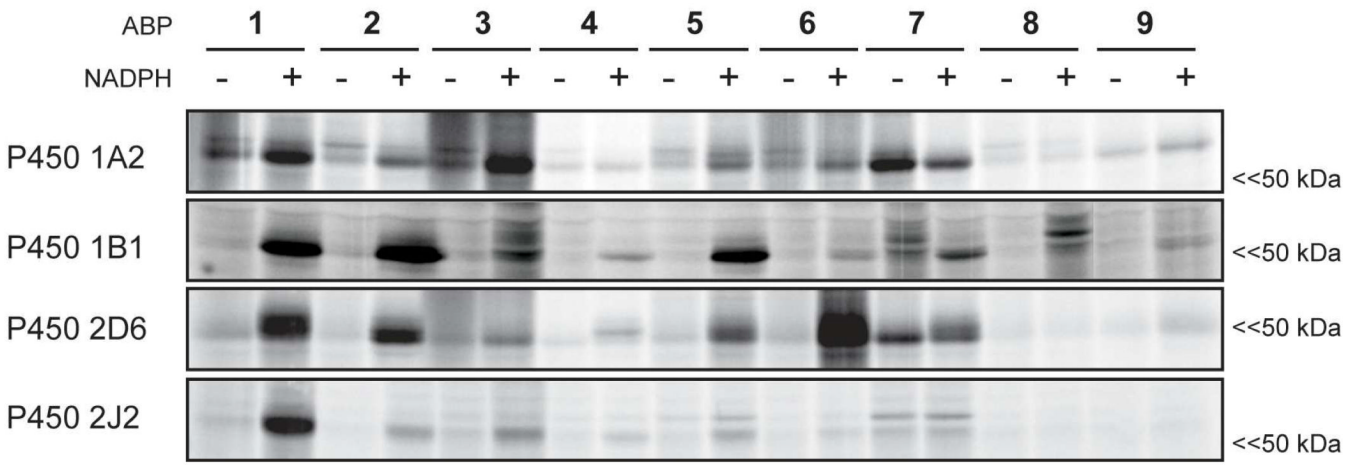


Figure 3.

Probe suite labeling of four representative members of the 14-member P450 panel. Full SDS-PAGE separation was performed, but only the 45–55 kDa region is shown, where NADPH-dependent probe-labeled proteins were detected (consistent with the molecular masses of P450 enzymes). See Supporting Figure 3 for labeling profiles of the entire 14-member P450 panel.

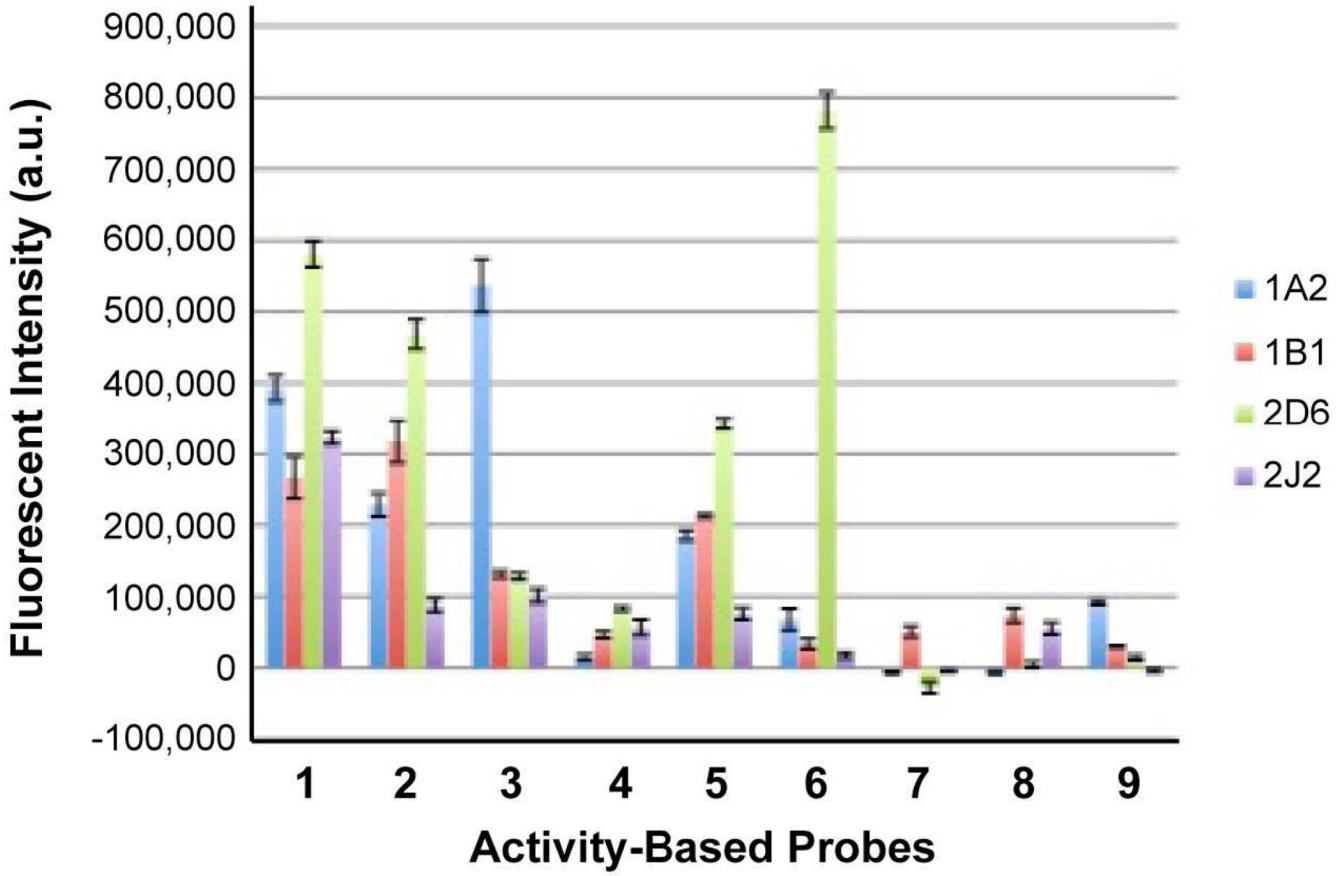


Figure 4.

Extracted quantitative data from SDS-PAGE gels of probe labeling, measured as fluorescent intensity (a.u. – arbitrary units). The reported fluorescent intensity values correspond to measurements of P450 bands observed in the gel lanes containing + NADPH samples minus the equivalent size regions of gel lanes containing – NADPH samples. These measurements were restricted to well-defined boundaries around the fluorescent band signals. Data represent the average ± standard error of three independent experiments per P450 for each ABP. See Supporting Figure 4 for quantitative analysis of labeling profiles for the entire 14-member P450 panel.

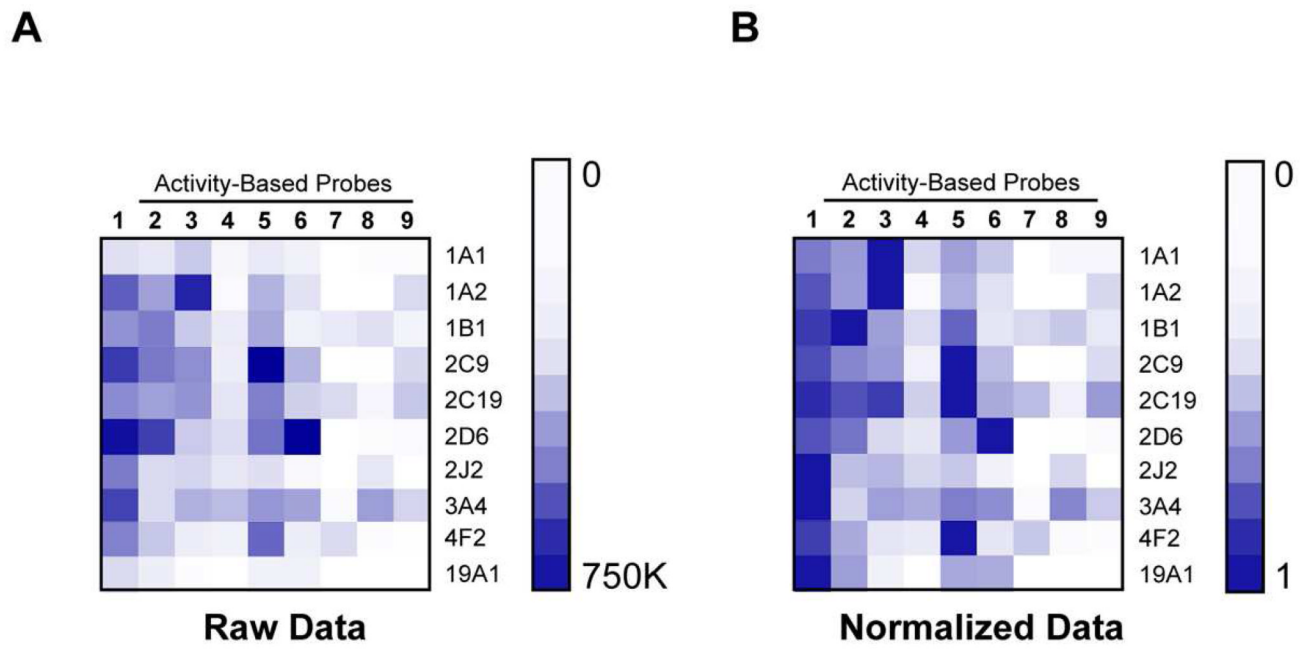


Figure 5.

Heat maps illustrating probe labeling profiles for individual human P450 enzymes. (A) Absolute fluorescence signals of probe labeling events. (B) Normalized fluorescence signals of probe labeling events, where data for each P450 enzyme is shown as a ratio of the strongest labeling signal for that enzyme. A ‘1’ as being the strongest binding event for an individual P450. Negative values in both the raw data and normalized data (reflecting less labeling in the presence versus absence of NADPH) have been assigned ‘0.’ Fluorescence intensity values are in arbitrary units and represent the mean of three independent experiments per P450.

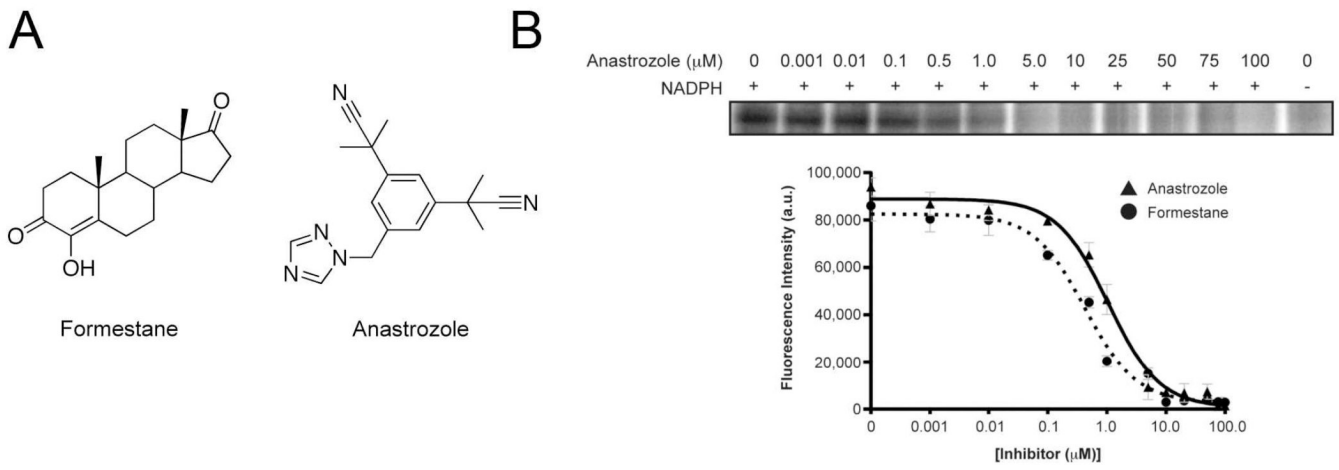


Figure 6.

Blockade of probe **1** labeling of P450 19A1 (aromatase) by small-molecule inhibitors. (A) Type I (steroidal, formestane) and type II (non-steroidal, anastrozole) aromatase inhibitors. (B) Treatment of insect cell proteomes expressing P450 19A1 with increasing concentrations of aromatase inhibitors (0–100 μM) resulted in concentration dependent blockade of **1** labeling. The IC_{50} curves for inhibition of **1** labeling of P450 19A1 by both inhibitors are shown. Data represent the average ± standard error of three independent experiments per inhibitor.

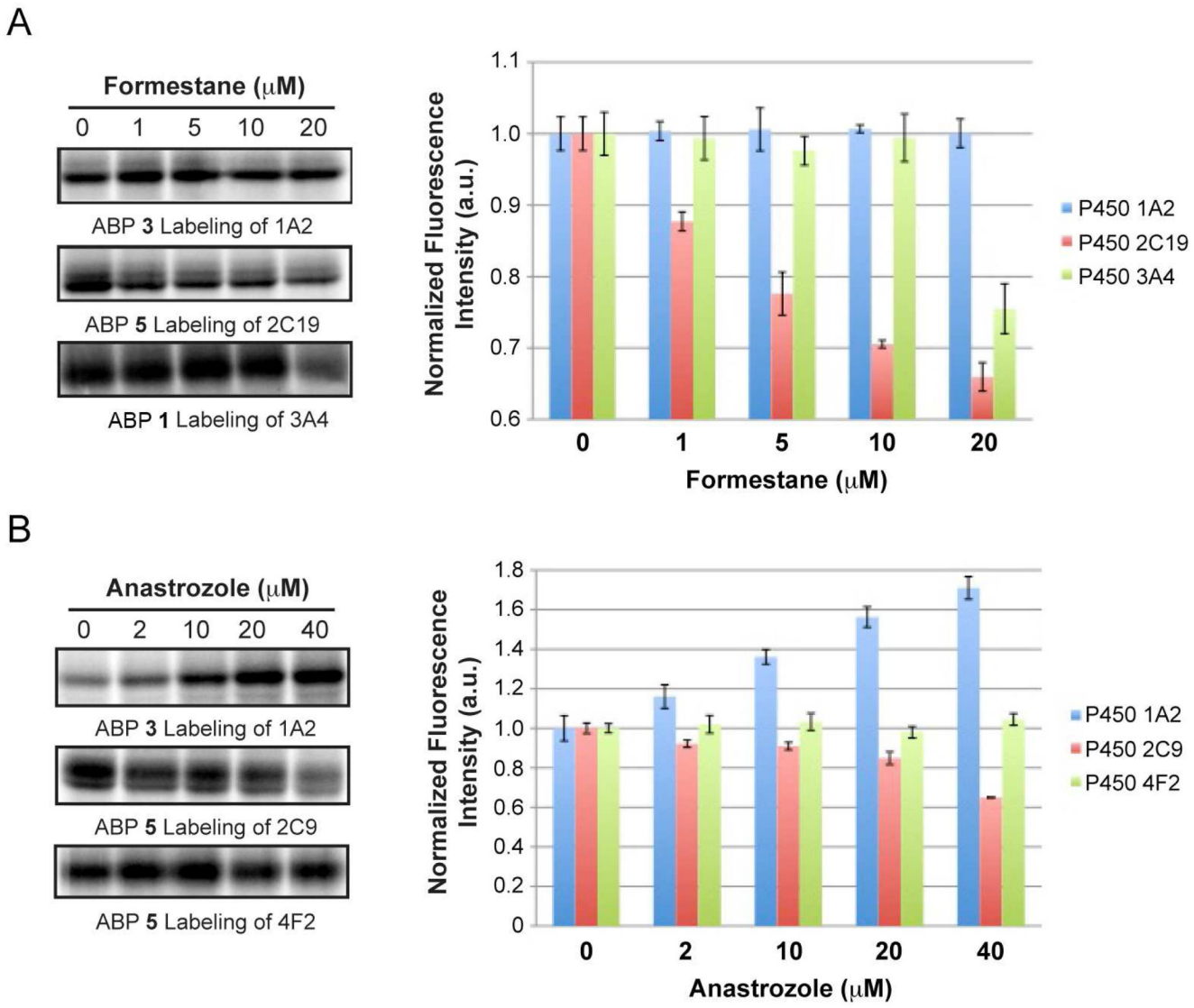
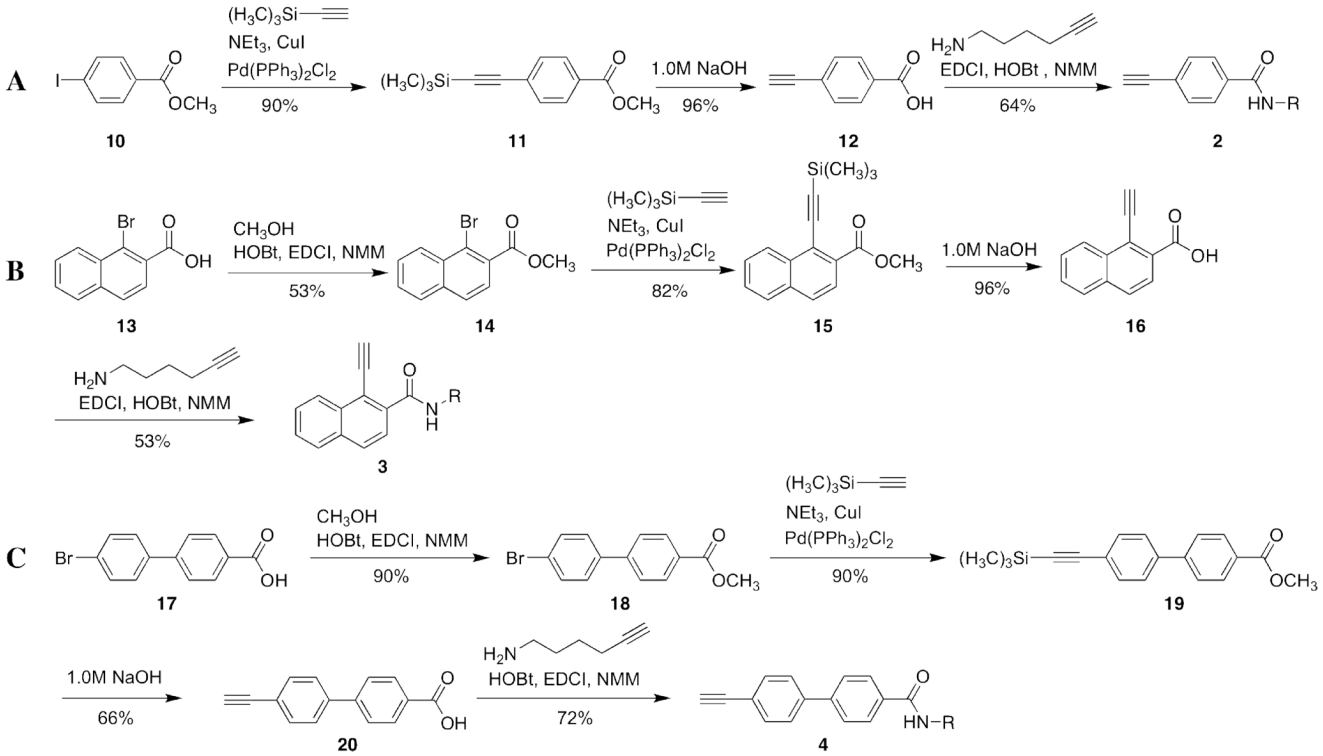
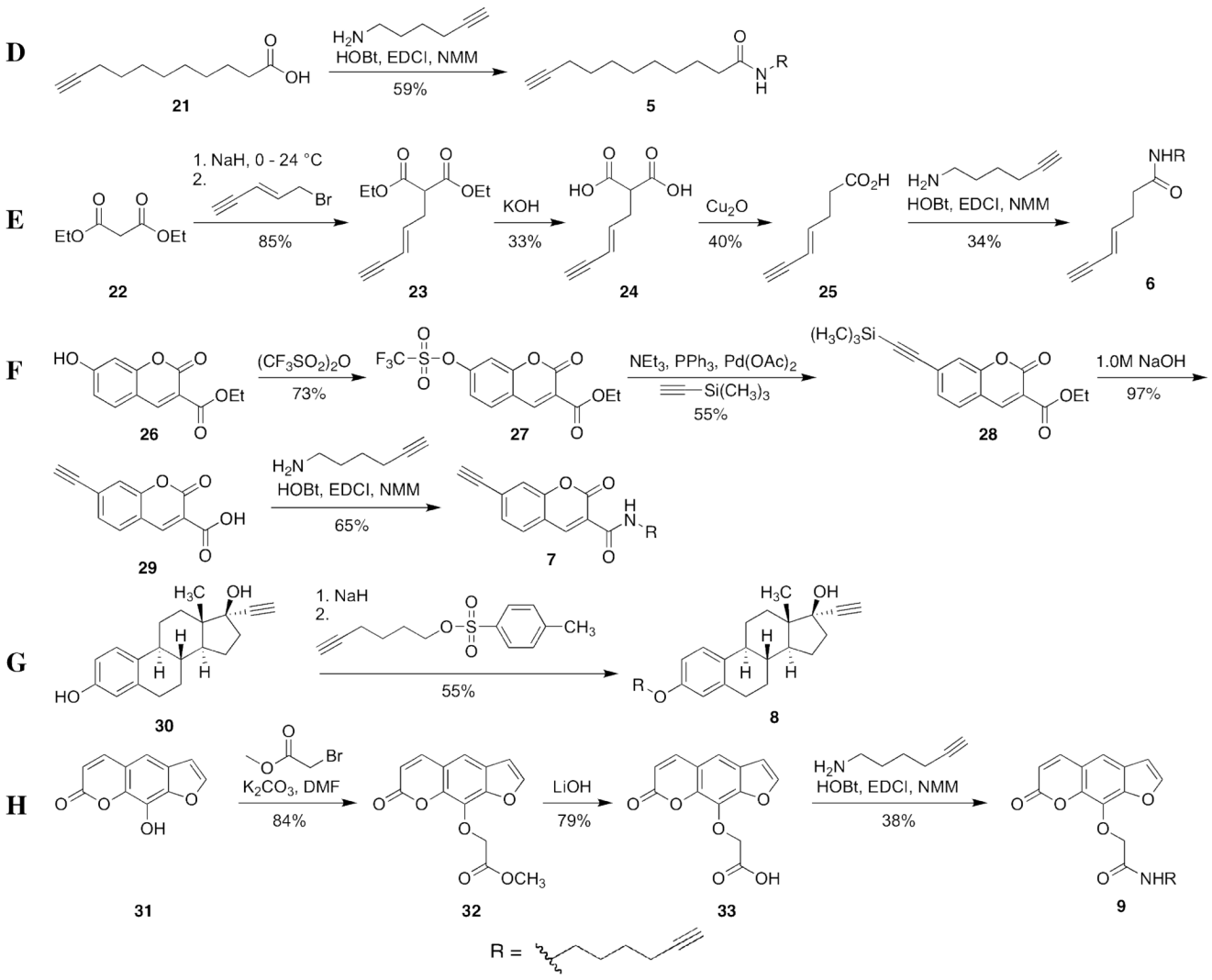


Figure 7.

Effects of aromatase inhibitors on P450 activity. (A) Treatment of human P450s with formestane (2×, 10×, 20×, and 40× the IC_{50} of aromatase inhibition), followed by probe labeling (20 μM), resulted in inhibition (2C19, 3A4) or unchanged (1A2) P450 activity. (B) Treatment of human P450s with anastrozole (2×, 10×, 20×, and 40× the IC_{50} of aromatase inhibition) resulted in increased (1A2), decreased (2C9) or unchanged (4F2) P450 activity. Quantitative values from the gels are normalized to the intensity value without inhibitor treatment. Data represent the average ± standard error of three independent experiments per inhibitor.





Scheme 1.
P450 ABP syntheses.