Cytochrome P450 4A Isoform Inhibitory Profile of *N*-Hydroxy-*N′*-(4-butyl-2-methylphenyl)-formamidine (HET0016), a Selective Inhibitor of 20-HETE Synthesis

Takayuki SEKI,*[a] Mong-Heng WANG,[b] Noriyuki MIYATA,[a] and Michal LANIADO-SCHWARTZMAN[c]

[a] *Medicinal Pharmacology Laboratory, Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd.; 1–403 Yoshino-cho, Kita-ku, Saitama, Saitama 331–9530, Japan:* [b] *Department of Physiology, Medical College of Georgia; Augusta, GA 30912, U.S.A.: and* [c] *Department of Pharmacology, New York Medical College; Valhalla, NY 10595, U.S.A.*
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We examined the effect of *N*-hydroxy-*N′*-(4-butyl-2-methylphenyl)-formamidine) (HET0016), an inhibitor of 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) synthesis on the *ω*-hydroxylation and epoxidation of arachidonic acid (AA) catalyzed by recombinant cytochrome P450 4A1 (CYP4A1), CYP4A2 and CYP4A3, and characterized the enzyme inhibitory profile of HET0016. The IC_{50} values of HET0016 for recombinant CYP4A1-, CYP4A2- and CYP4A3-catalyzed 20-HETE synthesis averaged 17.7 nM, 12.1 nM and 20.6 nM, respectively. The IC_{50} value for production of 11,12-epoxy-5,8,14-eicosatrienoic acid (11,12-EET) by CYP4A2 and 4A3 averaged 12.7 nM and 22.0 nM, respectively. The IC_{50} value for CYP2C11 activity was 611 nM which was much greater than that for CYP4As. The initial velocity study showed the K_i value of HET0016 for CYP4A1 was 19.5 nM and a plot of V_{max} *versus* amount of recombinant CYP4A1 added shows HET0016 is an irreversible non-competitive inhibitor. These results indicate that HET0016 is a selective, non-competitive and irreversible inhibitor of CYP4A.

Key words cytochrome P450 A4; 20-hydroxy-5,8,11,14-eicosatetraenoic acid; HET0016; kinetics

The metabolism of arachidonic acid (AA) by cytochrome P450 (CYP) enzymes in various cells can be divided into two major categories, *i.e.* *ω*-hydroxylases that produce 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) and epoxygenases that catalyze the formation of epoxyeicosatrienoic acids (EETs). In the rat, there are four different CYP4A isozymes that can catalyze the *ω*-hydroxylation of AA to 20-HETE. CYP4A1, CYP4A2, CYP4A3 and CYP4A8 are all expressed in rat kidneys.[1—3)] Kinetic profile of the rat CYP4 isoforms on AA metabolism was shown by Nguyen *et al*.[4)] While CYP4A2 and CYP4A3 catalyzed both *ω*-/*ω*-1-hydroxylation and 11,12-epoxidation of AA, CYP4A1 catalyzed only *ω*-/*ω*-1-hydroxylation and recombinant CYP4A8 showed little if any *ω*-hydroxylation activity.[5)] A number of studies have demonstrated that 20-HETE has potent biological activities which contribute to the regulation of renal tubular and vascular function and the long-term control of arterial blood pressure.[6—10)] 20-HETE is also a potent vasoconstrictor in the cerebral microcirculation and a regulator of vascular tone and autoregulation of cerebral blood flow.[11—14)] On the other hand, EETs have been known as vasodilators and inhibitors of sodium and water transport in the kidney.[15,16)] The CYP4A enzymes are considered to be the major AA *ω*-hydroxylases in the rat tissues and thereby the primary contributors of 20-HETE synthesis. Studies using antisense oligonucleotids against CYP4A1 and CYP4A2 further support a role for these proteins in the regulation of vascular tone and blood pressure.[17)]

HET0016 (*N*-hydroxy-*N′*-(4-butyl-2-methylphenyl)-formamidine (Fig. 1) was discovered using a high throughput screening of the compound library of the Taisho Pharmaceutical Co., Ltd. We reported that HET0016 is a potent and selective inhibitor of 20-HETE synthesis in rat and human renal microsomes.[18)] Kehl *et al*. reported that HET0016 inhibited 20-HETE synthesis by human isoforms of CYP4F2, CYP4F3 and CYP4A11.[19)] However, little is known regarding the inhibitory profile of HET0016 on the other CYP4A isoforms. In the present study, we examined the effect of HET0016 on 20-HETE synthesis catalyzed by rat recombinant CYP4A1, 4A2, and 4A3 and characterized its inhibitory profile on these enzymes.

MATERIAL AND METHODS

Reagents [1-^{14}C]-AA (56 mCi/mmol) was purchased from Dupont-New England Nuclear (Boston, MA, U.S.A.). Purified recombinant human NADPH-P450 oxidoreductase (OR) (specific activity, 58 *μ*mol/min/mg) was obtained from Oxford Biomedical Reseach Inc. (Oxford, MI, U.S.A.). Emulgen E911 was purchased from KAO Atlas (Tokyo, Japan). Purified rat liver cytochrome b_5 (specific content, 40 nmol/mg) was obtained from Panvera Corp. (Madison, WI, U.S.A.) and NADPH was from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). HPLC grade solvents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of the highest grade commercially available.

Preparation of Recombinant CYP4A Cell Membranes CYP4A proteins were expressed in the baculovirus-Sf9 insect cell expression system as described previously.[5)] The CYP4A cDNAs were kindly provided by Dr. Richard Roman (Medical College of Wisconsin). Sf9 cells were infected with either CYP4A1, CYP4A2, or CYP4A3 recombinant viruses. Insect culture media were fortified with 5 *μ*g/ml of hemin at the time of infection. After 72 h, the cells were harvested, washed twice with PBS, and resuspended in sucrose buffer

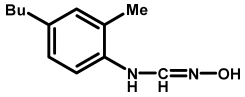

Fig. 1. Chemical Structure of HET0016 (*N*-Hydroxy-*N′*-(4-butyl-2-methylphenyl)-formamidine)

* To whom correspondence should be addressed. e-mail: takayuki.seki@po.rd.taisho.co.jp

(50 mM potassium phosphate, pH 7.4, 0.4 M sucrose). Cell lysates were prepared by brief sonication (4—5 bursts of 4 s duration), and the membrane fraction was obtained by high-speed centrifugation (100000 ***g***) for 60 min. The membrane pellet was then resuspended in sucrose buffer and stored at −80 °C. The protein concentration was determined using the Bradford method. The concentration of CYP was determined by CO-reduced difference spectral method of Omura and Sato with an extinction coefficient $\epsilon_{450-490\,nm}=91\,mM^{-1}\cdot cm^{-1}$.[20]

Inhibition Assay of HET0016 on Recombinant CYP Enzymes and 20-HETE Synthesis Rat recombinant CYP4A1, CYP4A2, or CYP4A3 (10 pmol) membranes were mixed on ice with OR and cytochrome b_5 at a molar ratio of 1 : 7 : 4 for CYP4A1; 1 : 14 : 4 for CYP4A2, CYP4A3; 1 : 2 : 1.8 for CYP2C11. CYP2C11 was purchased from GENTEST (Woburn, MA, U.S.A.). The mixture was incubated with or without HET0016 [10^{-9}—10^{-6} M for CYP4A1, CYP4A2, and CYP4A3; 10^{-8}—10^{-5} M for CYP2C11] and [1-^{14}C]-AA (0.4 μCi) for 5 min at 37 °C in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$. Reduced nicotinamide adenine dinucleotide phosphate (NADPH; 1 mM) was added to the mixture to initiate the reaction and incubation continued for 20 min at 37 °C. The reaction was terminated by acidification to pH 3.5 to 4.0 with 20 μl of 2 M formic acid, and the metabolites were extracted with ethyl acetate. The combined extracts were evaporated under nitrogen and the residue was re-suspended in 150 μl of methanol. Metabolites of AA were separated on a Reverse-phase HPLC which was performed on a 5 μm octadecyl silane-Hypersil column (4.6×200 mm; Hewlett-Packard, Palo Alto, CA, U.S.A.) at a flow rate of 1 ml/min using a gradient elusion ranging from acetonitrile : water : acetic acid (50 : 50 : 0.1) to acetonitrile : water : acetic acid (100 : 0 : 0.1) over a 30-min period.

Radio-labeled metabolites were monitored using a radioactive flow detector (In/Us System, Tampa, FL, U.S.A.). The identity of each metabolite was confirmed by comigration with a reference standard. The experiments were repeated several times for each enzyme and HET0016 concentration.

HET0016 was dissolved in 100% dimethyl sulfoxide (DMSO). The final concentration of DMSO in the test medium was 1% and did not affect any enzyme activity. Results were expressed as a percent of the control peak area which was obtained with the 1% DMSO test medium without HET0016. The percent of the control activity (peak area) was displayed graphically. Curve-fitting and parameter estimation were carried out by using Origin 6.0 J (OriginLab Corp., MA, U.S.A.).

RESULTS

Effects of HET0016 on Recombinant CYP4A-Catalyzed Arachidonate Oxidation HET0016 (10^{-9}—10^{-6} M) inhibited the formation of 20-HETE by all three isoforms in a concentration-dependent manner (Figs. 2A—C). Inhibitory effects of HET0016 on epoxidation of AA by Sf9-expressed CYP4A2 and CYP4A3 were similar to those of ω-hydroxylation by CYP4A2 and CYP4A3 isoforms (Figs. 2B, C). The IC_{50} values of HET0016 for recombinant CYP4A1-, CYP4A2- and CYP4A3-catalyzed 20-HETE synthesis averaged 17.7 nM, 12.1 nM, and 22.3 nM, respectively. The IC_{50} value for production of 11,12-EET by CYP4A2 and 4A3 averaged 12.7 nM and 26.6 nM, respectively triplicate observations.

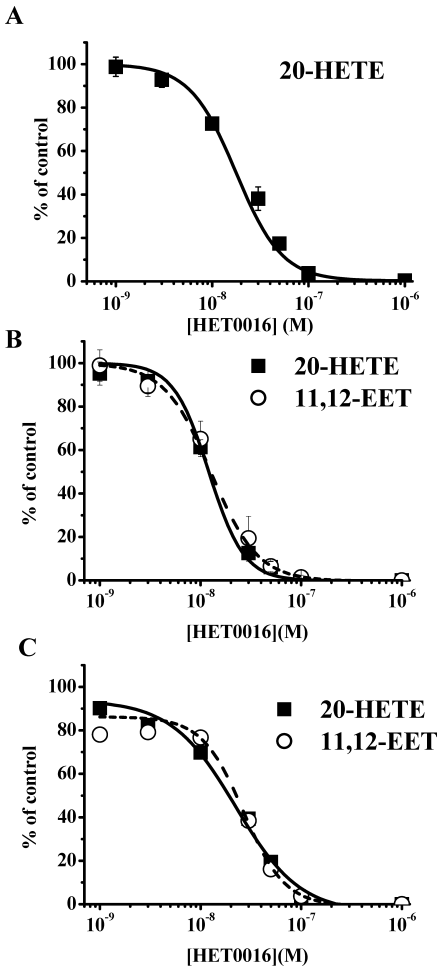

Fig. 2. Effect of HET0016 on the Production of 20-HETE and 11,12-EET by Rat Recombinant CYP4A1 (A), -4A2 (B) and -4A3 (C)

Cell membranes containing 10 pmol of expressed CYP4A1 (A), -4A2 (B) and -4A3 (C) were reconstituted with OR (70 or 140 pmol) and b_5 (40 pmol) and incubated with 0.4 μCi (7 nmol) of [1-^{14}C]-AA in presence of NADPH (1 mM). Reactions were carried out for 20 min at 37 °C, and metabolites were extracted and separated by HPLC. Results are expressed as percent of control and each point represents an average of triplicate observations. Control activity of CYP4A1, -4A2, -4A3 were 4.0, 1.2, 0.7 nmol 20-HETE formed/min/pmol P450 respectively. And control activity of CYP4A2, -4A3 were 0.3, 0.2 nmol 11,12-EET formed/min/pmol P450 respectively.

HET0016 also inhibited the formation of 11,12-EET by rat recombinant CYP2C11 (Fig. 3). The IC_{50} value was 611 nM with triplicate observations. The IC_{50} of HET0016 for CYP2C11-mediated epoxidation was about 30 times greater than those of ω-hydroxylation catalyzed by CYP4A1, 4A2 and 4A3.

Initial Velocity Kinetic Studies We used the CYP4A1 isoform to analyze the enzyme inhibitory profile of HET0016 on 20-HETE synthesis. CYP4A1 enzyme kinetic analyses were performed under optimal reconstitution conditions. Table 1 shows the Michaelis constant (K_m) and maximal velocity (V_{max}) values of CYP4A1 for AA in the absence or presence of different concentrations of HET0016.

HET0016 caused a marked decrease in the velocity of recombinant CYP4A1-catalyzed AA oxidation. To estimate

Table 1. Initial Velocity Kinetic Constants of Baculovirus-Expressed CYP4A1 with Arachidonic Acid in the Presence of HET0016

Inhibitor concentration	V_{max} (nmol/min/nmol P450)	K_m (μM)	V_{max}/K_m (nM$^{-1}\cdot$min^{-1})	K_i (nM)
Control	8.5	12.1	766	—
HET0016 (20 nM)	4.3	11.0	388	20.0
HET0016 (40 nM)	2.7	12.7	213	18.9

Results are the mean of 4—8 reactions.

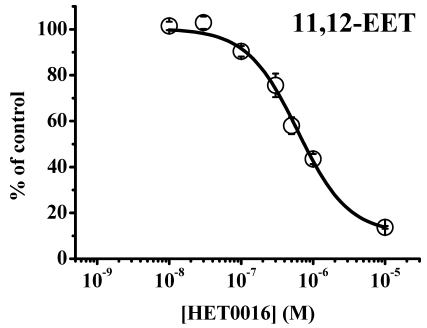

Fig. 3. Effect of HET0016 on the Production of 11,12-EET by Rat Recombinant CYP2C11

Cell membranes containing 10 pmol of CYP2C11 was reconstituted with OR and b_5 and incubated with 0.4 μCi (7 nmol) of [1-^{14}C]-AA in presence of NADPH (1 mM). Reactions were carried out for 20 min at 37 °C, and metabolites were extracted and separated by HPLC. Results are expressed as percent of control and each point represents an average of triplicate observations. Control activity of CYP2C11 was 5.3 nmol 11,12-EET formed/min/pmol P450.

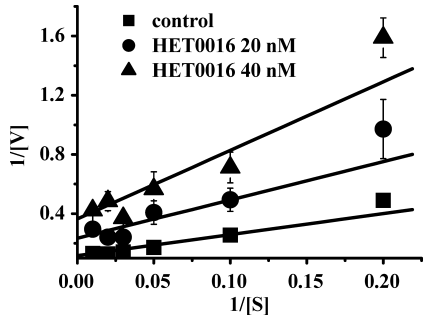

Fig. 4. Kinetic Analysis of 20-HETE Formation from AA by Recombinant CYP4A1 A Plot of 1/Velocity (V) *versus* 1/Substrate (S) Concentrations

Cell membranes containing 10 pmol of expressed CYP4A1 was reconstituted with OR (70 pmol) and b_5 (40 pmol) and incubated with 1—16 nmol of [1-^{14}C]-AA in presence of NADPH (1 mM). Reactions were carried out for 20 min at 37 °C, and metabolites were extracted and separated by HPLC. Results are mean±S.E. (n=4—8).

precise values for inhibition of the formation of 20-HETE from AA, we examined two different concentrations of HET0016 (20, 40 nM) and calculated K_m, V_{max} and K_i (Table 1, Fig. 4). The K_i value of 20 and 40 nM HET0016 for CYP4A1-catalyzed AA metabolize to 20-HETE were very similar (20.0 *vs*. 18.9 nM) (Table 1). This initial velocity study showed the K_i value of HET0016 for CYP4A1 was found to be 19.2 nM. The apparent K_m and V_{max} values given in Table 1 were calculated by fitting the results to a single-component Michaelis–Menten equation. The K_i was calculated based on the formula $V^I_{max}=V_{max}/1+[I]/K_i$, where [I] is concentration of inhibitor (HET0016). We also examined various concentration of CYP4A1 (2.5—10 pmol) added to the assay mixture in the absence or presence 20 nM of HET0016 to confirm the mode of enzyme inhibitory profile of HET0016. According to the plot, the line of control was

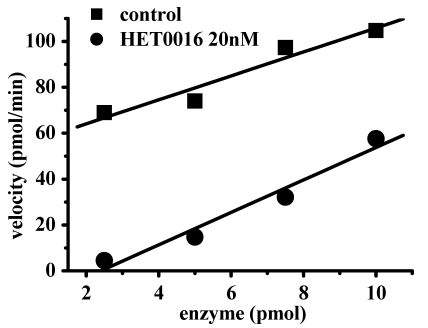

Fig. 5. Irreversibility of Inhibition-Form for HET0016 A Plot of V_{max} *versus* Amount of CYP4A1 Added (pmol P450).

Cell membranes containing 2.5—10 pmol of expressed CYP4A1 was reconstituted with OR and b_5 kept the ratio (1 : 7 : 4) and incubated with 0.4 μCi (7 nmol) of [1-^{14}C]-AA in presence of NADPH (1 mM). Reactions were carried out for 20 min at 37 °C, and metabolites were extracted and separated by HPLC. Results are from one experiment run in duplicates.

not cross the line of containing 20 nM HET0016 (two lines were parallel). This plot of V_{max} *versus* amount of recombinant CYP4A1 added showed HET0016 is an irreversible non-competitive inhibitor (Fig. 5).

DISCUSSION

The present study characterizes CYP4A isoform inhibitory profile of HET0016, a potent 20-HETE synthesizing enzyme inhibitor. HET0016 inhibited AA conversion to 20-HETE by all three CYP4A isoforms in a concentration-dependent manner. The IC_{50} values of HET0016 for recombinant CYP4A1, 4A2, and 4A3-catalyzed 20-HETE syntheses averaged around 10—20 nM. Formation of 20-HETE from AA by recombinant CYP4A1 exhibited simple Michaelis–Menten kinetics. Furthermore the plot of maximal initial velocity (V_{max}) *versus* the amount of enzyme added showed that HET0016 is an irreversible inhibitor. These results indicate that HET0016 is a non-competitive and irreversible inhibitor of CYP4 and thereby may be used to specifically target 20-HETE synthesis.

We have previously reported that HET0016 inhibited the formation of 20-HETE by rat renal microsomes.[18] In the present study, we examined the effect of HET0016 on the baculovirus-Sf9 cell-expressed CYP4A1, CYP4A2 and CYP4A3 membranes, which catalyzed AA to 20-HETE and 11,12-EET to clarify the CYP4A isoform specificity of HET0016. CYP4A1 membranes exhibited only ω-hydroxylation activity of AA. However, both CYP4A2 and CYP4A3 membranes, which exhibit >97% amino-acid sequence homology,[21,22] catalyzed the 11,12-epoxidation activity of AA in addition to ω-hydroxylation activity. These findings are agreement with previous report by Wang *et al*.[5] and Nguyen *et al*.[4] HET0016 equally inhibited the formation of 20-HETE catalyzed by CYP4A1, CYP4A2 and CYP4A3 (Fig.

2). HET0016 also inhibited the 11,12-EET formation by CYP4A2 and CYP4A3 in the same concentration range. These results indicate that HET0016 is a potent inhibitor of CYP4A isoforms.

CYP2C23, CYP2J2 and CYP2C11 are known to catalyze AA metabolite to 11,12-EET in kidney.[23—25] In the cerebral circulation, formation of 11,12-EET catalyzed by CYP2C11 plays a critical role in the regulation of cerebral blood flow and neuroprotection against ischemic stroke.[26,27] On the other hand, 20-HETE catalyzed by CYP4A has potent vasoconstrictor effect in cerebral artery and contributes to reduction of cerebral blood flow after subarachnoid hemorrhage.[19] To examine the selective inhibition of HET0016 for CYP4A isoforms, we also examined the ability of HET0016 to inhibit the production of 11,12-EET by CYP2C11. HET0016 inhibited the formation of 11,12-EET by rat recombinant CYP2C11 with IC_{50} values 611 nM which is about 30 times greater than that for CYP4A isoforms (Fig. 3). We have previously indicated that HET0016 exhibited a high degree of selectivity in inhibiting the formation of 20-HETE in renal microsomes from spontaneously hypertensive rats (SHR). The IC_{50} value for 20-HETE formation averaged 35 nM, whereas the IC_{50} value for inhibition of the formation of EETs by HET0016 averaged 2800 nM. The current study with recombinant CYP4A and CYP2C11 further establishes that the HET0016 acts as a selective inhibitor of CYP4A-catalyzed reactions and supports its use for selective inhibition of 20-HETE *in vitro* and *in vivo*.[19]

In the present study, we also examined the kinetic analysis of enzyme inhibitory profile to HET0016 by using the CYP4A1 with optimal reconstitution conditions. To estimate precise values for the inhibition of the formation of 20-HETE from AA, we examined two different concentrations (20, 40 nM) of HET0016. The apparent K_m and V_{max} values were calculated by fitting the results to a single-component Michaelis–Menten equation. These results indicate that HET0016 is a non-competitive inhibitor of CYP4A1 isoform (Fig. 4). To evaluate the mode of inhibitory action of HET0016 against CYP4A1, we examined the various amount of CYP4A1 (2.5—10 pmol) added to the assay mixtures in the absence or presence of 20 nM HET0016, and a plot of V_{max} *versus* amount of recombinant CYP4A1 added shows HET0016 is an irreversible non-competitive inhibitor.

In conclusion, the present study indicates that HET0016 is a non-competitive and irreversible inhibitor of CYP4A isoforms and thereby can be used to specifically target the 20-HETE synthesis.

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