CYTOCHROME P450 (CYP) PHENOTYPING OF AFICAMTEN, A NEXT-IN-CLASS INHIBITOR OF CARDIAC MYOSIN, USING HUMAN LIVER MICROSOMES AND HUMAN RECOMBINANT CYP ENZYMES

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Abstract and Objective

Aficamten, also known as CK-3773274, is a next-in-class small molecule cardiac myosin inhibitor being developed for the chronic oral treatment of patients with symptomatic hypertrophic cardiomyopathy (HCM).

The objective of the present studies was to identify the major cytochrome P450 (CYP) enzymes responsible for the in vitro metabolism of aficamten using two distinct approaches: 1) heterologous expressed human recombinant CYP enzymes (rCYPs) and 2) human liver microsomes (HLM) in the presence and absence of CYP enzyme-selective inhibitors.

Aficamten is primarily metabolized in human liver microsomes (HLM) through hydroxylation, resulting in an ω-1-hydroxylated metabolite of the ethyl group on the ethyl-1,2,4-oxadiazol-3-yl moiety, forming metabolite M1 (a mixture of stereoisomers M1a and M1b, **Figures 1-3**). Due to aficamten's high metabolic stability, the formation rate of metabolite M1 was measured and used to determine intrinsic clearance (CL_{int}). These CYP phenotyping methods focused on eight common CYP isoforms: CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5.

In the human rCYP phenotyping test system, the relative activity factor (RAF) approach was employed using RAF-characterized Supersomes™ prepared from insect cells engineered to over-express cDNA for human CYPs. Incubation conditions were chosen based on preliminary time- and *aficamten*-concentration studies to ensure that M1 formation in RAF studies was performed under first-order kinetic conditions. CL_{int} in recombinant P450s ($CL_{int,rP450}$) was calculated for *aficamten* and extrapolated to human liver microsomal (HLM) clearance for the P450 of interest for an estimate of CYP fraction metabolized (percent $f_{m,CYP}$).

In the second approach using HLM incubations with CYP-selective chemical inhibitors (HLM-CI), *aficamten* (1000 nM) was incubated from 0- to 120-minutes with HLM (0.25 mg/mL) and a panel of seven CYP-selective chemical inhibitors, namely: α-naphthoflavone (CYP1A2), 2-phenyl-2-(1-piperidinyl)propane (CYP2B6), montelukast (CYP2C8), sulfaphenazole (CYP2C9), (-)-*N*-3-benzylphenobarbital (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A) for the measurement of M1 formation and estimation aficamten $CL_{int,P450}$. The percent $f_{m,CYP}$ was determined by the calculated HLM $CL_{int,P450}$ in the presence and absence of each CYP-selective inhibitor using the equation: $f_{m,CYP} = ((CL_{int,\ no\ inhibitor} - CL_{int,CYP\text{-}selective\ inhibitor})/CL_{int,\ no\ inhibitor})*100$.

Results from these in vitro CYP phenotyping studies indicate that aficamten undergoes metabolism contributed by several CYP enzymes, including CYP2D6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4. Importantly, none of these individual CYP isoforms were found to be responsible for more than 50% of aficamten's metabolism.

METHODS

CYP Phenotyping in human recombinant CYP enzymes

A range of eight CYP enzymes that included CYPs 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5 were examined for the estimation of CYP fraction metabolized ($f_{m,CYP}$). Aficamten was incubated in triplicate with rCYPs (Supersomes, 0.25 mg/mL) at 37 °C with aficamten concentrations, rCYP concentrations, and over an incubation timeframe as indicated in **Table 1**. Incubations were performed on a Tecan automated incubation platform in 100 mM sodium phosphate buffer (pH 7.4). Reactions were terminated by adding 25 µL of the reaction mixture to 125 µL of 100% acetonitrile containing stable-labeled [$^2H_5$]aficamten internal standard (5 nM). Sample were vortex-mixed, centrifuged, and the supernatants prepared for LC-MS/MS bioanalysis.

Contributions in Supersomes™ of the individual CYPs to total CYP-mediated clearance in HLMs were estimated by a relative activity factor (RAF) scale-up approach (Nakajima *et al.*, 1999).

CYP Phenotyping in human liver microsomes

An in vitro study was conducted with HLMs (mixed gender, 200 donor pool) and with selective inhibitors of CYP enzymes to determine the formation rate of metabolite M1 for the range of CYPs tested. Aficamten (1 µM) was incubated from 0 to 120 minutes with HLM (0.25 mg/mL) and NADPH and with a panel of seven CYP-selective chemical inhibitors, namely, α-naphthoflavone (CYP1A2), 2-phenyl-2-(1-piperidinyl)propane (PPP, CYP2B6), montelukast (CYP2C8), sulfaphenazole (CYP2C9), (-)-*N*-3-benzylphenobarbital (N3BP, CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A).

Samples were processed for LC-MS/MS bioanalysis for metabolite M1 formation (M1a and M1b are detected with equal response by LC-MS/MS detection [Figure 3]).

Contributions of the individual CYPs to total CYP-mediated clearance in HLMs were estimated using CYP-specific chemical inhibitors (Obach and Walsky, 2004).

All studies were conducted at Q² Solutions, Indianapolis, IN

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Obach RS, Walsky RL (2004) Validated Assays for Human Cytochrome P450 Activities. *Drug Metab. Dispos.* 32:647-660.

Sukhun R, Cremin P, Xu D, Zamora J, Cheung J, Ashcraft L, Grillo MP, Morgan BP. In Vitro and In Vivo Pharmacokinetic Preclinical Characterization of Aficamten, a Small Molecule Cardiac Myosin Inhibitor. *Xenobiotica* 2024 (DOI: 10.1080/00498254.2024.2389407).

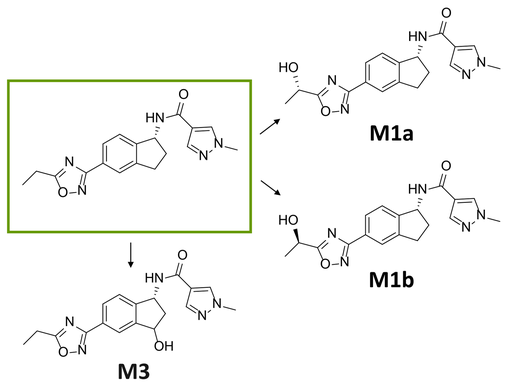

Figure 1. Chemical structures of *aficamten,* metabolite M1 (mixture of stereoisomers M1a and M1b), and metabolite M3 formed in incubations with human liver microsomes (HLM, Sukhun *et al.*, 2024)

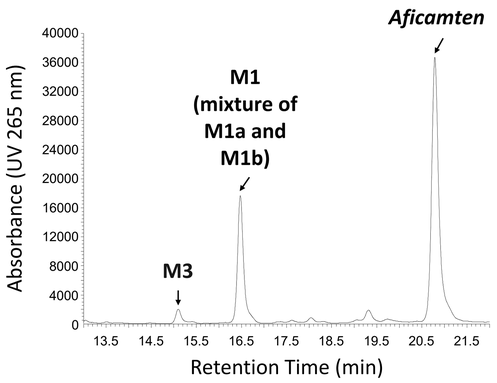

Figure 2. Representative LC-UV (265 nm) chromatogram of a 60-minute incubation extract of aficamten (20 µM) with human liver microsomes (Sukhun *et al.*, 2024).

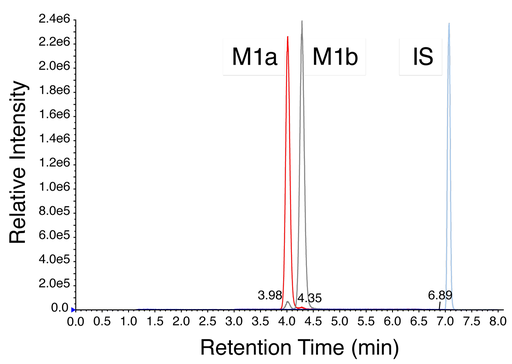

Figure 3. LC-MS/MS analysis of authentic standards M1a and M1b using chiral chromatography showing equal detection response.

Matrices	Substrate Concentrations (µM)	rCYP Concentrations (pmol/mL)	Time (minutes)
rCYP1A2	1.95-250	2.5, 5, 10	2, 4, 6
rCYP2B6	1.95-250	5, 10, 15	10, 15, 20
rCYP2C8	1.95-250	5, 10, 20	15, 30, 60
rCYP2C9	0.750-96	40	30
rCYP2C19	0.750-96	10	60
rCYP2D6	0.391-50	1.5, 3, 6	2, 4, 6
rCYP3A4	3.13-400	4.38, 8.75, 17.5	2.5, 5, 7.5
rCYP3A5	1.56-200	1, 2, 4	15, 30, 45

Table 1. Examination of CYP enzymes mediating the metabolism aficamten: Aficamten was incubated in triplicate with rCYPs (Supersomes™, 0.25 mg/mL) at 37 °C with varying concentrations of aficamten and rCYP over the times indicated in the table.

RESULTS

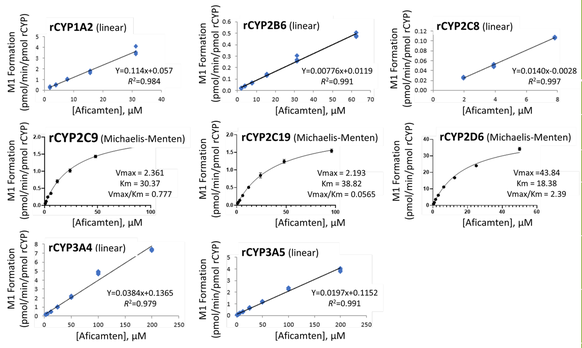

Figure 4. Kinetics plots for metabolite M1 formation from *aficamten* incubations with rCYP1A2, rCYP2B6, rCYP2C8, rCYP3A4, and rCYP3A5 Supersomes using linear slope fitting and with rCYPs 2C9, 2C19, and 2D6 using Michaelis-Menten fitting.

rCYP	Fitting	CL_{int} (µL/min/pmol P450)	RAF (pmol P450/mg)	Scaled CL_{int} (µL/min/mg)[D]	Scaled $f_{m,CYP}$ (%)
1A2	Linear	0.114	2.59	0.295	5.03
2B6	Linear	0.00776	2.95	0.0229	0.391
2C8	Linear	0.0140	172	2.41	41.1
2C9	Michaelis-Menten	0.0777	17.8	1.38	23.5
2C19	Michaelis-Menten	0.0565	1.63	0.0921	1.57
2D6	Michaelis-Menten	2.39	0.180	0.429	7.32
3A4	Linear	0.0384	31.7	1.22	20.8
3A5	Linear	0.0197	0.748	0.0147	0.251

Table 2. Determination of *aficamten* CL_{int} using rCYPs and determination of aficamten $f_{m,CYP}$ using RAF.

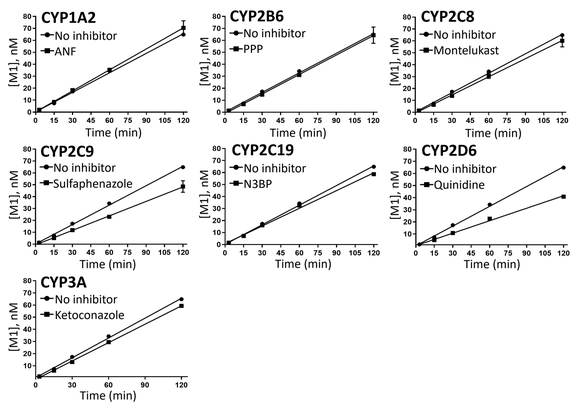

Figure 5. Metabolite M1 formation in incubations of *aficamten* (1000 nM) with HLMs in the absence and presence of CYP-selective inhibitors.

CYP	Inhibitor	R^2	k (min^{-1})	Velocity (pmol* min^{-1}*mg^{-1})	CL_{int} (µL/min/mg)	$f_{m,CYP}$ (%)
No Inhibitor	no inhibitor	0.998	0.545	1.09	1.09	N/A
1A2	α-naphthoflavone	0.992	0.586	1.17	1.17	0.00
2B6	2-phenyl-2-(1-piperidinyl)propane (PPP)	0.987	0.543	1.09	1.09	0.421
2C8	montelukast	0.990	0.508	1.02	1.02	7.79
2C9	sulfaphenazole	0.988	0.406	0.812	0.812	29.3
2C19	(-)-*N*-3-benzylphenobarbital (N3BP)	0.996	0.491	0.982	0.982	11.4
2D6	quinidine	0.996	0.343	0.686	0.686	42.5
3A	ketoconazole	0.999	0.504	1.01	1.01	8.63
Total $f_{m,CYP}$						87.2

Table 3. Determination of aficamten CL_{int} and $f_{m,CYP}$ in HLM using CYP-selective chemical inhibitors.

Conclusions

- Results from studies with RAF characterized rCYPs indicated the estimated relative contribution of the tested CYPs to the metabolism of aficamten as percent $f_{m,CYP}$ to be 5.03% for CYP1A2, 0.391% for CYP2B6, 41.1% for CYP2C8, 23.5% for CYP2C9, 1.57% for CYP2C19, 7.32% for CYP2D6, 20.8% for CYP3A4, and 0.251% for CYP3A5 (**Figure 4**, **Table 2**).
- Results from HLM incubations with aficamten and CYP-selective chemical inhibitors showed that the estimated percent $f_{m,CYP}$ values for each CYP compared to the total fraction metabolized (87.2%) were 0.00% for CYP1A2, 0.421% for CYP2B6, 7.79% for CYP2C8, 29.3% for CYP2C9, 11.4% for rCYP2C19, 42.5% for CYP2D6, and 8.63% for CYP3A (**Figure 5**, **Table 3**).
- Overall, results from these in vitro CYP phenotyping studies indicate that aficamten undergoes metabolism contributed by several CYP enzymes, including CYP2D6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4. Importantly, none of these individual CYP isoforms were found to be responsible for more than 50% of aficamten's metabolism and suggests a rather low probability of aficamten as the object of DDI from a concomitant medication that would inhibit or induce any single CYP isoform.

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