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Pyranoflavones: A Group of Small-molecule Probes for Exploring the Active Site Cavities of Cytochrome P450 Enzymes 1A1, 1A2, and 1B1

Jiawang Liu[†], Shannon F. Taylor[†], Patrick S. Dupart[†], Corey L. Arnold[†], Jayalakshmi Sridhar[†], Quan Jiang[†], Yuji Wang[‡], Elena V. Skripnikova[§], Ming Zhao[‡], and Maryam Foroozesh^{†,*}

[†]Department of Chemistry, Xavier University of Louisiana, New Orleans, Louisiana 70125, United States

[‡]College of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, PR China

[§]Cell and Molecular Biology Core, College of Pharmacy, Xavier University of Louisiana, New Orleans, Louisiana 70125, United States

Abstract

Selective inhibition of P450 enzymes is the key to block the conversion of environmental procarcinogens to their carcinogenic metabolites in both animals and humans. To discover highly potent and selective inhibitors of P450s 1A1, 1A2, and 1B1, as well as to investigate active site cavities of these enzymes, 14 novel flavone derivatives were prepared as chemical probes. Fluorimetric enzyme inhibition assays were used to determine the inhibitory activities of these probes towards P450s 1A1, 1A2, 1B1, 2A6, and 2B1. A highly selective P450 1B1 inhibitor, 5-hydroxy-4'-propargyloxyflavone (5H4'FPE) was discovered. Some tested compounds also showed selectivity between P450s 1A1 and 1A2. Alpha-naphthoflavone-like and 5-hydroxyflavone derivatives preferentially inhibited P450 1A2, while beta-naphthoflavone-like flavone derivatives showed selective inhibition of P450 1A1. On the basis of structural analysis, the active site cavity models of P450 enzymes 1A1 and 1A2 were generated, demonstrating a planar long strip cavity and a planar triangular cavity, respectively.

INTRODUCTION

Cytochrome P450s are a ubiquitous enzyme superfamily and play a predominant role in the metabolism and detoxification of endogenous and xenobiotic substances.^{1, 2} However, these versatile enzymes are also involved in the bioactivation of environmental pollutants leading to certain types of cancers.³ Many procarcinogens (such as polycyclic aromatic hydrocarbons) are metabolically activated by certain P450 enzymes into active intermediates which covalently bond to DNA and/or proteins to form DNA- and/or protein-adducts, resulting in DNA mutations and cancer formation.⁴⁻⁷ P450s 1A1, 1A2, and 1B1 are representative family I enzymes which carry out procarcinogen bioactivation reactions inducing subsequent mutagenesis and tumorigenesis.^{3, 4} Therefore, the development of potent and selective P450 enzyme inhibitors has attracted considerable attention over the

*To whom correspondence should be addressed Department of Chemistry Xavier University of Louisiana 1 Drexel Dr. New Orleans, LA 70125 Tel: 504-520-5078 Fax: 504-520-7942 mforooze@xula.edu.

Supporting Information Available: ¹H and ¹³C NMR spectra, elemental analysis results, and activity data of all the tested compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

years, and inhibiting family I P450 enzymes specifically has become an important cancer prevention target.⁸⁻¹⁶

The project reported here focuses on the development of selective inhibitors towards P450 family I enzymes, specifically P450s 1A1, 1A2, and 1B1.¹⁵⁻¹⁸ In our previous work, we obtained considerable information about P450 family I enzyme inhibitors belonging to various structural cores, and found that the inhibitors with flavone backbone possess very high potency, leading to follow-up flavone structure modification projects.^{18, 19} Due to the structural similarities of P450s 1A1, 1A2, and 1B1 (P450 1A1 shares 80% amino acid sequence identity with P450 1A2 and about 38% with P450 1B1),^{20, 21} highly potent and selective inhibitors of these enzymes have not yet been discovered.

In recent years, several research groups have been working on developing chemical probes containing a reactive moiety (such as an acetylenic group) in order to investigate the details of P450-ligand interactions.²²⁻²⁵ In this study, we aimed to explore the size and shape of P450 enzyme active site cavities by using chemical probes with a rigid and inert structure. The rigidity of the molecule gives fewer conformers for consideration and the lack of reactive functional groups provides a clear picture of the pure enzyme-ligand binding interactions. In brief, our strategy can be described as 1) selecting a structure core (flavone); 2) incorporating the rigid functional group(s) (pyranyl and 5-hydroxyl groups) into the core to obtain the chemical probes; 3) evaluating the inhibitory activities of the newly designed probes on various enzymes (P450s 1A1, 1A2, 1B1, 2A6, and 2B1); and 4) constructing the enzyme active site cavity models with the activity data obtained. Since the inhibitory activity of a rigid and inert probe positively correlates with the enzyme-probe affinity, valuable information about the shape of the active site cavity can be obtained by studying the most potent probes. As depicted in Figure 1, when probes A and B show high inhibition (high affinity) of the target enzyme, the shape of the enzyme's active site cavity can be generated based on the structures of these probes.

To investigate the differences among the active site cavities of P450s 1A1, 1A2, and 1B1, a series of α -naphthoflavone-like, β -naphthoflavone-like, and flavone C-ring extensional pyranoflavone derivatives were designed and synthesized. An additional 5-hydroxyl functional group was incorporated into some of the probes in order to verify the recent observation that the presence of a hydroxyl group in position 5 of flavone increases inhibitory activity towards P450 1A2.^{18, 26} Since the pyranoflavone probes have similar hydrophobic properties and electronic parameters, only the steric properties (molecular size and shape) need to be taken into consideration during the structure-activity analysis. This largely simplified the comparison of probes' 3D structures and accelerated the generation of compound models that best fit in the enzyme active sites.

RESULTS AND DISCUSSION

Synthetic Strategy for Pyranoflavones

In order to extend the flavone core, a propargyl moiety was used to construct a new six-membered ring adjacent to the flavone core through an etherification and an annulation reaction. (Scheme 1) The first reaction step, the formation of flavonyl propargyl ether from hydroxyflavone, has been well-documented.²⁶ In brief, the starting material hydroxyflavone is deprotonated by sodium hydride (NaH) before reacting with propargyl bromide to form the corresponding propargyl ether. The purpose of using a strong base (NaH) is to completely deprotonate the reactant, even in case of the hydroxyl group of 5-hydroxyflavone. The second reaction step is a Claisen rearrangement followed by a nucleophilic addition.^{27, 28} The propargyl ether undergoes a Claisen rearrangement to form an unstable cumulated diene intermediate,²⁸ leading to the ring closure product,

pyranoflavone. For each mono-hydroxy-substituted flavone, only one major thermal ring closure product was observed.

The starting materials in this synthetic route were 5-hydroxyflavone (5HF), 6-hydroxyflavone (6HF), 7-hydroxyflavone (7HF), 2'-hydroxyflavone (2'HF), 3'-hydroxyflavone (3'HF), and 4'-hydroxyflavone (4'HF). Six flavone propargyl ether (5FPE, 6FPE, 7FPE, 2'FPE, 3'FPE, and 4'FPE) intermediates and six pyranoflavone products were synthesized through the two facile reactions, respectively. Reagents and conditions: a) Propargyl bromide, NaH in dimethyl sulfoxide (DMSO) (25 °C); b) *N,N*-Diethylaniline, reflux.

Mono-etherification of Dihydroxyflavones 5,7dHF, 5,3'dHF, and 5,4'dHF

Since the 5-hydroxyl hydrogen in 5-hydroxyflavone forms an intramolecular H-bond with the oxygen of the carbonyl group at position 4, this compound is less polar and more difficult to deprotonate compared to the other hydroxyflavones. 5-hydroxyflavone also selectively inhibits P450 1A2 over P450 1A1 (about 10-time more selective).^{18, 26} In order to keep the 5-hydroxyl group intact in the dihydroxyflavone derivatives, mono-etherification was performed. Due to the intramolecular H-bond of the 5-hydroxy and 4-position carbonyl groups, using a mild base (K₂CO₃) deprotonated the other hydroxyl group on the flavone without affecting the hydroxyl group at position 5 (Scheme 2, step a), thus selective etherification succeeded in high yields (64-80%).

5,7-Dihydroxyflavone, 5,3'-dihydroxyflavone, and 5,4'-dihydroxyflavone were the starting materials used in this synthetic route. To obtain the mono-etherified products (5H7FPE, 5H3'FPE, and 5H4'FPE), a mild basic environment was applied for the etherification reaction. Due to the introduction of the 5-hydroxyl group, the regioselectivity of cyclization reaction decreased. Reagents and conditions: a) Propargyl bromide, potassium carbonate in acetone (40 °C); b) *N,N*-Diethylaniline, reflux.

Synthesis of 5-Hydroxypyranoflavones

As shown in Scheme 2, through the aforementioned thermal ring closure reaction, 5-hydroxypropargyloxyflavones (5H7FPE, 5H3'FPE, and 5H4'FPE) were converted to 5-hydroxypyranoflavones in acceptable yields. However, the presence of the 5-hydroxyl group decreased the regioselectivity of Claisen rearrangement. Thus, multiple annulation products (like 5H76PF and 5H3'4'PF) were produced and used as chemical probes to investigate the active site cavities of P450 enzymes.

Regioselectivity of Claisen Rearrangement Reaction on the Flavone Core

As mentioned above, the regioselectivity of Claisen rearrangement in an unsymmetrical system is an important concern. In recent years, the Claisen rearrangement of allyl aryl ethers has been investigated through synthetic experiment data, NMR techniques, and computational calculations.^{29, 30} Generally, an electron-withdrawing environment, a favored reactant conformation, and low product energy are beneficial to this reaction. Our work here was focused on the Claisen rearrangement of aryl propargyl ethers, and interestingly we have found that the higher chemical shift value (in the proton NMR spectrum) of the corresponding hydrogen in a reactant correlates with the product formed. For instance, 6- and 8-positions are the theoretical reaction sites for the Claisen rearrangement of 7-flavonyl propargyl ether (7FPE). However since the chemical shift value of H-8 is higher than that of H-6, the 7,8-pyranoflavone was observed to be the predominant product. This general rule was observed for all of the Claisen rearrangement reactions performed for this project. (Table 1) The chemical shift of a hydrogen reflects the surrounding chemical environment, and a high chemical shift correlates to an electron-withdrawing environment. Therefore, our

results support the conclusion that electron-withdrawing environments favor Claisen rearrangement.²⁹ We plan to collect more data in relation to the prediction of Claisen rearrangement products (such as favorable reactant conformations) and report them in a separated publication.

Determination of 5-Hydroxy-7,8-pyranoflavone and 5-Hydroxy-7,6-pyranoflavone by NOE Experiments

The decrease of regioselectivity accompanied with the introduction of the 5-hydroxyl group gives us opportunities to create miscellaneous small molecule probes, however, some of these compounds are isomeric and hard to analyze by regular methods. For example, the Claisen rearrangement of 5-hydroxy-7-propargyloxyflavone has poor regioselectivity and produces two ring closure products, 5-hydroxy-7,8-pyranoflavone (5H78PF, major product) and 5-hydroxy-7,6-pyranoflavone (5H76PF, minor product). Due to the structural similarity of the two isomers, their normal 1D and 2D NMR spectra are almost identical (Figure 2). Therefore, 1D NOESY spectra were used to identify the structures of these two isomers. Figure 2 shows the ¹H NMR spectra of 5H78PF and 5H76PF and the corresponding 1D NOESY spectra by selectively exciting H β and H-2', respectively. Through analyzing the steric relationship of H β and H-2', the structures of 5H78PF and 5H76PF were clearly elucidated.

Inhibitory Activities of the Chemical Probes towards P450s 1A1, 1A2, and 1B1

The inhibitory activities of the 14 chemical probes towards P450s 1A1, 1A2, and 1B1 were evaluated through the standard fluorimetric enzyme inhibition assays as described previously.^{9, 16, 31} Figure 3 shows the results observed for the inhibition of P450 1A1-dependent deethylation of resorufin ethyl ether, P450 1A2-dependent demethylation of resorufin methyl ether, and P450 1B1-dependent deethylation of resorufin ethyl ether activities. Chrysin (5,7-dihydroxyflavone, 57DHF), α -naphthoflavone (α NF), and β -naphthoflavone (β NF) were used as positive controls.^{10, 12, 18} The data is presented here as a volume chart, with various compounds on the horizontal axis and IC₅₀ values on the vertical axis.

P450s 1A1, 1A2, and 1B1 share a large number of substrates and inhibitors due to their structural similarities. Finding selective inhibitors for each of these enzymes is quite challenging. Based on our data, the compounds tested showed selectivity towards P450 1B1 over P450s 1A1 and 1A2. Especially, 5H4'FPE showed very high selectivity for P450 1B1 (120 times higher than shown for P450 1A1 and more than 250 times higher than observed for P450 1A2). As the data in Figure 3 shows, 0.1 μ M of 5H4'FPE inhibited the dealkylation activity of P450 1B1 by 50% without having any significant effect on P450s 1A1 and 1A2. This high selectivity makes 5H4'FPE an extremely useful compound in studying the structure and function of P450 1B1. The effect of 5H4'FPE on aryl hydrocarbon receptor (AhR) activation in yeast strain MYA-3637 was determined and the results are included in the Supporting Information.

For convenience, the ordinate axis was plotted on a logarithmic scale. The IC₅₀ values are represented as the mean $\pm$ SE μ M of three independent experiments. The IC₅₀ value of 5H4'FPE for 1A2 inhibition is higher than 20 μ M.

When studying the inhibitory activities of our chemical probes towards P450s 1A1 and 1A2, some selectivity was observed. C-ring extension did not increase the selectivity between P450s 1A1 and 1A2, while B-ring extension showed an obvious trend of selectivity. The data showed that the α -naphthoflavone-like derivatives, 7,8-pyranoflavone (78PF) and 5-hydroxy-7,8-pyranoflavone (5H78PF), have a high inhibitory activity for P450 1A2 (IC₅₀

values of 0.059 and 0.014 μM , respectively), and a relatively low inhibitory activity for P450 1A1 (IC_{50} values of 0.27 and 0.11 μM , respectively). In contrast, β -naphthoflavone-like derivatives, 5,6-pyranoflavone (56PF) and 6,5-pyranoflavone (65PF), showed higher inhibition of P450 1A1 (IC_{50} values of 0.32 and 0.15 μM , respectively) compared to P450 1A2 (IC_{50} values of 1.13 and 0.76 μM , respectively). Results of further investigation of these two types of inhibitors are presented below in the section titled "Ligand Models of P450s 1A1 and 1A2".

Investigation of the mechanism of inhibition showed that most of the target compounds are competitive inhibitors of these P450 enzymes. However, 5-hydroxy-3'-propargyloxyflavone (5H3'FPE) is a mechanism-based inhibitor of P450s 1A1 and 1A2. Kinetic calculations showed that 5H3'FPE reacts rapidly with P450 1A1 (Limiting k_{inact} 0.19 min^{-1} , K_{I} 0.13 μM), before inactivating the enzyme. The reaction rate between 5H3'FPE and P450 1A2 was relatively slow (Limiting k_{inact} 0.022 min^{-1} , K_{I} 0.071 μM), but still lead to irreversible inhibition of the enzyme. In our previous work, we have shown that the reactive site of 5H3'PF with P450 enzymes is the acetylenic group.

Inhibitory Activities of the Probes towards P450s 2A6 and 2B1

Generally, the tested chemical probes have good selectivity against P450s 2A6 and 2B1. Most of these compounds do not possess any activity towards these enzymes. However, the C-ring modified derivatives (2',3'-pyranoflavone, 3',2'-pyranoflavone, and 4',3'-pyranoflavone) have relatively low inhibitory activities towards the P450s 1A1 and 1A2, while showing a mild inhibition of P450 2B1 (IC_{50} s of 6.29 - 7.12 μM). This means that modification of flavone C-ring could increase the inhibitory activities on P450 2B1.

Ligand Models of P450s 1A1 and 1A2

The type I probes, α -naphthoflavone-like or 5-hydroxy flavone derivatives, showed selective inhibition towards P450 1A2 (Figure 4). This group of inhibitors included αNF , 78PF, 5H78PF, 5H7FPE, 57HF, and 5HF (the activity data for 5HF is cited from our recent paper).^{18, 26} 5H78PF showed the highest potency and notable selectivity for P450 1A2 (about 8 time higher than observed for P450 1A1). Due to the intramolecular hydrogen bond, the 5-hydroxyl group of flavone is not as polar as a regular phenol group, and the whole molecule exhibits relatively non-polar and hydrophobic properties similar to a regular flavone. Therefore, the introduction of 7,8-pyranyl and 5-hydroxyl groups mainly changes the molecular size and shape of the flavone molecule without much effect on the hydrophobic and electronic properties.

The type II chemical probes, β -naphthoflavone-like derivatives, showed selective inhibition of P450 1A1 (Figure 5). This group of inhibitors consisted of βNF , 56PF, 65PF, and 4'-propargyloxy- β -naphthoflavone ($\beta\text{NF4'PE}$) (data cited from a recent paper¹⁸). These compounds exhibit a stretched structural characteristic, and possess 5-14 times higher selectivity for P450 1A1 over P450 1A2. Especially, the longest molecule $\beta\text{NF4'PE}$ showed the highest potency and selectivity for P450 1A1.

Even though both 5H78PF and $\beta\text{NF4'PE}$ are planar hydrophobic molecules with a common flavone core, they show completely opposite selectivity for P450s 1A1 and 1A2. Thus, investigating the molecular shapes of 5H78PF and $\beta\text{NF4'PE}$ could disclose the differences between the active site cavities of P450s 1A1 and 1A2. The molecular surface images (wire mesh mode) of the two representatives, 5H78PF and $\beta\text{NF4'PE}$, were generated in UCSF Chimera 1.6.2. (UCSF San Francisco, CA) after energy minimization using the conjugate gradient method with CHARMM force field. P450 1A2-favored ligand (5H78PF) showed a planar heart-shape structure (with a 9.1 Å long side and a 7.0 Å short side) due to the

incorporation of a 7,8-pyranyl and a 5-hydroxy groups into the flavone core. While, P450 1A1-favored ligand (β NF4'PE) formed a fish fillet shape conformation with 15.8 Å in length and 4.6 Å in width. Thus, it can be concluded that P450 1A1 has a narrow and long cavity, and can accommodate a planar long strip molecule (with the maximum width of the strip less than that of P450 1A2). On the contrary, the P450 1A2 owns a compact active site cavity, and it can accommodate a triangular molecule. This active site cavity information will be the foundation of our future optimization. By employing the molecular probes, we have obtained critical structural characteristics of the active site cavities of P450s 1A1 and 1A2, while discovering selective inhibitors among the probes.

CONCLUSION

In order to explore the shape and dimensions of P450 active site cavities and further discover selective inhibitors of P450 enzymes, we carried out the design, synthesis, and evaluation of a group of small molecule probes. In this work, we focused on family I P450 enzymes 1A1, 1A2, and 1B1, and created a group of novel pyranoflavones as probes based on our previous studies. These probes showed varied inhibitory activities towards different P450 enzymes. Most tested compounds showed high inhibition of family I P450s, while showing weak or no inhibition activities towards P450s 2A6 and 2B1. The comparison of inhibition activities among P450s 1A1, 1A2, and 1B1 demonstrated that most of these probes possess higher inhibitory potential towards P450 1B1 compared to P450s 1A1 and 1A2. Especially, the compound 5H4FPE showed a 100 times higher selectivity towards P450 1B1 over 1A1, and no inhibition of P450 1A2. Even for the most similar P450 enzymes 1A1 and 1A2, some selectivity was observed with certain compounds. α -Naphthoflavone-like and 5-hydroxy-incorporating probes preferentially inhibited P450 1A2, while the β -naphthoflavone-like probes were selective towards P450 1A1. According to these and previous results, we have found remarkable differences in the preference for the ligand structures between the active site cavities of P450s 1A1 and 1A2, which share around 80% amino acid sequence identity. In summary, employing small molecule probes is a promising method to gain insight into the internal structure of P450 enzymes.

EXPERIMENTAL SECTION

Chemistry

The hydroxyflavone starting materials were purchased from INDOFINE Chemical Company, Inc (Hillsborough, NJ), and other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO) and Fisher Scientific International, Inc. (Hampton, NH). Mass spectral data were determined using an Agilent 6890 GC with a 5973 MS. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Fourier 300 MHz FT-NMR spectrometer. 1D NOE experiments were performed on a Bruker Avance III 400 MHz NMR spectrometer, and the data was processed with a MestReNova NMR software (School of Chemistry, University of Bristol, Bristol, UK). Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

General Procedure A: Synthesis of Flavonyl Propargyl Ethers (FPEs)

The synthetic procedure for flavonyl propargyl ethers has been well-described in our previous work.²⁶ Briefly, 500 mg (2.10 mmol) of the hydroxyflavone starting material was dissolved in 40 mL of dry dimethyl sulfoxide (DMSO) under nitrogen atmosphere, followed by the slow addition of 100 mg (2.52 mmol) of 60% sodium hydride (w/w) in mineral oil. After 5 min, 0.5 mL (4.50 mmol) of 80% propargyl bromide solution in toluene was added and the reaction mixture was left to stir under nitrogen atmosphere at room temperature for 24 h. An equal volume of water was then added and the mixture was left to stir for an

additional 12 h. The resulting precipitate was filtered, washed with water, and air-dried on a filter paper overnight. The crude product was purified by recrystallization from ethanol to give pure flavonyl propargyl ether as crystal with a yield of 72-80%. All analytical data for FPEs were reported in the aforementioned paper.

General Procedure B: Synthesis of Pyranoflavones (PFs)

In a reaction flask, 200 mg (0.72 mmol) of flavonyl propargyl ether was dissolved in 20 mL of N,N-diethylaniline. The reaction was refluxed under nitrogen atmosphere until the starting material completely disappeared on TLC (thin layer chromatography). The reaction solution was then cooled down to room temperature and 100 mL of dichloromethane (DCM) was added. The crude was washed successively with 5% hydrochloric acid (50 mL $\times$ 8), brine (50 mL $\times$ 2), saturated sodium bicarbonate (50 mL $\times$ 3), and brine (50 mL $\times$ 2). The DCM layer was then dried over anhydrous magnesium sulfate and concentrated under vacuum. The residue was purified by column chromatography using silica gel (solvent system varied based on the products) to provide pure pyranoflavone (Yield, 44 - 66%).

8-Phenylpyrano[2,3-*f*]chromen-10(2*H*)-one (5,6-pyranoflavone, 56PF)

Starting with 5-flavonyl propargyl ether, 122 mg (Yield, 62%) of 8-phenylpyrano[2,3-*f*]chromen-10(2*H*)-one (5,6-pyranoflavone, 56PF) was prepared by the General Procedure B. Column chromatography solvent system: DCM/methanol 20:1. mp 156-158°C. GC/MS: 276 (M^+ , 100%), 247 (8), 173 (30). 1H NMR ($CDCl_3$, 300 MHz) δ = 7.87 (m, 2H), 7.51 (m, 3H), 7.22 (d, J = 8.4 Hz, 1H), 7.02 (d, J = 8.4 Hz, 1H), 6.70 (s, 1H), 6.41 (d, J = 9.9 Hz, 1H), 5.80 (dt, J = 9.9 Hz, J = 3.6 Hz, 1H), 5.06 (dd, J = 3.3 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 178.12, 161.26, 157.21, 154.08, 131.40, 131.34, 131.21, 128.95, 126.02, 123.65, 120.97, 118.63, 114.07, 109.85, 108.54, 66.18. Anal. Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 77.77; H, 4.35.

3-Phenylpyrano[3,2-*f*]chromen-1(8*H*)-one (6,5-pyranoflavone, 65PF)

Starting with 6-flavonyl propargyl ether, 120 mg (Yield, 60%) of this product was prepared by the General Procedure B. Column chromatography solvent system: DCM. mp 142-144°C. GC/MS: 276 (M^+ , 100%), 247 (35), 174 (8). 1H NMR ($CDCl_3$, 300 MHz) δ = 8.12 (ddt, J = 10.2 Hz, J = 0.6 Hz, J = 1.8 Hz, 1H), 7.90 (m, 2H), 7.52 (m, 3H), 7.35 (d, J = 9.0 Hz, 1H), 7.16 (dd, J = 9.0 Hz, J = 0.6 Hz, 1H), 6.76 (s, 1H), 5.97 (dt, J = 10.2 Hz, J = 3.3 Hz, 1H), 4.81 (dd, J = 3.9 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 180.61, 161.99, 152.36, 151.28, 131.51, 131.48, 129.01, 126.17, 123.58, 123.04, 122.47, 120.56, 118.68, 118.29, 108.10, 64.85. Anal. Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 77.70; H, 4.41.

2-Phenylpyrano[2,3-*f*]chromen-4(8*H*)-one (7,8-pyranoflavone, 78PF)

Starting with 7-flavonyl propargyl ether, 130 mg (Yield, 65%) of this compound was prepared by the General Procedure B. Column chromatography solvent system: Petroleum ether/ethyl acetate 3:1. Mp 143-144°C. GC/MS: 276 (M^+ , 100%), 247 (13), 173 (40), 146 (18), 102 (18), 89 (20). 1H NMR ($CDCl_3$, 300 MHz) δ = 7.97 (d, J = 8.7 Hz, 1H), 7.87 (m, 2H), 7.53 (m, 3H), 7.00 (d, J = 10.2 Hz, 1H), 6.84 (d, J = 8.7 Hz, 1H), 6.78 (s, 1H), 5.90 (m, J = 10.2 Hz, 1H), 4.98 (dd, J = 3.6 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 177.87, 162.76, 158.53, 152.15, 131.89, 131.56, 129.11, 126.18, 126.12, 121.73, 118.08, 117.51, 114.61, 110.57, 107.29, 66.10. Anal. Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 78.13; H, 4.41.

2'H,4H-[2,8'-Bichromen]-4-one (2',3'-pyranoflavone, 2'3'PF)

Starting with 2'-flavonyl propargyl ether, 110 mg (Yield, 55%) of this product was prepared by the General Procedure B. Column chromatography solvent system: Petroleum ether/DCM 1:2. mp 132-134°C. GC/MS: 276 (M^+ , 100%), 247 (15), 155 (30), 121 (18). 1H NMR ($CDCl_3$, 300 MHz) δ = 8.20 (dd, J = 8.1 Hz, J = 1.5 Hz, 1H), 7.71 (dd, J = 7.8 Hz, J = 1.8 Hz, 1H), 7.66 (m, 1H), 7.50 (dd, J = 8.4 Hz, J = 0.6 Hz, 1H), 7.38 (m, 1H), 7.14 (s, 1H), 7.06 (dd, J = 7.5 Hz, J = 1.8 Hz, 1H), 6.96 (t, J = 7.8 Hz, J = 7.5 Hz, 1H), 6.44 (dt, J = 9.9 Hz, J = 1.8 Hz, 1H), 5.84 (dt, J = 9.9 Hz, J = 3.6 Hz, 1H), 4.93 (dd, J = 3.6 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 178.92, 160.34, 156.40, 152.89, 133.60, 129.50, 128.33, 125.58, 124.95, 123.75, 123.24, 122.42, 121.17, 119.57, 118.03, 112.43, 65.99. Anal. Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 78.16; H, 4.28.

2'H,4H-[2,5'-Bichromen]-4-one (3',2'-pyranoflavone, 3'2'PF)

Starting with 3'-flavonyl propargyl ether, 132 mg (Yield, 66%) of this compound was prepared by the General Procedure B. Column chromatography solvent system: DCM. mp 120-122°C. GC/MS: 276 (M^+ , 100%), 259 (15), 247 (28), 156 (60), 121 (35). 1H NMR ($CDCl_3$, 300 MHz) δ = 8.25 (dd, J = 7.8 Hz, J = 1.5 Hz, 1H), 7.70 (td, J = 7.2 Hz, J = 1.5, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.44 (t, J = 7.8 Hz, J = 7.2 Hz, 1H), 7.27~7.17 (m, 2H), 6.98 (d, J = 7.8 Hz, 1H), 6.68 (d, J = 10.2 Hz, 1H), 6.49 (s, 1H), 5.92 (dt, J = 10.2 Hz, J = 3.9 Hz, 1H), 4.83 (dd, J = 3.9 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 178.09, 163.91, 156.54, 154.93, 133.91, 130.07, 129.06, 125.80, 125.40, 123.81, 123.30, 122.17, 121.87, 120.90, 118.68, 118.17, 112.72, 64.74. Anal. Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 77.94; H, 4.23.

2'H,4H-[2,6'-bichromen]-4-one (4',3'-pyranoflavone, 4'3'PF)

Starting with 4'-flavonyl propargyl ether, 88 mg (Yield, 44%) of this compound was prepared by the General Procedure B. Column chromatography solvent system: DCM. mp 126-127°C. GC/MS: 275 ($[M-1]^+$, 100%), 247 (8), 155 (40), 124 (20). 1H NMR ($CDCl_3$, 300 MHz) δ = 8.21 (dd, J = 7.8 Hz, J = 1.5 Hz, 1H), 7.71~7.65 (m, 2H), 7.54 (dd, J = 8.4 Hz, J = 0.6 Hz, 1H), 7.48 (d, J = 2.4 Hz, 1H), 7.40 (td, J = 7.2 Hz, J = 1.2 Hz, 1H), 6.85 (d, J = 8.7 Hz, 1H), 6.74 (s, 1H), 6.47 (dt, J = 9.9 Hz, J = 1.8 Hz, 1H), 5.84 (dt, J = 9.9 Hz, J = 3.3 Hz, 1H), 4.94 (dd, J = 3.3 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 178.34, 163.39, 157.26, 156.16, 133.66, 127.64, 125.65, 125.16, 124.66, 124.47, 123.81, 123.70, 122.94, 122.35, 117.97, 116.38, 106.05, 66.23. Anal. Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 77.78; H, 4.45.

General Procedure C: Synthesis of 5-Hydroxypropargyloxyflavone (5HFPEs)

To a solution of 500 mg (1.97 mmol) of 5,7-dihydroxyflavone, 5,3'-dihydroxyflavone, or 5,4'-dihydroxyflavone in 40 mL of anhydrous acetone, 500 mg (3.62 mmol) of anhydrous potassium carbonate was added. When the reaction solution turned yellow, 0.5 mL (4.50 mmol) of 80% propargyl bromide solution in toluene was added. The reaction solution was heated to 50°C using a heating mantle for 2 h. Once the starting material spot disappeared on TLC, the reaction was cooled down to room temperature, passed through a silica pad, and then concentrated under vacuum to give the crude product. The pure product was obtained as crystals by recrystallization from anhydrous ethanol (Yield, 64-82%).

5-Hydroxy-2-phenyl-7-(propargyloxy)-4H-chromen-4-one (5-hydroxy-7-propargyloxyflavone, 5H7FPE)

Starting with 5,7-dihydroxyflavone, this compound was prepared by the General Procedure C (Yield, 73%). mp 154-155°C. GC/MS: 292 (M^+ , 100%), 263 (20), 207 (20), 189 (20). 1H NMR ($CDCl_3$, 300 MHz) δ = 12.64 (br, 1H), 7.80 (m, 2H), 7.49 (m, 3H), 6.59 (s, 1H), 6.50

(d, $J = 2.4$ Hz, 1H), 6.36 (d, $J = 2.4$ Hz, 1H), 4.69 (d, $J = 2.4$ Hz, 2H), 2.53 (t, $J = 2.4$ Hz, 1H). ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = 182.50, 164.14, 163.33, 162.17, 157.62, 131.93, 131.18, 129.11, 126.32, 106.18, 105.90, 98.94, 93.54, 77.41, 56.20$. Anal. Calcd for $\text{C}_{18}\text{H}_{12}\text{O}_4$: C, 73.97; H, 4.14. Found: C, 73.89; H, 4.00.

5-Hydroxy-2-(3-(propargyloxy)phenyl)-4H-chromen-4-one (5-hydroxy-3'-propargyloxyflavone, 5H3'FPE)

Starting with 5,3'-dihydroxyflavone, this compound was prepared by the General Procedure C, with a yield of 82%. mp 116-118°C. GC/MS: 292 (M^+ , 100%), 263 (18), 207 (45), 156 (40), 137 (30). ^1H NMR (CDCl_3 , 300 MHz) $\delta = 12.50$ (br, 1H), 7.53-7.37 (m, 4H), 7.12 (d, $J = 6.9$ Hz, 1H), 6.93 (d, $J = 8.1$ Hz, 1H), 6.76 (d, $J = 8.4$ Hz, 1H), 6.65 (s, 1H), 4.75 (d, $J = 2.1$ Hz, 2H), 2.59 (t, $J = 2.1$ Hz, 1H). ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = 183.48, 163.99, 160.71, 157.91, 156.29, 135.43, 132.49, 130.23, 119.59, 118.35, 113.00, 111.44, 110.80, 107.07, 106.23, 77.99, 76.29, 56.02$. Anal. Calcd for $\text{C}_{18}\text{H}_{12}\text{O}_4$: C, 73.97; H, 4.14. Found: C, 73.43; H, 4.38.

5-Hydroxy-2-(4-(propargyloxy)phenyl)-4H-chromen-4-one (5-hydroxy-4'-propargyloxyflavone, 5H4'FPE)

Starting with 5,4'-dihydroxyflavone (the starting material 5,4'-dihydroxyflavone purchased from INDOFINE Chemical Company, Inc was purified before use), this compound was prepared by the General Procedure C, with a yield of 64%. Decomposed before melting. GC/MS: 292 (M^+ , 100%), 155 (25). ^1H NMR (CDCl_3 , 300 MHz) $\delta = 12.64$ (s, 1H), 7.89 (m, 2H), 7.54 (t, $J = 8.4$ Hz, 1H), 7.11 (m, 2H), 6.98 (dd, $J = 8.4$ Hz, $J = 0.9$ Hz, 1H), 6.81 (dd, $J = 8.4$ Hz, $J = 0.9$ Hz, 1H), 6.67 (s, 1H), 4.79 (d, $J = 2.4$ Hz, 2H), 2.58 (t, $J = 2.4$ Hz, 1H). ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = 183.52, 164.39, 160.82, 160.56, 156.39, 135.26, 128.21, 124.33, 115.46, 111.41, 110.77, 106.98, 104.87, 55.94$. Anal. Calcd for $\text{C}_{18}\text{H}_{12}\text{O}_4$: C, 73.97; H, 4.14. Found: C, 73.60; H, 4.12.

Preparation of 5-Hydroxy-7,8-pyrano[4,3-f]chromen-4-one and 5-Hydroxy-7,6-pyrano[4,3-f]chromen-4-one

In a reaction flask, 200 mg (0.68 mmol) of 5-hydroxy-7-propargyloxyflavone was dissolved in 40 mL of *N,N*-diethylaniline. The reaction was refluxed under nitrogen atmosphere until the starting material completely disappeared on TLC. When the reaction solution was cooled down to room temperature, 150 mL of dichloromethane (DCM) was added. The solution was washed successively with 5% hydrochloric acid (50 mL $\times$ 10), brine (50 mL $\times$ 2), saturated sodium bicarbonate (50 mL $\times$ 3), and brine (50 mL $\times$ 2). The DCM layer was dried with anhydrous magnesium sulfate and concentrated under vacuum. The residue was purified by column chromatography on silica gel (petroleum ether/DCM 1:1) to provide 73 mg of 5-hydroxy-7,8-pyrano[4,3-f]chromen-4-one (Yield, 37%) and 20 mg of 5-hydroxy-7,6-pyrano[4,3-f]chromen-4-one (Yield, 10%) as yellow crystals, respectively.

5-Hydroxy-2-phenylpyrano[2,3-f]chromen-4(8H)-one (5-hydroxy-7,8-pyrano[4,3-f]chromen-4-one, 5H78PF)

mp 196-198°C. GC/MS: 292 (M^+ , 100%), 264 (15), 189 (25). ^1H NMR (CDCl_3 , 300 MHz) $\delta = 12.81$ (s, 1H), 7.86 (m, 2H), 7.55 (m, 3H), 6.88 (d, $J = 10.2$ Hz, 1H), 6.65 (s, 1H), 6.27 (s, 1H), 5.75 (dt, $J = 9.9$ Hz, $J = 3.6$ Hz, 1H), 4.93 (dd, $J = 3.3$ Hz, $J = 1.8$ Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = 182.53, 163.54, 161.82, 160.45, 151.79, 131.89, 131.32, 129.14, 126.16, 118.40, 117.11, 105.82, 105.72, 102.17, 99.89, 66.27$. Anal. Calcd for $\text{C}_{18}\text{H}_{12}\text{O}_4$: C, 73.97; H, 4.14. Found: C, 73.27; H, 4.31.

5-Hydroxy-2-phenylpyrano[3,2-*g*]chromen-4(8*H*)-one (5-hydroxy-7,6-pyranoflavone, 5H76PF)

mp 172-174°C. GC/MS: 292 (M^+ , 100%), 263 (40), 189 (18). 1H NMR ($CDCl_3$, 300 MHz) δ = 13.00 (s, 1H), 7.87 (m, 2H), 7.53 (m, 3H), 6.80 (dtd, J = 10.2 Hz, J = 1.8 Hz, J = 0.6 Hz, 1H), 6.65 (s, 1H), 6.40 (d, J = 0.9 Hz, 1H), 5.74 (dt, J = 10.2 Hz, J = 3.6 Hz, 1H), 4.93 (dd, J = 3.6 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 182.52, 163.85, 160.38, 157.14, 156.35, 131.83, 131.26, 129.09, 126.27, 118.96, 117.76, 106.36, 105.94, 105.78, 94.66, 66.39. Anal. Calcd for $C_{18}H_{12}O_4$: C, 73.97; H, 4.14. Found: C, 73.42; H, 4.34.

Preparation of 5-Hydroxy-3',2'-pyranoflavone and 5-Hydroxy-3',4'-pyranoflavone

In a reaction flask, 200 mg (0.68 mmol) of 5-hydroxy-3'-propargyloxyflavone was dissolved in 40 mL of *N,N*-diethylaniline. The reaction was refluxed under nitrogen atmosphere until the starting material completely disappeared on TLC. When the reaction solution was cooled down to room temperature, 150 mL of DCM was added. The solution was then washed successively with 5% hydrochloric acid (50 mL $\times$ 10), brine (50 mL $\times$ 2), saturated sodium bicarbonate (50 mL $\times$ 3), and brine (50 mL $\times$ 2). The DCM layer was dried with anhydrous magnesium sulfate and concentrated under vacuum. The residue was purified by column chromatography on silica gel (petroleum ether/DCM 1:1) to provide 136 mg of 5-hydroxy-3',2'-pyranoflavone (Yield, 68%) and 32 mg of 5-hydroxy-3',4'-pyranoflavone (Yield, 16%) as yellow crystals, respectively.

5-Hydroxy-2'*H*,4*H*-[2,5'-bichromen]-4-one (5-hydroxy-3',2'-pyranoflavone, 5H3'2'PF)

mp 161-163°C. GC/MS: 292 (M^+ , 100%), 263 (15), 156 (50), 137 (30). 1H NMR ($CDCl_3$, 300 MHz) δ = 12.52 (s, 1H), 7.54 (t, J = 8.4 Hz, 1H), 7.24 (t, J = 7.8 Hz, J = 6.3 Hz, 1H), 7.16 (d, J = 6.6 Hz, 1H), 6.96 (m, 2H), 6.83 (d, J = 8.1 Hz, 1H), 6.65 (d, J = 9.9 Hz, 1H), 6.40 (s, 1H), 5.93 (dt, J = 9.9 Hz, J = 3.9 Hz, 1H), 4.82 (dd, J = 3.6 Hz, J = 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 183.26, 165.12, 160.87, 156.72, 154.98, 135.53, 129.49, 129.12, 123.55, 121.97, 121.89, 120.93, 119.02, 111.57, 111.15, 110.75, 107.14, 64.74. Anal. Calcd for $C_{18}H_{12}O_4$: C, 73.97; H, 4.14. Found: C, 72.54; H, 4.36.

5-Hydroxy-2'*H*,4*H*-[2,7'-bichromen]-4-one (5-hydroxy-3',4'-pyranoflavone, 5H3'4'PF)

mp 166-167°C. GC/MS: 292 (M^+ , 100%). 1H NMR ($CDCl_3$, 300 MHz) δ = 12.55 (s, 1H), 7.50 (t, J = 8.4 Hz, 1H), 7.34 (dd, J = 7.8 Hz, J = 1.5 Hz, 1H), 7.22 (s, 1H), 7.00 (d, J = 7.8 Hz, 1H), 6.93 (d, J = 8.4 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 6.61 (s, 1H), 6.41 (d, J = 9.9 Hz, 1H), 5.87 (dt, J = 9.9 Hz, J = 3.6 Hz, 1H), 4.88 (dd, J = 3.0 Hz, J = 1.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 183.49, 163.87, 160.72, 156.29, 154.35, 135.34, 131.48, 126.96, 125.69, 124.72, 123.63, 119.49, 113.41, 111.36, 110.85, 107.03, 105.68, 65.89. Anal. Calcd for $C_{18}H_{12}O_4$: C, 73.97; H, 4.14. Found: C, 72.34; H, 4.15.

5-Hydroxy-2'*H*,4*H*-[2,6'-bichromen]-4-one (5-hydroxy-4',3'-pyranoflavone, 5H4'3'PF)

In a reaction flask, 200 mg (0.68 mmol) of 5-hydroxy-4'-propargyloxyflavone was dissolved in 40 mL of *N,N*-diethylaniline. The reaction was heated at 195°C under nitrogen atmosphere until the starting material completely disappeared on TLC (about 48 h). When the reaction solution was cooled down to room temperature, 150 mL of DCM was added. The solution was washed successively with 5% hydrochloric acid (50 mL $\times$ 10), brine (50 mL $\times$ 2), saturated sodium bicarbonate (50 mL $\times$ 3), and brine (50 mL $\times$ 2). The DCM layer was dried with anhydrous magnesium sulfate and concentrated under vacuum. The residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 3:1) followed by recrystallization with acetone to provide 86 mg of 5-hydroxy-4',3'-pyranoflavone (Yield, 43%) as colorless crystals. Decomposed higher than 180°C. GC/MS: 292 (M^+ , 100%), 155 (18). 1H NMR ($CDCl_3$, 300 MHz) δ = 12.66 (s, 1H), 7.66 (dd, J = 8.7

Hz, $J = 2.1$ Hz, 1H), 7.53 (t, $J = 8.7$ Hz, 1H), 7.48 (d, $J = 2.1$ Hz, 1H), 6.98 (d, $J = 8.1$ Hz, 1H), 6.86 (d, $J = 8.4$ Hz, 1H), 6.80 (d, $J = 8.1$ Hz, 1H), 6.62 (s, 1H), 6.48 (d, $J = 9.9$ Hz, 1H), 5.85 (dt, $J = 9.9$ Hz, $J = 3.3$ Hz, 1H), 4.96 (dd, $J = 3.0$ Hz, $J = 1.8$ Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = 183.47, 164.43, 160.80, 157.59, 156.35, 135.18, 127.81, 124.76, 123.95, 123.60, 123.05, 122.39, 116.47, 111.36, 110.76, 106.95, 104.56, 66.29$. Anal. Calcd for $\text{C}_{18}\text{H}_{12}\text{O}_4$: C, 73.97; H, 4.14. Found: C, 73.87; H, 4.24.

Fluorimetric Enzyme Inhibition Assays of P450s 1A1, 1A2, 1B1, 2A6, and 2B1

The inhibition activities of tested compounds on P450s 1A1, 1A2, 1B1, 2A6, and 2B1-dependent reactions were tested through standard methods as previously described.^{31, 32} These studies included P450 1A1-dependent deethylation of resorufin ethyl ether, P450 1A2-dependent demethylation of resorufin methyl ether, P450 1B1-dependent deethylation of resorufin ethyl ether, P450 2B1-dependent depropylation of resorufin propyl ether, and P450 2A6-dependent coumarin 7-hydroxylation assays. In brief, potassium phosphate buffer (1760 μL of a 0.1 M solution, pH 7.6) was placed in a 1.0 cm quartz cuvette, and 10 μL of a 1.0 M MgCl_2 solution, 10 μL of a 1.0 mM corresponding resorufin or coumarin substrate solution (final concentration of 5 μM) in dimethyl sulfoxide (DMSO), 10 μL of the microsomal P450 protein (final concentration of 5 nM), and 10 μL of an inhibitor solution in DMSO were added. For the controls, 10 μL of pure DMSO was added in place of the inhibitor solution. The reaction was initiated by the addition of 200 μL of a NADPH regenerating solution. The regenerating solution was prepared by combining 797 μL of a 0.10 M potassium phosphate buffer solution (pH 7.6), 67 μL of a 15 mM NADP^+ solution in buffer, 67 μL of a 67.5 mM glucose-6-phosphate solution in buffer, and 67 μL of a 45 mM MgCl_2 solution, and incubating the mixture for 5 minutes at 37°C before the addition of 3 units of glucose 6-phosphate dehydrogenase/mL and a final 5 minute incubation at 37°C. The final assay volume was 2.0 mL. The production of resorufin anion was monitored by a spectrofluorimeter (OLIS DM 45 Spectrofluorimetry System) at 530 nm excitation and 585 nm emission, with a slit width of 2 nm. The production of 7-hydroxycoumarin was monitored at 338 nm excitation and 458 nm emission, with a slit width of 2 nm. The reactions were performed at 37°C. For each inhibitor, a number of assay runs were performed using varying inhibitor concentrations (ranging from 0.1 to 100 μM). At least four concentrations of each inhibitor showing 20-80% inhibition were tested.

Data Analysis.³³

IC_{50} values—The initial data obtained from the above assays were a series of reaction progress curves (the time-course of product formation) in the presence of various inhibitor concentrations and in the absence of the inhibitor as the control. The Microsoft Excel Program was used to fit these data (fluorescence intensity vs. time) in order to obtain the parameters of the best-fit second-order curves ($y = ax^2 + bx + c$). The coefficient b in the above second-order equation represented enzymatic activity (v). Dixon plots were used (by plotting the reciprocals of the enzymatic activity ($1/v$) vs. inhibitor concentrations $[I]$) in order to determine IC_{50} values (x -intercepts) for the inhibitors. The results based on the first 6 minutes of enzymatic reaction are tabulated in Table S1 of Supporting Information. The data are represented as the mean $\pm$ SE μM of three independent experiments. Figure 3 shows the IC_{50} values for the tested compounds on P450s 1A1, 1A2, and 1B1 as a column chart. The data for P450s 2A6 and 2B1 are shown in Table 2 (in text).

K_i and limiting k_{inact} values—The first-order derivatives ($y = 2ax + b$) of the above second-order curves ($y = ax^2 + bx + c$) represent the enzymatic activity over time. The semilog plots of the percent relative activity ($Y = \log [(y/y_0) \cdot 100]$) versus time demonstrate the loss of enzymatic activity with time. The linear portions of the above semilog plots were used to determine $t_{1/2}$ ($0.693/k_{\text{inact}}$) values at various concentrations for the observed time-

dependent losses of activity. To obtain K_I and limiting k_{inact} , values of $1/k_{inact}$ were plotted versus reciprocals of the inhibitor concentration ($1/[I]$) (Kitz-Wilson plots). The limiting k_{inact} values were obtained from the abscissa intercepts of the plots, and the K_I values were calculated from the ordinate intercepts ($-1/K_I$). The K_I and limiting k_{inact} values of the mechanism-based inhibitor 5H7FPE against P450 1A1 and 1A2 are tabulated in Table S2 of Supporting Information, and also described in the text.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

NaH	sodium hydride
DMSO	dimethyl sulfoxide
DCM	dichloromethane
TLC	thin layer chromatography

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Figure 1.

A flow diagram for the determination of the enzyme's active site cavity shape using small molecule probes. This strategy includes four steps: 1) determination of an active structure core, 2) modification of the core with rigid functional group(s), 3) evaluation of the inhibitory activity of probes on the target enzyme, and 4) determination of the enzyme's active site cavity shape based on structural analysis of the most potent probes.

Figure 2.

The ^1H NMR and 1D NOESY spectra of 5H78PF (A) and 5H76PF (B) used for structural determination. The 1D NOESY spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer by exciting $\text{H}\beta$ and H-2' , respectively. The two-way arrows on the molecular structures indicate the NOE effects between the corresponding hydrogens.

Figure 3.
The IC₅₀ values of the chemical probes for inhibition of P450s 1A1, 1A2, and 1B1

Figure 4.
α-Naphthoflavone-like and 5-hydroxy flavone derivatives (Type I) and their IC₅₀ values for inhibition of P450s 1A1 and 1A2

Figure 5.
 β -Naphthoflavone-like derivatives (Type II) and their IC₅₀ values for inhibition of P450s 1A1 and 1A2.

Figure 6. The molecular surface images (wire mesh mode) of 5H78PF (A) and β NF4'PE (B) generated from UCSF Chimera. The size of the molecules were measured and labeled around them.

Scheme 1.
Synthesis of pyranoflavones from monohydroxyflavones

Scheme 2.
Synthesis of 5-hydroxypyranoflavones from dihydroxyflavones

Table 1¹H NMR chemical shift values of the aromatic hydrogens next to the propargyl group

Reactant	Structure	¹ H NMR Chemical Shift (ppm)					
		Position					
		5	6	7	8	2'	4'
6-flavonyl propargyl ether (6FPE)		7.65		7.31			
7-flavonyl propargyl ether (7FPE)			7.06		7.10		
3'-flavonyl propargyl ether (3'FPE)						7.55	7.16
5-hydroxy-7-propargyloxyflavone (5H7FPE)			6.36		6.50		
5-hydroxy-3'-propargyloxyflavone (5H3'FPE)						7.47	7.12

Table 2

The IC₅₀ values of tested chemical probes for the inhibition of P450s 2A6 and 2B1

	IC ₅₀ (μM)			IC ₅₀ (μM)	
	P450 2A6	P450 2B1		P450 2A6	P450 2B1
Chrysin	>25	>25	5H7FPE	>25	>25
aNF	>25	13.0±2.6	5H78PF	>25	>25
bNF	>25	>25	5H76PF	>25	>25
56PF	>25	>25	5H3'FPE	>25	4.08±0.48
65PF	>25	>25	5H3'2'PF	>25	>25
78PF	>25	>25	5H3'4'PF	>25	>25
2'3'PF	>25	6.29±0.59	5H4'FPE	>25	>25
3'2'PF	>25	7.12±0.62	5H4'3'PF	>25	>25
4'3'PF	>25	6.62±0.50			

The IC₅₀ values are represented as the mean ± SE μM of three independent experiments.