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Cytochrome P-450 2A6 Inhibitor Based on the Indole Moiety

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Abstract : The cytochrome P-450 enzymes (CYP 2A6) regulate many endogenous signaling molecules and drugs. Aryl alkynes such as 2-ethynylnaphthalene are important P450 inhibitors which have been extensively studied as medicines or as an effective chemical probes for profiling mouse liver microsomal P-450. Here we have synthesized indole-based novel P450 inhibitor, 5-ethynyl indole 3, and showed that it has successfully inhibited CYP 2A6 by chemical inhibition reaction. By using HPLC equipped with a photo diode array(PDA) detector, all of the peaks derived from the enzymatic reaction have been characterized.

Keywords : Cytochrome P-450 2A6, aryl alkyne, P-450 inhibitor, chemical inhibition, 5-ethynyl indole.

1. Introduction

The P450s catalyze a wide spectrum of enzymatic reactions, for example, hydroxylations, epoxidations, dehalogenations, dealkylations, oxidations, oxidative deamination and isomerizations. The human P450s are well studied to investigate the metabolism of exogenous compounds such as drugs, environmental pollutants, and a variety of pesticides as well as the endogenous metabolism of fatty acids, steroids, eicosanoids, bile acids, and fat-soluble vitamins[1]. These heme-containing enzymes play a key role in the metabolism of a variety of compounds. Human liver microsomes contain the CYP isoenzymes involved in drug metabolism, such as CYP 1A2, 2A6, 2B6, 2C8, 2C19, 2D6, 2E1 and 3A4[2].

To determine the role of specific human P450s in drug metabolism, an array of *in vitro* metabolism techniques including the use of chemical inhibitors and specific substrates with diverse structures has been developed[3]. For chemical inhibition, mechanism and structure of the inhibitors are important for consideration. Mechanism-based inhibitions are defined by covalent bonding between the substrate and the enzyme irreversibly. It has been reported that aryl alkyne serves an effective probe for profiling mouse liver microsomal P450s. CYP-specific aryl alkyne inhibitors are employed to block

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or decrease enzyme activities of a single CYP. Mechanism proposed for the inactivation of CYP P450s by aryl alkyne is shown in Fig. 1. Activated oxygen from Heme of CYP may be transferred to terminal alkyne which leads to ketene intermediate formation. This ketene metabolite can readily undergo acetylation of the CYP protein or react with water to be carboxylic acid. Formation of heme adduct by oxidation of the terminal alkyne moiety have also been reported[4-6].

Although aryl alkyne are effective chemical inhibitors, each P450s show distinctive substrate and inhibitor specificities, which requires multiple probe structures to cover diverse P450 enzymes family. We hypothesize that our indole-based alkyne may also be an effective mechanism-based inhibitor for P450s. We have chosen one of typical P450 enzymes, CYP 2A6 as a model to investigate inhibition effect of a new inhibitor. Crystal structure of human microsomal P450 2A6 with coumarin bound has shown in Fig. 2. Human microsomal cytochrome P450 2A6 contributes extensively to nicotine detoxication but also activates tabacco-specific precarcinogen to mutagenic products. The CYP 2A6 structure shows a compact, hydrophobic active site with one hydrogen bonding donor, Asn297, that orients coumarin for regioselective oxidation[7].

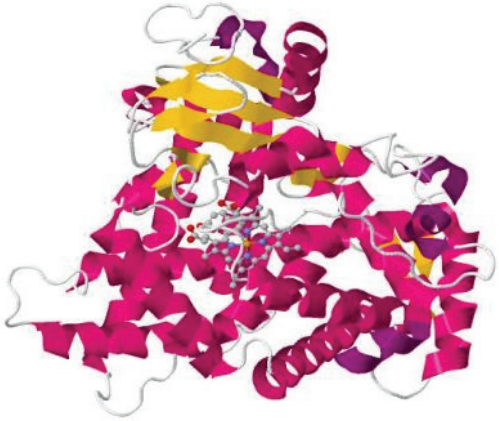


Fig. 2. Crystal structure of human microsomal P450 2A6 with coumarin bound. PDB ID: 1Z10.

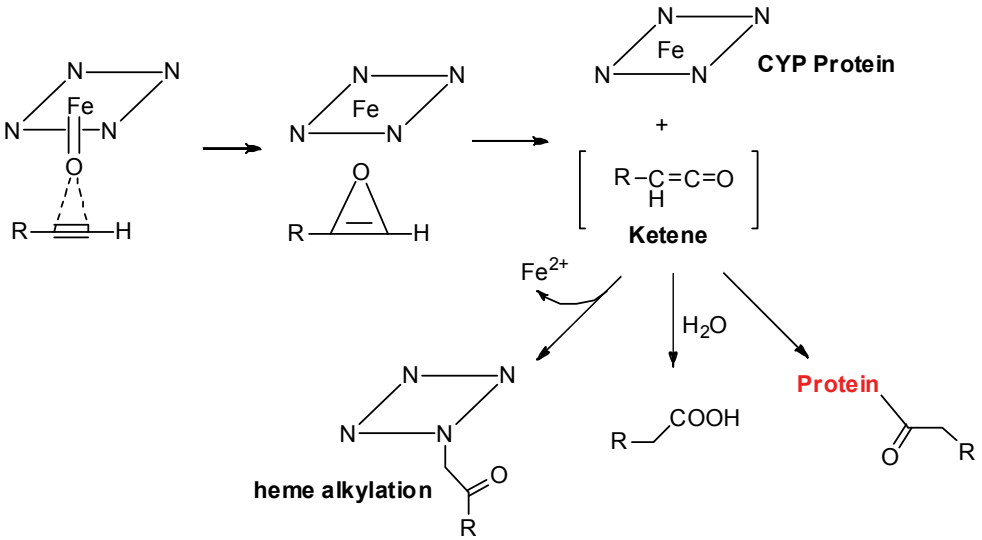


Fig. 1. Mechanism proposed for the inactivation of CYP P450s by aryl alkyne.

Table 1. General Marker Reactions and Substrates that can be Employed in The Course of Cytochrome P450 Experiments.

CYP	Sub6strate	Marker Reaction
1A2	7-ethoxyresorufin	7-ethoxyresorufin *O*-deethylation
2A6	coumarin	coumarin 7-hydroxylation
2B6	*S*-mephenytoin	*S*-mephenytoin *N*-demethylation
2C8	paclitaxel	paclitaxel 6a-hydroxylation
2C9	diclofenac	diclofenac 4'-hydroxylation
2D6	bufuralol	bufuralol 1'-hydroxylation
2E1	chlorzoxazone	chlorzoxazone 6-hydroxylation
3A4	testosterone	testosterone 6b-hydroxylation

General marker reactions and substrates that can be employed in the course of cytochrome P450 experiments are shown in Table 1. Among many P450s, CYP 2A6 transforms coumarin into 6-hydroxycoumarin by enzymatic oxidation reaction.

An aryl alkyne CYP P450 inhibitor, 2-ethynylnaphthalene based compound (2EN-ABP), has been proved capable of profiling human P450 activities in both *in vitro*- and *in vivo* systems in order to characterize new classes of human enzymes. Although 2EN-ABP showed to label several P450 enzymes, it is not universal inhibition compound for all mammalian P450s so that varieties of activity-based probes need to be synthesized[8-12].

We have designed an indole-based inhibitor, which has similar aromatic ring size to 2-ethynylnaphthalene(2EN). Indole-based inhibitors may have an advantage over 2-ehtylnynaphthalene from the fact that the alkyne functional group can be introduced to any position of the indole ring where it has designated. Thus various position of alkyne functional group along indole ring would show different reactivity in the confined enzyme active site such as in the plant P450 enzyme[13-15]. By comparing the metabolism rates of inhibitor treated CYP 2A6 enzyme with that of untreated control enzyme, coumarin 6-hydroxylase activity from CYP 2A6 was examined. We now report the successful synthesis of indole based chemical inhibitor, 5-ethynyl indole, and its inhibitory effect on CYP 2A6 enzymatic by using comparative HPLC analysis equipped with PDA detector.

2. Experimental

2.1. Materials

The cytochrome P450 enzymes (CYP 2A6) was purchased from BD Biosciences. This product is baculovirus-insect cell expressed with supplemental cDNA expressed human reductase and human cytochrome *b*5. Total protein concentration is 5.3 mg/ml (0.5 nmole P450). 5-Iodoindole, trimethylsilylacetylene, $Pd(PPh_2)Cl_2$, CuI, Tetrabutylammonium fluoride(TBAF), NH_4Cl and triethylamine were purchased from Sigma Aldrich. THF, MeOH, EtOAc and Hexanes were purchased

from Daehan Science and used without further purification. Mass analysis was performed on an Acquity Ultra Performance BEH C18 column with a 1.7 mm particle size, 2.1 x 100 mm dimension, and a flow rate of 0.6 ml min^{-1} in tandem with a Micromass LCT Premier TOF Mass Spectrometer with an ESI source (Waters Corporation). The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) were taken with a Bruker 300 NMR spectrometer. Chemical shifts are reported in ppm (δ).

2.2. P-450 2A6 index reaction assay

Index reaction of P450 2A6 was measured using an high-performance liquid chromatography(HPLC). Microsomal CYP 2A6(final concentration, 1.5 mg/mL) in the presence of 1 mM NADPH(final concentration) and 25 mM tris-HCl buffer(pH 7.4) was treated with coumarin(final concentration 75 μM). The incubation was carried out at 37 ℃ in a shaking incubator(60 rpm). After 60 min of incubation, the reaction was stopped by the addition of 3 μM HCl (50 μL) and the mixture was extracted with ethyl acetate. Organic layer was then evaporated to dryness under vacuum. The residue was reconstituted in 200 μL of 30 % methanol prior to HPLC injection. The reconstituted sample was directly injected onto an analytical HPLC (Youngrin Co., YL9100 HPLC system) with C18 column (Agilent HC-C18(2), 5μm column) using a solvent gradient of 10 % to 70 % acetonitrile in 0.1 % aqueous trifluoroacetic acid. The UV absorbance of enzymatic metabolite, 7-hydroxyl coumarin was detected by HPLC photo diode array(PDA) detector.

2.3. Chemical inhibition study of P-450 2A6 with inhibitor 3.

Microsomal CYP 2A6 (final concentration, 1.5 mg/mL) in the presence of 1mM NADPH(final concentration) and 25 mM tris-HCl buffer (pH 7.4) was treated with coumarin(final concentration 75 μM). The incubation was carried out at 37 ℃ in a shaking incubator(60 rpm). After 60 min of preincubation, 0.5 μl of inhibitor **3**(20 μM final) taken from the stock solution in DMSO was added to enzymatic reaction mixture. The reaction was stopped by the addition of 3 μM HCl (50 μL) and the mixture was extracted with ethyl acetate. Organic layer was then evaporated to dryness under vacuum. The residue was reconstituted in 200 μL of 30 % methanol prior to injection and then directly injected on HPLC.

2.4. Synthetic method for 5-ethynyl-1H-indole 3.

A mixture of 5-iodoindole (200 mg, 0.82 mmol), trimethylsilylacetylene (182 mL, 1.28 mmol), $Pd(PPh_2)Cl_2$ (58 mg, 0.082 mmol), CuI (8 mg, 0.042 mmol), and triethylamine (230 μL) in acetonitrile (880 μL) was refluxed for 4 h. After cooling, water was added to reaction mixture and extracted with EtOAc twice. Then the organic layer was filtered through Celite and washed with brine. And it was dried over anhydrous Na_2SO_4 and concentrated under the reduced pressure. The residue was dissolved in THF (10 mL) and MeOH (10 mL) and cooled to 0 ℃. Tetrabutylammonium fluoride (1.0 M solution of THF, 10 mL, 10.0 mmol) was added drop wise for 15 min. After stirring at 0 ℃ for 2 h, it was poured into saturated aquous NH_4Cl solution and extracted with EtOAc and washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography with EtOAc and Hexane (2:8) to give the desired product, 5-ethynyl-1*H*-indole **3**. ^{1}H-NMR (300 MHz, DMSO-d_6) 3.37(1H, s), 6.48-6.50 (1H, m),

7.29 (1H, d, J_1 = 3.0 Hz), 7.44 (2H, dd, J_1 = 3.0 Hz, J_2 = 3.0 Hz), 7.85 (1H, d, J_1 = 3.0 Hz), 11.40 (1H, s). ^{13}C NMR (75 MHz, DMSO-d_6) 83.1, 84.1, 101.52, 110.8, 112.1, 124.9, 125.1, 127.0, 127.6, 136.1, Mass (ESI) 142 ($M+H^+$).

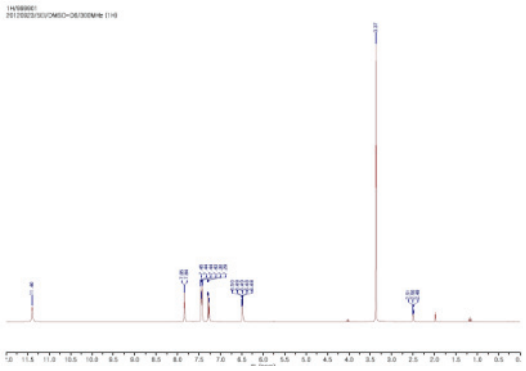


Fig. 3. H^1-NMR spectrum of 5-ethynyl-1*H*-indole **3**.

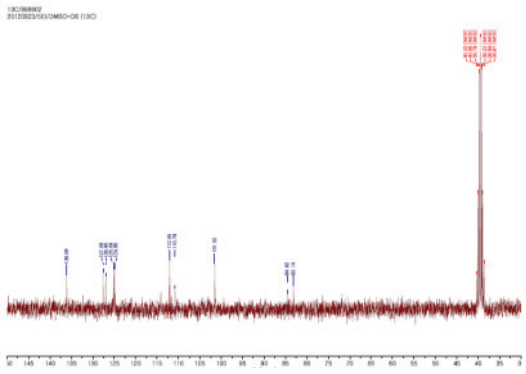


Fig. 4. C^{13}-NMR spectrum of 5-ethynyl-1*H*-indole **3**.

3. Results and Discussion

3.1. New P-450 chemical inhibitor (5-ethynyl indole 3) design and synthesis.

We present a new aryl alkyne inhibitor **3** designed to inhibit of P450 enzymes in human metabolism. We constructed an indole-based inhibitor and tested it in the most typical human P450 enzyme 2A6.

The synthesis of compound was carried out from the starting material, 5-iodo indole **1**, and trimethylsilylacetylene intermediate **2** was achieved from trimethylsislyacethylene and iodo-indole by Sonogashira cross-coupling reaction[16-18]. This cross-coupling reaction of iodo indole and trimethylsilylacetylene using $Pd(PPh_3)Cl_2$ as the catalyst gave trimthylsilylacetylene compound **2** in 85~95%. Then using Tetrabutylammonium fluoride in MeOH deprotection reaction occurred which results in final product 5-ethynyl indole compound **3** in 49% yield. Compound 3 was confirmed by NMR and mass spectrometer.

3.2. HPLC analysis of enzymatic reaction of P-450 2A6 with the substrate 4.

Cytochrome P450 2A6 is one of the most abundant human P450s. The most characteristic and specific reaction of this P450 2A6 is coumarin 7-hydroxylation as shown in Fig. 6. Before we carry out the inhibition assay with our new inhibitor **3**, the enzymatic activity of CYP 2A6 was

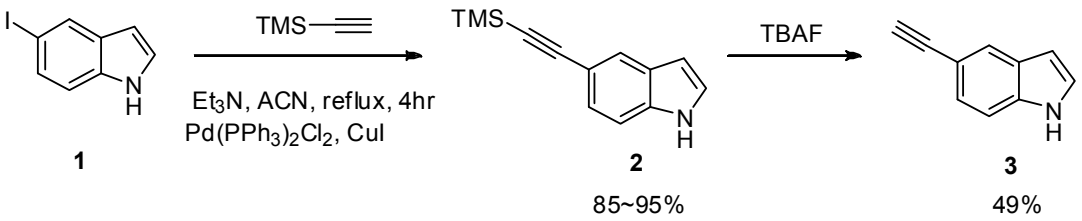


Fig. 5. Synthesis of 5-ethynyl indole **3**.

evaluated with the substrate, coumarin **4**, for coumarin 7-hydroxylation reaction.

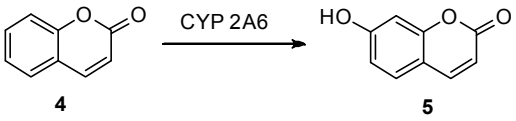


Fig. 6. Coumarin 7-hydroxylation reaction by CYP 2A6.

Reactivity of CYP 2A6 was measured using a high-performance liquid chromatography(HPLC) assay. A solution of microsomal CYP 2A6 (final concentration, 1.5 mg/mL) in the presence of 1mM NADPH(final concentration) was treated with coumarin and incubated in 25 mM tris-HCl buffer (pH 7.4) at 37 ℃ in a shaking incubator (60 rpm).

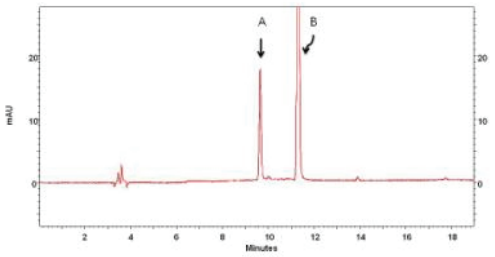
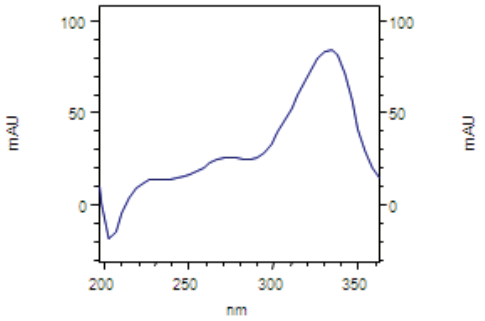
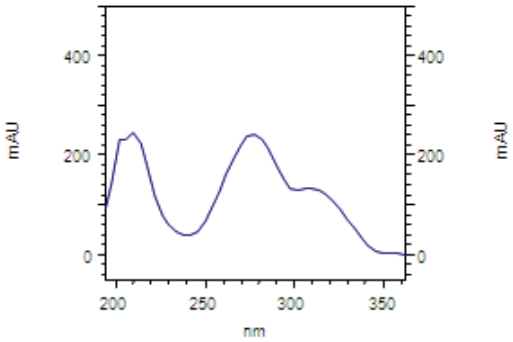


Fig. 7. HPLC chromatogram of the enzymatic reaction with human P450 2A6 showing 7-hydroxylase activity. A: 7-hydroxycoumarin appears at 9.8 min, B: the substrate, coumarin, appears at 11.2 min. Gradient, 10-70% acetonitrile in water with 0.1% trifluoroacetic acid over 20 min, monitoring at 280 nm.

Peak (A) in Fig. 7 at 9.8 min represents the product, 7-hydroxycoumarin **5** (peak A), which is derived from hydroxylation reaction of CYP 2A6 with the substrate coumarin **4**. Peak (B) at 11.2 min is the substrate **4**. This enzymatic product (peak A) was eluted earlier than the starting material, coumarin (peak B), due to the hydrophilic hydroxyl group at 7 position of indole ring. Moreover, 7-hydroxycoumarin **5** shows maximum UV-absorbance at 335 nm due to the n-p* excitation of additional hydroxyl group on indole ring, whereas coumarin shows UV-absorbance for n-p* excitation at much shorter wavelength, 275 nm.



A: Product (7-hydroxycoumarin **5** at 9.8min)



B: Starting material (Coumarin at 11.2 min)

Fig. 8. UV absorption spectra of 7-hydroxycoumarin (A) and coumarin (B) were obtained by HPLC photo diode array (PDA) detector.

3.3. HPLC analysis of Inhibitory effect on P-450 2A6 with the inhibitor 3.

To confirm inhibition activity of 5-ethynyl indole **3**, the inhibitor was preincubated with cytochrome P450 2A6 and NADPH for 60 min prior to the addition of substrate, coumarin **4**. The metabolite peak(A) showed

at 9.8 min as in control incubated without the inhibitor was disappeared in this assay whereas the substrate peak(B) still existed at 11.0 min. As a result of this analysis for the comparison with and without the inhibitor, metabolic product, 7-hydroxycoumarin **5**, mediated by P450 2A6 was not observed even after 24 hours' incubation which strongly suggest that our new indole-based inhibitor **3**, has inhibitory activity over CYP 2A6 as shown in Fig 9.

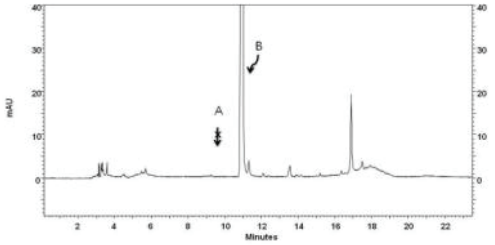


Fig. 9. HPLC chromatogram of inhibition of 7-hydroxylation activity with P450 2A6 using chemical inhibitor **3**. **A**: the metabolite peak A disappeared at 9.8 min. **B**: the substrate, coumarin peak B, still exists at 11.0 min. Gradient, 10-70% acetonitrile in water with 0.1% trifluoroacetic acid over 20 min, monitoring at 280 nm.

4. Conclusions

Synthesis of the compound **3** was successfully achieved from 5-iodo indole and trimethylsilylacetylene by Sonogashira cross-coupling reaction using $Pd(PPh_3)Cl_2$ as the catalyst which results in final product, 5-ethynyl indole compound **3**, in good yield. CYP 2A6 produced 7-hydroxycoumarin **5** as a metabolite from the substrate coumarin. However when the inhibitor **3** was preincubated with P450 2A6 prior to the addition of **4**, 7-hydroxycoumarin **5** has not shown primarily due to the inhibition of **3**. 7-Hydroxycoumarin (peak B) showed at 9.8 min on HPLC chromatogram from the control whereas the peak B was disappeared when CYP 2A6 was incubated with the inhibitor. As a result of this analysis for the comparison of the assay with and without the inhibitor, it is strongly suggesting that our new indole-based inhibitor, 5-ethynyl indole **3**, has inhibitory activity over CYP 2A6.

The future studies lay in adding a chemical handle such as azide onto this indole scaffold, so that, once covalently modified, the CYP proteomes could be selectively labeled with a dye or biotin for profiling enzyme activities *in vivo* using click chemistry methods.

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