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Potent and Selective Inhibitors of Long Chain L-2-Hydroxy Acid Oxidase Reduced Blood Pressure in DOCA Salt-Treated Rats

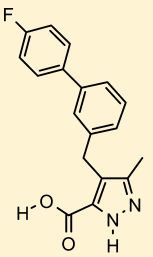
Dinesh A. Barawkar,*[,†] Ashwin Meru,[†] Anish Bandyopadhyay,[†] Abir Banerjee,[†] Anil M. Deshpande,[†] Chandrashekhar Athare,[†] Chandrasekhar Koduru,[†] Goraksha Khose,[†] Jayasagar Gundu,[†] Koshu Mahajan,[†] Pradeep Patil,[†] Sachin R. Kandalkar,[†] Sanjay Niranjan,[†] Shubhangi Bhosale,[†] Siddhartha De,[†] Sudit Mukhopadhyay,[†] Sumit Chaudhary,[†] Summon Koul,[†] Umesh Singh,[†] Anita Chugh,[†] Venkata P. Palle,[†] Kasim A. Mookhtiar,[†] Joseph Vacca,[‡] Prasun K. Chakravarty,[‡] Ravi P. Nargund,[‡] Samuel D. Wright,[‡] Sophie Roy,[‡] Michael P. Graziano,[‡] Sheo B. Singh,[‡] Doris Cully,[‡] and Tian-Quan Cai[‡]

[†]Drug Discovery Facility, Advinus Therapeutics Limited, Quantum Towers, Plot-9, Phase-I, Rajiv Gandhi InfoTech Park, Hinjewadi, Pune 411 057, India

[‡]Merck Research Laboratories, Rahway, New Jersey 07065, United States

Supporting Information

ABSTRACT: L-2-Hydroxy acid oxidase (Hao2) is a peroxisomal enzyme with predominant expression in the liver and kidney. Hao2 was recently identified as a candidate gene for blood pressure quantitative trait locus in rats. To investigate a pharmacological role of Hao2 in the management of blood pressure, selective Hao2 inhibitors were developed. Optimization of screening hits **1** and **2** led to the discovery of compounds **3** and **4** as potent and selective rat Hao2 inhibitors with pharmacokinetic properties suitable for in vivo studies in rats. Treatment with compound **3** or **4** resulted in a significant reduction or attenuation of blood pressure in an established or developing model of hypertension, deoxycorticosterone acetate-treated rats. This is the first report demonstrating a pharmacological benefit of selective Hao2 inhibitors in a relevant model of hypertension.



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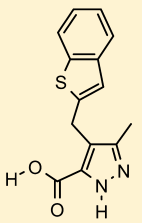

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KEYWORDS: *Hao2, hypertension, pyrazolecarboxylic acid, DOCA rat*

Human hypertension is a complex, multifactorial disorder resulting from the interplay of multiple environmental and genetic factors, and this common disorder can lead to an increased risk of heart attack, stroke, and renal failure. The mechanisms underlying the initiation and maintenance of the hypertensive process remain unclear.[1] Almost one-third of the U.S. adult population has high blood pressure (BP), which increases the risk of cardiovascular and renal disease and shortened life expectancy.[2−4] Various antihypertensive drugs have been developed, including diuretics, beta blockers, calcium channel blockers (CCBs), renin inhibitors,[5] angiotensin-converting enzyme (ACE) inhibitors, and angiotensin II receptor blockers (ARBs).[6,7] However, these drugs either lack sufficient efficacy or are associated with significant adverse effects. In a search for a novel antihypertensive target, we have identified L-2-hydroxy acid oxidase (Hao2)[8] as a potential target for pharmacological intervention.

L-2-Hydroxy acid oxidases are flavin mononucleotide (FMN)-dependent peroxisomal enzymes, which are members of the flavoenzyme family that are responsible for the oxidation of a number of L-2-hydroxy acids to ketoacids at the expense of molecular oxygen, resulting in the formation of hydrogen peroxide. Several examples of such enzymes have been identified in different organisms, e.g., glycolate oxidase from plants, lactate oxidase from *Mycobacterium* (L-lactate 2-monooxygenase), flavocytochrome b_2 from yeasts (L-lactate cytochrome *c* oxidoreductase), and mandelate dehydrogenase from *Pseudomonas putida*.[9] In mammals, this family of enzymes was first identified as an L-amino acid oxidase in the kidney and the liver of rats and later found to have activity similar to that of L-2-hydroxy acid.[10,11] Two α-hydroxy acid oxidases were also reported from hog renal cortex, named long chain L-α-hydroxy acid oxidase (Hao2) and short chain L-α-hydroxy acid oxidase (Hao1), because of their substrate specificity toward long and short carbon chain L-α-hydroxy acids, respectively. In both prokaryotes and eukaryotes, all the members of the hydroxy oxidase family are highly conserved in terms of both nucleotide and amino acid sequences. Human Hao2 has 351 amino acids with a predicted molecular mass of 39 kDa, while human Hao1 comprises 370 amino acids and has a predicted molecular mass of 41 kDa. Human Hao2 shares ∼50% identity with human Hao1 and 72−74% identity with rodent (rat and mouse) Hao2. Hao2 is predominantly expressed in the liver and kidney, with greatest potency for long chain 2-hydroxy acid substrates

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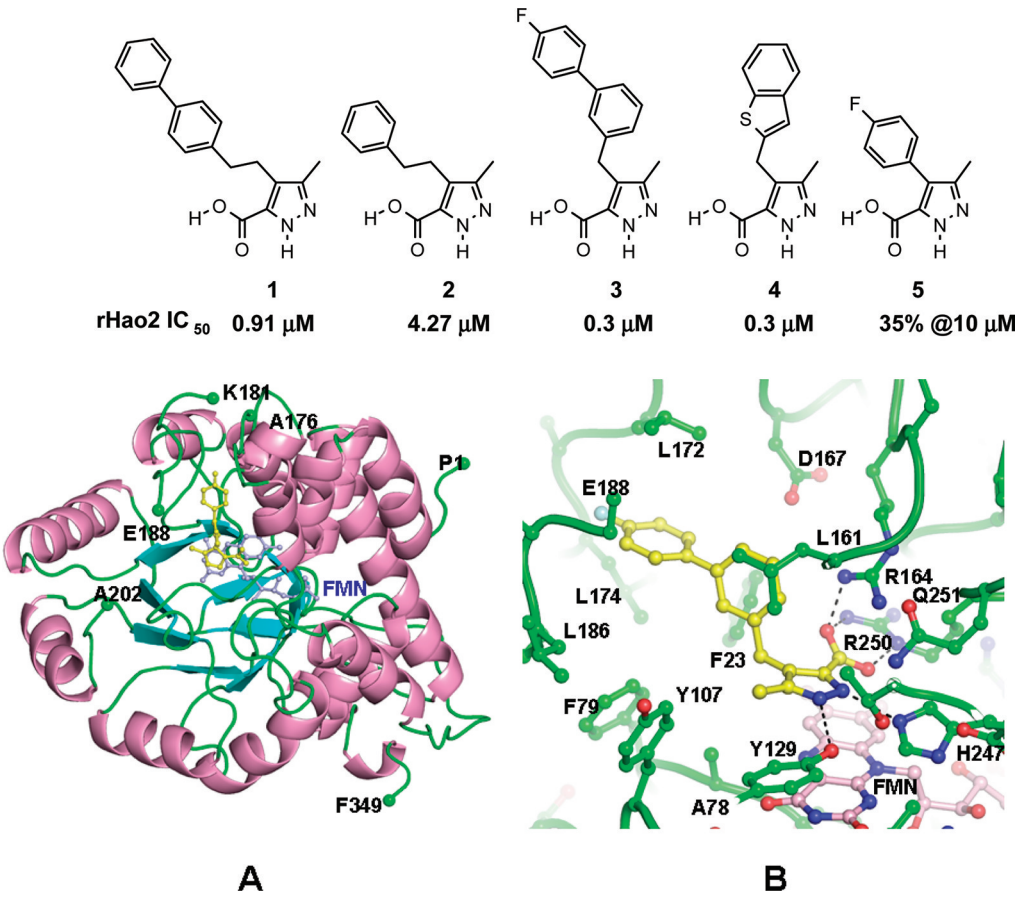

Figure 1. Structures of screening hits (**1** and **2**) and optimized leads (**3** and **4**). (A) Cartoon representation of docked compound **3** in the active site of rat Hao2 (Protein Data Bank entry 1TB3). The FMN ring is colored light blue. (B) Close-up view of docked compound **3** (yellow) in the active site of the enzyme. The FMN ring is colored light pink. The putative hydrogen bonds are shown as dashed lines.

Table 1. PK Properties of Lead Compounds 3 and 4[a]

Compound	3		4	
	in vitro			
RLM: MR (nmol/min/mg) @ 0.125 mg/mL protein	0.003		0.02	
Cytochrome p450 inhibition: 3A4, 2C9, 2D6, 2C19, 1A2	IC_{50} >10 μM		IC_{50} >10 μM	
Rat Plasma Protein Binding (%)	>99		>99	
Cytotoxicity in HepG2 cell line	IC_{50} >100 μM		IC_{50} >100 μM	
PXR induction	No induction at 100 μM		No induction at 100 μM	
	in vivo (rat)			
Route of admin	iv	Po	iv	Po
CL (mL/min/kg)	8 ± 3.2	NA	0.52 ± 0.02	NA
V_{ss} (L/kg)	0.6 ± 0.5	NA	0.14 ± 0.01	NA
C_{max} (μM)	NA	24 ± 0.3	NA	134 ± 17
t_{max} (h)	NA	0.25 ± 0.0	NA	0.25 ± 0.0
$AUC_{0\text{-}8h}$ (μM.h)	7.1 ± 2.4	27 ± 6	97 ± 3	517 ± 14
$t_{1/2}$ (h)	1.7 ± 0.4	1.5 ± 0.6	3.7 ± 0.3	4.1 ± 0.3
%F	NA	38	NA	53

[a]NA means not available. iv (1 mg/kg), po (10 mg/kg); vehicle: iv [dimethylacetamide (2%), crempohor (2%), PEG400 (2%), and Milli Q water (94%)], po [Tween 80 (0.5%) and 0.5% CMC (99.5%)].

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(displays the highest activity toward 2-hydroxypalmitic acid). Hao1 is expressed primarily in liver and pancreas and shows greatest potency for the two-carbon 2-hydroxy acid substrate (glycolic acid) but also displays activity on long chain 2-hydroxy fatty acids. Both Hao2 and Hao1 are capable of oxidizing 2-hydroxy fatty acids, but the endogenous physiological substrates remain to be identified.

Hao2 has been identified as a candidate gene for the systolic BP quantitative trait locus (QTL) in rats.[12−14] Genome-wide linkage analysis in humans locates a BP QTL in a defined region containing Hao2 (located in Ch. 1 at 119.6 cM), thus supporting a potential link between Hao2 and hypertension in humans, as well.[15]

To establish a pharmacological validation of Hao2 in blood pressure regulation, potent and selective rat Hao2 inhibitors were developed. Pyrazole carboxylic acids, **1** and **2** (Figure 1), were first identified as inhibitors of Hao2 by a focused enzyme screen of our compound collection. Subsequent optimization of these hits resulted in the discovery of compounds **3** and **4** as potent inhibitors of rat Hao2, each exhibiting an IC_{50} value of 0.3 μM.[16] These two compounds were further characterized in a spectrum of assays, including intervention studies in a well-established deoxycorticosterone acetate (DOCA) salt hypertension model.

Molecular modeling studies using the rat Hao2 crystal structure (Protein Data Bank entry 1TB3)[17] were used to understand the potential binding mode of the inhibitor (**3**) in the enzyme active site. The modeling suggests that the carboxylic acid moiety binds in the active site by forming salt bridge interactions with basic residues R250 and R164 (Figure 1). The interaction of the carboxylic acid moiety with these residues has also been observed in homologous enzymes of Hao2.[18,19] The NH group of the pyrazole moiety forms putative hydrogen bonds with the catalytic H247 and nearby Y129 residues. The biphenyl moiety resides in the hydrophobic pocket and is surrounded by residues such as F79, A185, L172, L174, E188, F23, and L161.

The model suggests that a one-carbon linker between the pyrazole and aromatic moiety (**3** and **4**) is optimal for filling the available space and placing the aromatic moiety in an appropriate orientation. A longer and flexible carbon linker may increase the conformational entropy of these structures (**1** and **2**), thereby decreasing their activity. Deletion of the carbon linker makes the molecules very rigid and leads to poor occupancy of the phenyl moiety in the active site. Hence, compound **5** without any linker shows substantially lower activity (Figure 1).

To assess selectivity and to demonstrate compounds **3** and **4** are selective inhibitors of rat Hao2, we profiled these two

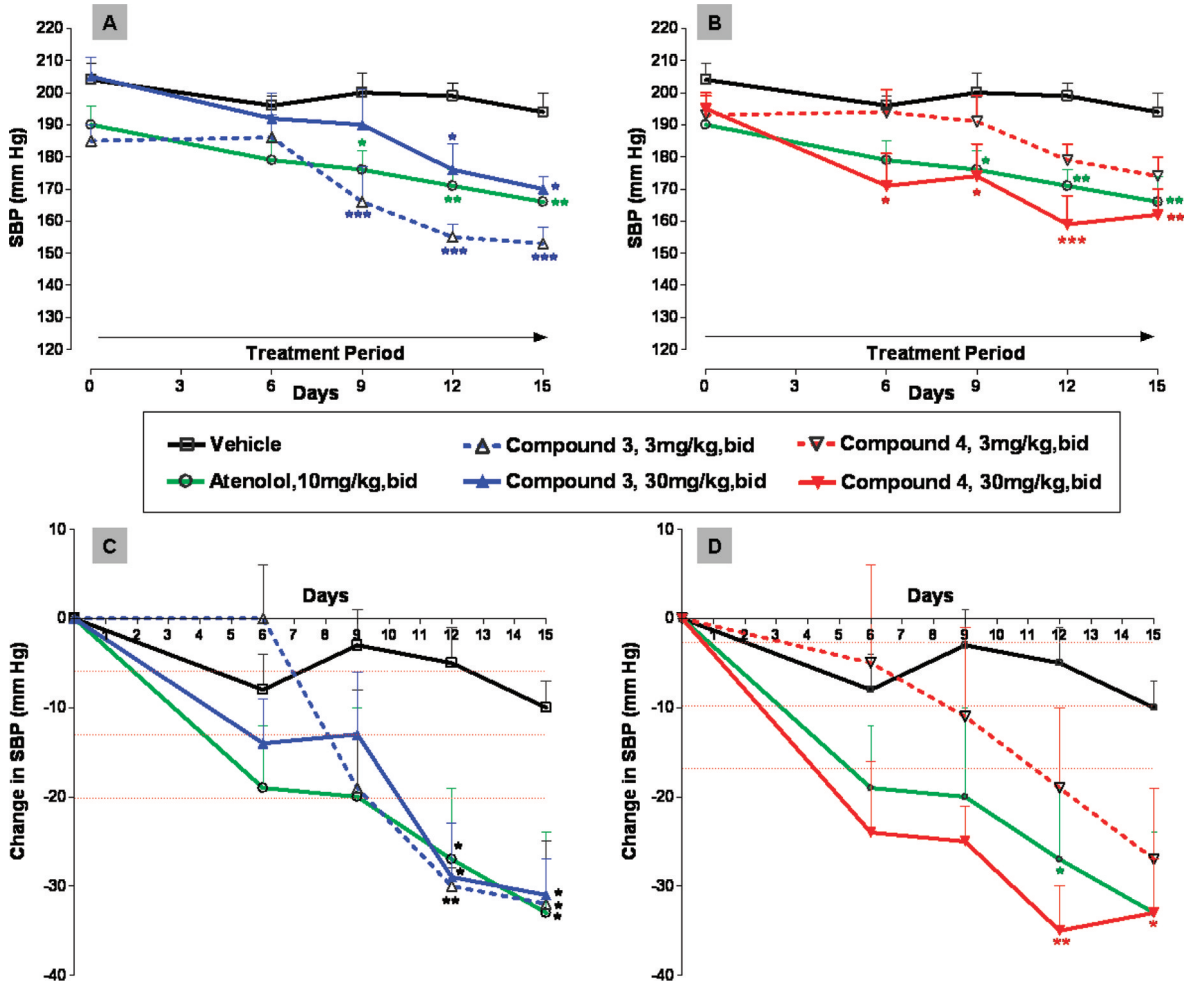

Figure 2. (A and B) Lowering of SBP and (C and D) change in SBP from baseline by Hao2 inhibitors **3** and **4**, respectively, in a DOCA model (n = 6−8). Compared to the control group on each day, *P < 0.05, **P < 0.01, and ***P < 0.001. Two-way analysis of variance (ANOVA) followed by a Bonferroni post test.

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Table 2. Drug Levels of Compounds 3 and 4 in Plasma and Kidney

		concn 1 h postdose (n = 3)		concn 12−15 h postdose (n = 3)		
compd	dose (mg/kg)	plasma (μM)	kidney (μmol/g)	plasma (μM)	kidney (μmol/g)	ΔSBP (mmHg) on day 15
3	3	2.2 ± 0.5	10 ± 1	0.2 ± 0.1	3 ± 1	32 ± 7
	30	8 ± 2	24 ± 3	1.9 ± 0.8	7 ± 2	31 ± 4
4	3	48 ± 11	34 ± 5	12 ± 2	13 ± 12	27 ± 8
	30	157 ± 9	113 ± 36	86 ± 13	NA[a]	33 ± 6

[a]Not available.

compounds in a range of in vitro assays. First, we tested their potential cross reactivity against a closely related enzyme, Hao1.[16] Compound **3** inhibited rat Hao1 with an IC_{50} of 45.7 μM, while compound **4** was completely inactive at 10 μM, indicating that compounds **3** and **4** have a minimum 150-fold selectivity against rat Hao1. Compounds **3** and **4** were further profiled against a panel of 125 targets (MDS PanLab Drug Matrix Screen), which includes most targets known to regulate blood pressure, and were shown to be completely inactive at 10 μM. Additionally, compounds **3** and **4** were screened against the GPR109a[20] receptor agonist assay, as they are structurally similar to the known high-affinity agonists 3-methylpyrazole-5-carboxylic acid and 3-*n*-butylpyrazole-5-carboxylic acid, and were found to be completely inactive at 10 μM.

The in vitro pharmacokinetic (PK) properties of compounds **3** and **4** are summarized in Table 1. Both leads showed low rates of oxidative metabolism in rat liver microsomes (RLM) and no cytotoxicity in the HepG2 cell line. They neither inhibit nor induce cytochrome P450 at 10 μM. Both were highly (>99%) bound to rat plasma protein.

The in vivo PK properties of **3** and **4** were examined in rats (Table 1). Both compounds exhibited low systemic plasma clearance (8 mL/min/kg for **3** and 0.52 mL/min/kg for **4**) and elimination half-lives (1.7 h for **3** and 3.7 h for **4**). At an oral dose of 10 mg/kg, compound **4** showed much higher plasma exposure (C_{max} = 134 μM) than compound **3** (C_{max} = 24 μM) with an $AUC_{0-8\,h}$ values of 517 and 27 μM h^{-1} for compounds **4** and **3**, respectively. Compounds **3** and **4** exhibited oral bioavailability of 38 and 53%, respectively. Thus, both leads were selected for investigation of their antihypertensive effect in the well-studied DOCA salt model of hypertension in treatment as well as prevention of hypertension mode.

The effect of Hao2 inhibitors in a DOCA salt rat model of hypertension (treatment mode) was examined. To investigate the potential effect of Hao2 inhibitors on blood pressure regulation, compounds **3** and **4** along with atenolol (as a positive control) were studied in a well-established rat model of hypertension, DOCA salt-treated Wistar rats.[16] Treatment with atenolol resulted in a significant reduction in systolic blood pressure (SBP) (Figure 2). Treatment with compound **3** or **4** reduced ∼30 mmHg of SBP from the baseline at a dose of 30 mg/kg. Compound **3** exhibited a similar reduction in blood pressure at a dose of 3 mg/kg, but the scale of blood pressure reduction by compound **4** was lower at a dose of 3 mg/kg.

To demonstrate the role of Hao2 inhibition in the observed lowering of SBP, the degree of inhibition of enzyme activity in vivo (target engagement) was assessed. Rats were sacrificed at the end of the treatment period, and their plasma and kidneys were collected 1 and 12−15 h postdosing for estimation of drug concentration and ex vivo inhibition of Hao2 activity. Appropriate drug concentrations for Hao2 inhibition were observed (Table 2). The ex vivo Hao2 activity was studied by monitoring the conversion of [^{3}H]-2-hydroxyoctanoic acid into 2-ketooctanoic acid with the release of [^{3}H]H_2O using kidney slices.[16] Both compounds (**3** and **4**) showed target engagement 1 h postdosing at 3 and 30 mg/kg, whereas 12−15 h postdosing, target engagement was observed at only the 30 mg/kg dose (Table 3). Demonstration of appropriate drug concentration (compound **3** or **4**) and target engagement data clearly indicate the role of Hao2 inhibition in the observed lowering of SBP.

The effect of Hao2 inhibitors in a DOCA salt rat model of hypertension (prevention mode) was examined. Compound **3** was further tested in a hypertension prevention model along with atenolol. In this study, DOCA salt (25 mg/kg, twice a week) was administered in uninephrectomized male Wistar rats continuously for 35 days.[21,22] A sham control group (surgery with no nephrectomy and without DOCA administration) was also included in this study. Compound treatment[23] was initiated on day 14 of DOCA administration and continued for the next 21 days. Blood pressure was measured in conscious rats on days 0, 7, 14, 28, 30, and 35 of DOCA administration by tail-cuff plethysmography 12−15 h after the last dose of compound had been administered. Treatment with compound **3** at 30 mg/kg significantly attenuated DOCA salt-induced BP elevation (Figure 3) from

Table 3. Target Engagement Study

	Conversion of [^{3}H]-2-Hydroxyoctanoate to [^{3}H]H_2O (% inhibition vs vehicle)			
	3		**4**	
dose (mg/kg)	1 h postdose	12−15 h postdose	1 h postdose	12−15 h postdose
3	29 ± 15	0 ± 12	75 ± 5	0 ± 55
30	38 ± 13	28 ± 16	93 ± 3	42 ± 9

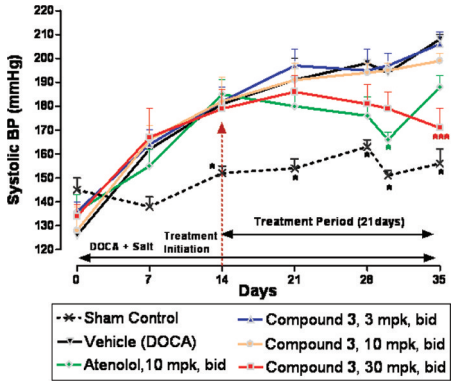

Figure 3. Prevention of DOCA-induced hypertension (n = 6). Compared to the control group on each day, *P < 0.05 and ***P < 0.001. Two-way analysis of variance (ANOVA) followed by a Bonferroni post test.

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day 14 to day 35, suggesting its role in preventing kidney damage.

In summary, we have successfully identified pyrazole-3-carboxylic acids, lead compounds **3** and **4**, as potent and selective inhibitors of rat Hao2. These compounds are metabolically stable, with good PK profiles, and demonstrate in vivo efficacy in the DOCA model of hypertension and for the first time validated that inhibition of Hao2 leads to lowering of blood pressure in a well-established rat hypertension model. We hope the development of such inhibitors will facilitate studies in understanding the mechanism of Hao2 in blood pressure regulation.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, analytical data for compounds **3**−**5**, expression and purification of recombinant proteins, in vitro screening protocol, protocol for the DOCA model, blood pressure measurement, and target engagement assay. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: 91-20-66539630. Fax: 91-20-6653 9620. E-mail: dinesh.barawkar@advinus.com.

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(23) Compound **3** was orally dosed with the same vehicle (0.5% Tween 80 and 0.5% sodium methylcellulose) at 3, 10, and 30 mg/kg, twice a day. Atenonol was used as the positive control in this study, with a dose of 10 mg/kg, twice a day.