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Soluble epoxide hydrolase inhibitor, *t*-TUCB, protects against myocardial ischemic injury in rats

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Introduction

Ischemic heart disease (IHD) is one of the major cardiovascular disorders and reported to be the single largest cause of death in the developed countries and a main contributor to the disease burden in developing countries.^[1] A majority of drugs used against myocardial ischemia such as nitrates, β blockers, calcium channel blockers and angiotensin converting enzyme inhibitors reduce imbalance between oxygen consumption and oxygen supply. Other drugs like anti-atherosclerotic and thrombolytic drugs reduces the chances of reoccurrence of the events and improve hemodynamic parameters, however, none prevent direct cell death that occurs during the ischemic injury. Reperfusion of viable myocardium would be beneficial for survival of the heart cells; paradoxically, however, the reperfusion of ischemic myocardium may lead to arrhythmias, myocardial stunning (i.e., reversible contractile dysfunction), or lethal reperfusion injury. Reperfusion injury is mediated in part by oxygen free radicals, neutrophil–endothelium interactions, apoptosis, and intracellular calcium overload.^[2-4] The current drugs used in the management of IHD, however, have no ability to prevent cell death that occurs during the ischemic reperfusion injury. The better management of IHD, therefore, warrants the development of novel agents.

Epoxyeicosatrienoic acids (EETs) are endogenous oxylipids reported to play an important role in cardioprotection. The cardiovascular effects of EETs range from vasodilation, anti-hypertension, pro-angiogenesis, anti-atherosclerosis, and anti-inflammatory to cytoprotection.^[5] The ability of EETs to prevent cell death during reperfusion injury is attributed to their anti-apoptotic, pro-mitogenic, and anti-migration effects at the cellular levels.^[5, 6] EETs are reported to reduce calcium overload during cardiac ischemia by activating ATP-sensitive K^+ channels (K_{ATP}).^[7-9] Opening of the K_{ATP} ATP channels allows a K^+ efflux, resulting in an increased rate of repolarization and a decreased period of

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Conflict of Interest: Sung Hee Hwang and Bruce D Hammock are authors on University of California patents on soluble epoxide hydrolase inhibitors.

Ca²⁺ influx during the plateau phase of the action potential.^[10, 11] In addition EETs are reported to be pro-mitogenic and attenuate reactive oxygen species level, mitochondrial dysfunction, caspase activation, cell migration and apoptosis. All the above effects of EETs are reported to play an important role in their cardioprotection against reperfusion injury.^[12-15]

The cytochrome P450 system catalyzes the formation of EETs from arachidonic acid. The EETs are later converted to biologically inactive or less active diols by soluble epoxide hydrolase (sEH) enzyme.^[16] One of the novel strategies, therefore, is to inhibit the enzyme sEH and thereby promote the cardioprotective benefits of EETs. A diverse class of agents such as amides, ureas, thioamides, thioureas, carbamates, acyl hydrazones, chalcone oxides, etc., have been reported to possess potential as both *in vitro* and *in vivo* sEH inhibitors.^[17] Among these, compounds containing either urea or amide moiety as central pharmacophore are reported to be the most potent inhibitors.^[18] Hammock et al., have synthesized and reported the structural activity relationships of various N, N'-disubstituted ureas.^[19, 20] They report the poor solubility and rapid *in vivo* metabolism of the early sEH inhibitors resulted in reduced *in vivo* activity.^[21] They have also carried out various modifications in the structure to improve their pharmacokinetics. Positioning of polar residues such as esters, sulfones, amides and carbamates roughly 5-7 Å from the central pharmacophore has been reported to improve binding without reducing the potency, however, some of these molecules were found to be metabolically unstable.^[20] To overcome these problems, conformationally restricted N, N'-disubstituted ureas having polar substitutions have been synthesized and evaluated for their *in vitro* sEH inhibition activity and also for their bioavailability. These derivatives have reported to show improved bioavailability and *in vitro* activity.^[22, 23] In the present study, one such molecule, *trans*-4-{4-[3-(4-trifluoromethoxyphenyl)ureido]cyclohexyloxy}benzoic acid (*t*-TUCB), synthesized by this group has been chosen for the evaluation of its *in vivo* cardioprotective activity against isoproterenol (ISO) induced myocardial infarction in rats. This molecule has a central N, N'-disubstituted urea pharmacophore in which a cyclohexane ring serves as a linker (between a urea and a polar group) and also as a template to restrict flexibility of the structure (Figure 1).

Materials and Methods

Animals

Male Wistar albino rats and Swiss albino mice were procured from in-house animal facility of J.S.S College of Pharmacy, Udhagamandalam. The animals were housed under laboratory conditions of temperature (18-24 °C) and relative humidity (55-75%) with a 12:12 h light-dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. All the experiments were performed with a prior approval from the Institutional Animal Ethics Committee of J.S.S College of Pharmacy, Udhagamandalam (Approval no. JSSCP/IAEC/M.PHARM/PH.COLOGY/01/2012-13).

Chemicals and reagents

The soluble epoxide hydrolase inhibitor, *t*-TUCB, was synthesized as previously described.^[19] Isoproterenol (ISO) was from Sigma Aldrich Ltd., India. The creatine kinase (CK-MB), lactate dehydrogenase (LDH) and calcium kits were from Labkit Ltd., India. All other chemicals and reagents used were of analytical grade.

Preparation of test compound

t-TUCB stock solutions (1/10 of the dose) were prepared in 20% PEG-400 in saline and administered at a dose volume of 10 ml/kg, body weight.

Acute oral toxicity study

Acute oral toxicity of *t*-TUCB was evaluated in Swiss albino mice as per OECD 423 guidelines. A limit test at a dose of 2000 mg/kg, body weight was carried out using 6 mice (3 per step). Following overnight fasting the first set of 3 animals were dosed with the test compound and observed for clinical signs and mortality at 30, 60, 120 and 240 min on day 0, and thereafter daily once for a period of 14 days. Based on 24h observation results of the first set, the second set of 3 animals were dosed with the same dose and observed. Body weights were recorded prior to administration and again on days 7 and 14. On day 15, all the animals were culled by deep ether anesthesia and subjected to gross necropsy analysis.

In vivo cardioprotective activity

Forty male Wistar rats were divided into 5 groups of 8 each. The group 1 and 2 animals received vehicle (10 mL/kg, p.o.) and served as normal and control, respectively. The group 3-5 animals received *t*-TUCB at 3, 10 and 30 mg/kg, p.o., respectively. On day 13 and 14 all the groups except the group 1 received subcutaneous ISO injection at a dose of 150 mg/kg, body weight. 24h after the last ISO injections EKG parameters were monitored from all the groups using PowerLab (AD Instruments, Australia). Later, blood was collected from retro-orbital sinus under light ether anesthesia and used for the estimation of the biochemical parameters (CKMB and LDH). Thereafter, rats were sacrificed by cervical dislocation and hearts were excised, weighed and immediately frozen in liquid nitrogen for the analysis of infarct size, myocardial calcium and antioxidant enzymes.

In vivo electrocardiographic (EKG) recordings

All electrocardiographic recordings were done with Animal Bio Amp (FE136, AD instruments, Australia). For *in vivo* recordings of EKG, rats were anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (15 mg/kg, i.m.). The positive and negative lead I electrodes were inserted into left and right forearms, respectively. The reference electrode was grounded to right hind limb. All the parameters of EKG were recorded using LabChart 7 Pro software (AD instruments, Australia).

Estimation of serum CK-MB and LDH

Blood was allowed to clot and serum was separated by centrifuging at 3000 rpm for 10 min in a cold centrifuge. The serum CK-MB and LDH levels were estimated in Merck-200 semi auto analyzer (Merck Ltd, Darmstadt, Germany) using Labkit diagnostic kits.

Infarct size analysis

Briefly, hearts were cut into thin cross sectional slices (1-2 mm) and kept in 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in phosphate buffer (0.1 M, pH 7.4) at 37 °C for 20 min. The individual slices were digitally photographed and the area of infarction was quantified by measuring stained (red, live tissue) and unstained (white, necrotic) regions using Image J, version 1.47 (NIH, USA) and the percentage infarct size was calculated.

Estimation of cardiac calcium and antioxidant parameters

One part of heart tissue was homogenized with 5 parts of phosphate buffer (0.1 M, pH 7.4) in Elvenjan homogenizer fitted with a Teflon plunger at 600 rpm for 20 minutes. The homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used to estimate Ca²⁺ levels and antioxidant parameters.

Calcium was estimated in tissue homogenate using calcium kit as per the manufacturer's protocol. Calcium with arsenazo III (1, 8-Dihydroxy-3, 6-disulpho-2, 7-naphthalene-bis (azo)-dibenzene-arsonic acid), at neutral pH, yields a blue colored complex. The intensity of the color formed is proportional to the calcium concentration in the sample. All the glass ware was pretreated with concentrated nitric acid overnight followed by extensive rinsing before the estimation. Lipid peroxide level in tissue homogenate was estimated by the method of Okhawa.^[24] Catalase was estimated according to the method of Beers and Sizer^[25] and SOD in tissue homogenate was estimated as previously described.^[26]

Statistical analysis

Values are expressed as the mean ± SEM. Statistical significance was determined by one way ANOVA followed by Dunnett's multiple comparison test to assess differences between the groups. Values were considered significant, if $p < 0.05$.

Results

Acute oral toxicity study

All the six mice treated with *t*-TUCB at 2000 mg/kg, p.o., showed no abnormal clinical signs during the study period and there were no mortalities. All the animals gained 1-2 g of body weight during the study period (Table 1). The gross necropsy analysis carried out at the end of the study showed no abnormal findings. Based on the above results the acute oral LD₅₀ of *t*-TUCB was, therefore, considered greater than 2000mg/kg body weight.

In vivo cardioprotective activity

Effect of *t*-TUCB on EKG—The results are given in Table 2 and Figure 2. Pretreatment with *t*-TUCB at 3, 10 and 30 mg/kg, p.o., significantly prevented ISO induced EKG abnormalities such as prolongation of QTc interval, ST height depression, pathological Q wave formation and T-wave inversion ($p < 0.05$).

Effect of *t*-TUCB on relative heart weight and serum CK-MB and LDH—The results are given in Table 3. Pretreatment with *t*-TUCB at 10 and 30 mg/kg, p.o.,

significantly prevented ISO induced increase in heart weight and elevation of CK-MB and LDH levels ($p < 0.05$), indicating its cardioprotective effect against ISO induced cardiac injury. At 3 mg/kg, p.o. *t*-TUCB only showed significant protection against heart weight changes.

Effect of *t*-TUCB on infarct size—The results are given in Table 4 and Figure 3. Pretreatment with *t*-TUCB at 3, 10 and 30 mg/kg, p.o., significantly reduced the ISO induced infarct size ($p < 0.05$) when compared to control. The calculated percentage reductions for these doses were found to be 15.90, 46.60 and 40.44%, respectively.

Effect of *t*-TUCB on myocardial calcium and antioxidant parameters—The results are given in Table 5. ISO treatment significantly increased the intracellular calcium and lipid peroxide levels, and decreased the SOD and catalase levels ($p < 0.05$). Pretreatment with *t*-TUCB at 3, 10 and 30 mg/kg, p.o., significantly prevented the above changes induced by ISO ($p < 0.05$), except, low dose showing a non-significant protection against lipid peroxide, catalase and SOD levels.

Discussion

t-TUCB, is a potent *in vitro* sEH inhibitor ($IC_{50} = 0.9$ nM), and is reported to show improved bioavailability and metabolic stability than previous urea derivatives.^[22, 27] It is in formal clinical trials in cats, dogs and horses, and a preliminary case study for a successful treatment of *Equine laminitis* has been published.^[28] In the current study we, therefore, selected this molecule for the investigation of its cardioprotective effects against ISO induced myocardial infarction in rats. ISO-induced MI injury model is a well-developed model for ischemia related ventricular remodeling and cardiac hypertrophy. Also, high dose treatment with ISO had been seen to produce myocardial infarction in multiple animal models.^[29-31] High dose ISO treatment triggers various cellular pathways and leads to activation of several apoptotic events. Most of its effects are attributed due to its ability to cause functional hypoxia and ischemia, coronary insufficiency, alterations in metabolism, decreased level of high-energy phosphate stores, intracellular Ca^{2+} overloading, changes in electrolyte contents and oxidative stress.

In this study, the typical changes induced with ISO treatment were EKG related abnormalities (QTc interval prolongation, ST height depression, appearance of pathological Q waves and T wave inversion), increased cardiac mass, infarct size, CK-MB and LDH levels in serum, myocardial Ca^{2+} and free radical generation. Our results demonstrated that treatment with *t*-TUCB significantly abolished development of EKG abnormalities, reduced the cardiac hypertrophy, infarct size, intracellular Ca^{2+} overloading, myocardial damage and inflammation and free radicals generation. Moreover, the use of sEHIs resulted in the prevention of electrical remodeling post MI and in so doing, prevents the propensity for the development of life threatening cardiac arrhythmias as assessed by electrophysiological studies *in vivo*.

In vivo EKG recordings revealed a dose dependent prevention of QTc interval prolongation and ST height depression, appearance of pathological Q waves and T wave inversion with

10 and 30 mg/kg doses being statistically significant for all the measured parameters while a non-significant protection was seen at 3 mg/kg dose. Ischemia presents a condition where there is abnormality in distribution of ions and ion channels. Prolonged ischemia leads to inactivation of delayed rectifier K⁺ channels. These channels are responsible for repolarization current after initiation of an action potential in ventricular cardiomyocytes.^[32] QT prolongation reflects a decreased repolarization following depolarization of the cardiac ventricles, whereas ST height abnormality provides a measure of ischemic injury. In acute myocardial infarction, ST-segment depression is often seen in leads opposite to the affected area. The ST segment may be depressed in subendocardial infarction (an infarction that does not involve the full thickness of the left ventricle) as in case of ISO induced MI, or during an exercise stress test in a patient suspected of having coronary ischemia. .

Cardiac biomarkers such as CK-MB and LDH have long been used as markers of cardiac injury and inflammation. We correlated our drug efficacy in terms of levels of these marker enzymes in serum. Animals treated with ISO showed marked increase in serum levels of these enzymes. When pre-treated with *t*-TUCB, for a period of 2 weeks, serum levels of these enzymes were not significantly elevated as that seen with ISO alone. Previous studies have suggested that the progressive deterioration in cardiac function post MI is associated with cellular apoptosis. In our study, *t*-TUCB has shown beneficial effects in preventing cellular apoptosis by decreasing Ca²⁺ overloading in cells and preventing the formation of oxygen free radicals. Our results show a dose dependent decrease in formation of lipid peroxide and increase in catalase and SOD levels. Also, protection against injury was seen by reduced infarct size in *t*-TUCB treated hearts at all doses.

Conclusions

Electrocardiographic, biochemical and histological changes associated with ISO treatment is well co-related in this study and treatment with our inhibitor, *t*-TUCB was able to protect the ischemic heart against all of these changes. Hence, the use of sEHIs appears to be an attractive opportunity to prevent post-ischemic injury.. In conclusion, our findings provide a strong evidence for *in vivo* efficacy of newer soluble epoxide hydrolase inhibitor, *t*-TUCB, in ameliorating myocardial ischemic injury.

Acknowledgments

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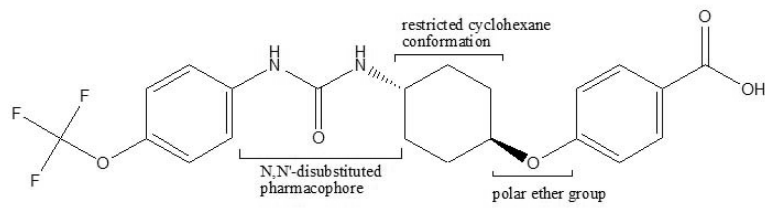


Figure 1.
trans-4-{4-[3-(4-Trifluoromethoxyphenyl)ureido]cyclohexyloxy}benzoic acid (*t*-TUCB)

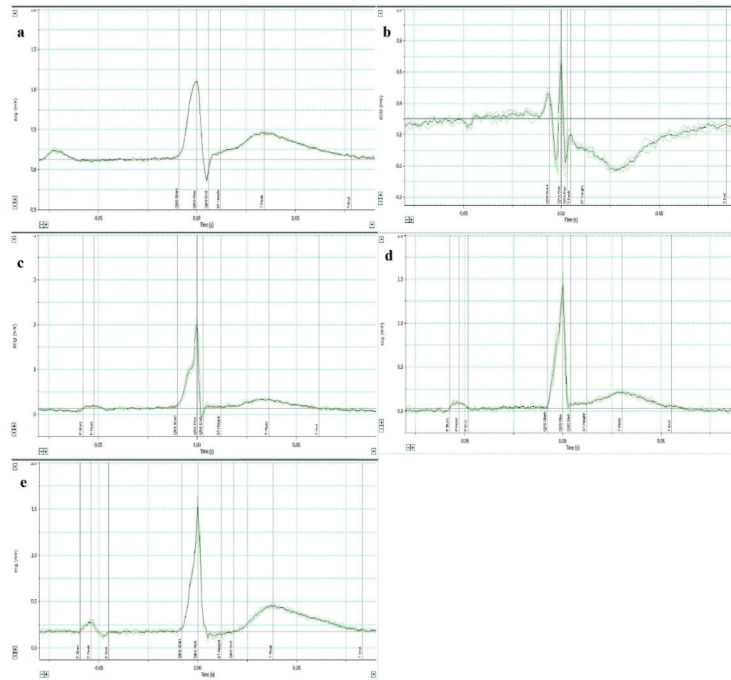


Figure 2. Effect of sEH inhibitor, *t*-TUCB, in preventing EKG abnormalities in rats
Representative images of EKG recordings in a) normal, b) control, c) *t*-TUCB (3 mg/kg), d) *t*-TUCB (10 mg/kg) and e) *t*-TUCB (30 mg/kg) groups using LabChart pro 7.0.

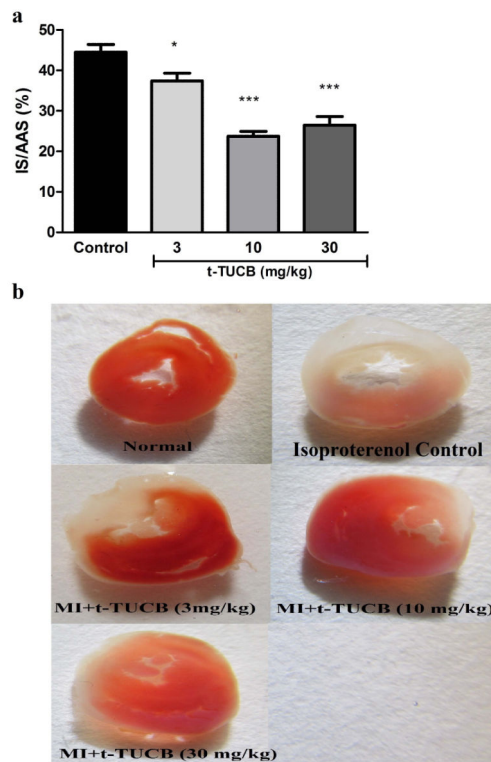


Figure 3. Effect of sEH inhibitor, *t*-TUCB, in ameliorating infarct size

a) Quantification of infarct size and b) Representative images of TTC staining in *t*-TUCB treated and control hearts. Surviving tissue stained red with TTC and infarcted white tissue. Bars represent mean \pm SEM; $n=3$ per group; * $p<0.05$; *** $p<0.001$ vs. Control rats. One way ANOVA followed by Dunnett's multiple comparison test.

Table 1

Acute oral toxicity study results of t-TUCB in female Swiss mice

Animal no.	Body weight (g)			Gross necropsy findings
	Initial	Day7	Day14	
1.	28	28	29	NAD
2.	26	27	27	NAD
3.	26	28	28	NAD
4.	25	25	26	NAD
5.	27	27	28	NAD
6.	24	26	26	NAD

NAD: No Abnormalities Detected

Table 2

Effect of t-TUCB treatment on ISO induced EKG changes in rats

Groups	QTc interval (s)	ST height (mV)	Q wave (mV)	T wave (mV)
Normal	0.147±0.015	0.036±0.032	0.023±0.010	0.206±0.040
Control	0.206±0.008*	-0.157±0.032*	-0.160±0.109*	-0.010±0.07*
t-TUCB (3 mg/kg)	0.191±0.009	0.015±0.002 [†]	0.059±0.007 [†]	0.221±0.04 [†]
t-TUCB (10 mg/kg)	0.164±0.006 [†]	0.050±0.021 [†]	0.034±0.008 [†]	0.223±0.05 [†]
t-TUCB (30 mg/kg)	0.163±0.010 [†]	-0.013±0.021 [†]	-0.100±0.121 [†]	0.161±0.043 [†]

Values represent mean ± SEM; n=6 per group;

* $p < 0.05$ vs. Normal;[†] $p < 0.05$ vs. Control rats. One way ANOVA followed by Dunnett's multiple comparison test.

Table 3Effect of *t*-TUCB treatment on ISO induced changes in relative heart weight and CK-MB and LDH levels.

Groups	Heart to body weight (mg/g)	CK-MB (IU/L)	LDH (U/L)
Normal	4.165±0.487	76.14±13.38	699.2±55.8
Control	5.461±0.716*	163.90±15.50*	1612.1±215.7*
<i>t</i> -TUCB (3 mg/kg)	4.586±0.410 [†]	116.70±17.68	1318.2±179.6
<i>t</i> -TUCB (10 mg/kg)	4.553±0.345 [†]	96.29±12.11 [†]	874.4±97.8 [†]
<i>t</i> -TUCB (30 mg/kg)	4.606±0.538 [†]	98.71±14.07 [†]	968.6±144.3 [†]

Values represent mean ± SEM; n=6 per group;

* $p < 0.05$ vs. Normal;[†] $p < 0.05$ vs. Control rats. One way ANOVA followed by Dunnett's multiple comparison test.

Table 4Effect of *t*-TUCB treatment on ISO induced infarct size

Groups	% infarct size	% protection fold in reducing infarct
Normal	---	---
Control	44.5±4.7	0.0
<i>t</i> -TUCB (3 mg/kg)	37.4±4.7*	15.9
<i>t</i> -TUCB (10 mg/kg)	23.7±3.0***	46.6
<i>t</i> -TUCB (30 mg/kg)	26.5±5.2***	40.4

Values represent mean ± SEM; n=3 per group;

* $p < 0.05$;*** $p < 0.001$ vs. Control rats. One way ANOVA followed by Dunnett's multiple comparison test.

Table 5Effect of *t*-TUCB treatment on ISO induced changes in intracellular Ca²⁺ ions and antioxidants levels.

Groups	Intracellular Ca ²⁺ concentration (mg/g tissue)	Lipid peroxidation (nM MDA/mg tissue)	Catalase (U/min/mg tissue)	SOD (U/min/mg tissue)
Normal	0.374±0.003	0.109±0.010	1.266±0.086	2.327±0.285
Control	0.454±0.012*	0.521±0.016*	0.499±0.089*	1.126±0.156*
<i>t</i> -TUCB (3 mg/kg)	0.419±0.011 [†]	0.381±0.067	0.668±0.102	1.366±0.172
<i>t</i> -TUCB (10 mg/kg)	0.419±0.005 [†]	0.332±0.067 [†]	1.015±0.159 [†]	1.938±0.169 [†]
<i>t</i> -TUCB (30 mg/kg)	0.418±0.006 [†]	0.325±0.041 [†]	1.006±0.128 [†]	1.920±0.142 [†]

Values represent mean ± SEM; n=3 per group;

* $p < 0.05$ vs. Normal;[†] $p < 0.05$ vs. Control rats. One way ANOVA followed by Dunnett's multiple comparison test.