

## Inhibition of soluble epoxide hydrolase contributes to the anti-inflammatory effect of antimicrobial triclocarban in a murine model

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### ABSTRACT

The increasing use of the antimicrobial triclocarban (TCC) in personal care products (PCPs) has resulted in concern regarding environmental pollution. TCC is a potent inhibitor of soluble epoxide hydrolase (sEH). Inhibitors of sEH (sEHIs) are anti-inflammatory, anti-hypertensive and cardio-protective in multiple animal models. However, the *in vivo* effects anticipated from a sEHI have not been reported for TCC. Here we demonstrated the anti-inflammatory effects *in vivo* of TCC in a murine model. TCC was employed in a lipopolysaccharide (LPS)-challenged murine model. Systolic blood pressure, plasma levels of several inflammatory cytokines and chemokine, and metabolomic profile of plasma oxylipins were determined. TCC significantly reversed LPS-induced morbid hypotension in a time-dependent manner. TCC significantly repressed the increased release of inflammatory cytokines and chemokine caused by LPS. Furthermore, TCC significantly shifted the oxylipin profile *in vivo* in a time-dependent manner towards resolution of inflammation as expected from a sEHI. These results demonstrated that at the doses used TCC is anti-inflammatory in the murine model. This study suggests that TCC may provide some benefits in humans in addition to its antimicrobial activities due to its potent inhibition of sEH. It may be a promising starting point for developing new low volume high value applications of TCC. However these biological effects also caution against the general over use of TCC in PCPs.

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### Introduction

Triclocarban (3,4,4'-trichlorocarbanilide, TCC) is an antimicrobial agent that has been used over 40 years in personal care products (PCPs) such as soaps and disinfectants (Roman et al., 1957; Black et al., 1975). TCC and other PCPs are currently used in very large amounts in the United States and Europe. Pharmacokinetics of TCC has been investigated in multiple species including mouse (Demetruilias et al., 1984), rat (Howes and Black, 1976; Jeffcoat et al., 1977; Warren et al., 1977; Birch et al., 1978), monkey (Birch et al., 1978; Hiles et al., 1978)

and human (Scharpf et al., 1975; Howes and Black, 1976; Birch et al., 1978; Hiles and Birch, 1978; Northroot et al., 1984; Schebb et al., 2011). Specifically, 1.1% of TCC was deposited on hairless mice skin by dermal exposure to TCC-containing soap bar for 10 min (Demetruilias et al., 1984), which is compared to the amount deposited onto the skin of human (Northroot et al., 1984). Parenteral injection or dermal use of TCC resulted in low levels of TCC in rat blood, indicating rapid metabolism of TCC in rodent models (Howes and Black, 1976). The principal metabolites of TCC common to rat, monkey and human were the sulfate and glucuronide conjugates of 2', 3'- and 6-hydroxy-TCC while the major urinary metabolites of TCC found in human and monkey were N- and N'-TCC glucuronides (Jeffcoat et al., 1977; Warren et al., 1977; Birch et al., 1978; Schebb et al., 2011). Oral administration of TCC to human at a dose of 2.2 μmol/kg body weight resulted in the maximum plasma level of 3.7 μM occurred 2.8 h after dosing. And the same study demonstrated that TCC was mainly excreted via fecal elimination (70% of dose) and urinary excretion (27% of dose) completed in 120 h and 80 h after dosing respectively (Hiles and Birch, 1978). In short, TCC is rapidly metabolized in both human and animal models. However, TCC biodegrades much more slowly in the environment than it does in humans and other animals (Gledhill, 1975; Ying et al., 2007). As an antimicrobial agent in PCPs, TCC enters the environment mainly by discharge of effluent from

**Abbreviations:** APAU, 1-(1-acetylpiperidin-4-yl)-3-adamantanyllurea; t-AUCB, trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid; CBA, Cytometric Bead Array; CIU, N-cyclohexyl-N'-(4-iodophenyl)urea; COX, cyclooxygenase; CUDA, 12-(3-cyclohexan-1-yl-ureido)-dodecanoic acid; DHET, dihydroxyeicosatrienoic acid; t-DPPO, [<sup>3</sup>H]-trans-1,3-diphenyl-trans-propene oxide; EET, epoxyeicosatrienoic acid; IFN-γ, Interferon-gamma; IL-6, interleukin-6; ip, intraperitoneally; K<sub>i</sub>, dissociation constant; K<sub>iapp</sub>, apparent inhibition constant; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LOX, lipoxygenase; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; PCP, personal care product; po, per oral; sEH, soluble epoxide hydrolase; sEHI, inhibitor of soluble epoxide hydrolase; TCC, triclocarban; TNF-α, tumor necrosis factor-alpha; TPAU, 1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea.

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wastewater treatment plants and disposal of biosolids on land. In addition, TCC is a hydrophobic chemical, capable of forming multiple hydrogen bonds. These physical properties lead TCC to partition from a waste water stream where it binds to sludge and becomes recalcitrant to microbial degradation (Heidler et al., 2006; Ying et al., 2007). This results in an accumulation of TCC in not only aqueous effluent but also the resulting biosolids. Thus TCC has been found to be one of the serious contaminants of water resources nationwide in the United States (Halden and Paull, 2005). Furthermore, TCC was recently found at higher concentrations to enhance the bioactivity of several hormones *in vitro* and *in vivo*, but it alone has little agonist activity (Ahn et al., 2008; Chen et al., 2008). Although the bioactivity and toxicity of TCC are still being explored, a general concern has been raised regarding the extensive use of TCC.

TCC, an *N,N'*-disubstituted urea, has been reported to be a potent inhibitor of recombinant murine and human soluble epoxide hydrolases (sEH) *in vitro* (Table 1) (Morisseau et al., 1999; McElroy et al., 2003; Morisseau et al., 2009). The sEH is the main enzyme that catalyzes the metabolism of epoxyeicosatrienoic acids (EETs) and other fatty acid epoxides into the more polar and usually less potent metabolites, dihydroxyeicosatrienoic acids (DHETs) and other corresponding diols (Morisseau and Hammock, 2005; Newman et al., 2005). EETs and other epoxy fatty acids have been demonstrated to be potent anti-inflammatory and cardio-protective mediators (Node et al., 1999; Spector et al., 2004; Dhanasekaran et al., 2006; Larsen et al., 2006a; Nithipatikom et al., 2006; Seubert et al., 2007; Li et al., 2009). The inhibitors of sEH (sEHIs) stabilize the endogenous EET levels by inhibiting sEH and thus reduce inflammation, cardiovascular diseases, nephrotoxicity and other diseases in multiple animal models (Jung et al., 2005; Schmelzer et al., 2005; Larsen et al., 2006b; Morisseau et al., 2006; Xu et al., 2006; Parrish et al., 2009). In addition, sEHs synergize the anti-inflammatory effect of non-steroidal anti-inflammatory drugs (indomethacin, celecoxib, and rofecoxib) (Schmelzer et al., 2006), aspirin and the 5-lipoxygenase activation protein inhibitor MK886 (Liu et al., 2010). Thus, we hypothesized that TCC may be beneficial in several models of inflammatory diseases in human and/or animals due to its sEH inhibition activity. Of course the unintentional or inappropriate inhibition of the sEH may lead to undesirable effects.

In this study, we address a possible health outcome *via* exposure either through the environment or personal use of products containing TCC by directly investigating its *in vivo* effects in a murine model of inflammation by lipopolysaccharide (LPS) challenge. The sEH inhibition demonstrated by TCC shows the expected alterations in oxylin profile *in vivo*. Moreover, TCC resulted in the reversal in LPS-challenged

hypotension as well as the amelioration of the increased release of the inflammatory cytokines similar to other sEHIs. Thus, TCC exposure may result in beneficial effects on human health such as anti-inflammation, reduced hypertension, and cardiovascular protection based on its sEH inhibitory activity. These effects should be particularly dramatic near the site of application of PCPs.

## Materials and methods

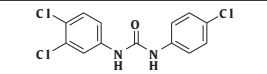
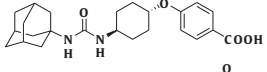
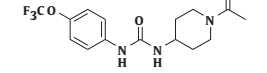
**Animals and chemicals.** All procedures and animal care were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee of the University of California, Davis. Male Swiss Webster mice were purchased from Charles River Laboratories. <sup>13</sup>C-labeled TCC (4'-chlorophenyl-<sup>13</sup>C<sub>6</sub>, 99%) used as an internal standard in drug level determination was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Acetonitrile and methanol used for liquid chromatography tandem mass spectrometry (LC/MS/MS) were purchased from Fisher Scientific (Pittsburgh, PA). Oleic oil (a mixture of oleic ester rich triglycerides) was purchased from Adams Vegetable Oils, Inc. (Arbuckle, CA). TCC, LPS (*Escherichia coli* serotype, L4130, 0111:B4), and polyethylene glycol (PEG400) were purchased from Sigma-Aldrich (St. Louis, MO). EDTA (K<sub>3</sub>) was purchased from Tyco Health Group LP (Mansfield, MA). Water (>18.0 MΩ) was purified by a NANO pure system (Barnstead, Newton, MA). Cytometric Bead Array (CBA) mouse inflammation kit was purchased from BD Biosciences (San Jose, CA). Oxylin standards were purchased from Cayman Chemical (Ann Arbor, MI). The sEHIs, *t*-AUCB (*trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid) and TPAU (1-trifluoromethoxyphenyl-3-(1-acetyl piperidin-4-yl) urea) were synthesized previously in house (Jones et al., 2006; Hwang et al., 2007).

**Measurement of sEH inhibition.** The inhibitory activities of TCC against recombinant human and murine sEHs shown in Table 1 were measured by using the fluorescent cyano(6-methoxy-naphthalen-2-yl) methyl *trans*-[(3-phenyloxyran-2-yl)methyl] carbonate (CMNPC) (Jones et al., 2005) or racemic [<sup>3</sup>H]-*trans*-1,3-diphenylpropene oxide (*t*-DPPO) (Borhan et al., 1995) as substrate. Results are presented as the average ± standard deviation (SD) of three separate measurements.

**Kinetic studies.** Dissociation constants (K<sub>i</sub>) were determined following a method (Dixon, 1972) for competitive tight binding inhibitors, using [<sup>3</sup>H]-1,3-diphenyl-*trans*-propene oxide (*t*-DPPO) as substrate (Borhan et al., 1995). Inhibitor concentrations between 0 and 500 nM for TCC were incubated in triplicate for 5 min in pH 7.4 sodium phosphate buffer at 30 °C with 100 μL of the enzyme (1 nM of human sEH). Substrate (7.5 ≤ [S]<sub>final</sub> ≤ 30 μM) was then added. Velocity was measured as described (Borhan et al., 1995). For each substrate concentration, the plots of the velocity as a function of the inhibitor concentration allowed the determination of an apparent inhibition constant (K<sub>iapp</sub>) (Dixon, 1972). The K<sub>iapp</sub> was calculated by non-linear fitting of the Dixon's equation to the obtained results using Sigma Plot version 11.0 (Systat Software Inc.; Chicago, IL). The linear plot of these K<sub>iapp</sub> as a function of the substrate concentration allows the determination of K<sub>i</sub> when [S] = 0. Results are presented as average ± standard error of K<sub>i</sub> calculation.

**Molecular modeling.** Molecular modeling was performed using "Scigress Explorer Standard ver. 7.7.0.49" software (Fujitsu Computer Systems Corporation). The atomic coordinates of the crystal structure of human sEH complex with CIU (*N*-cyclohexyl-*N'*-(4-iodophenyl) urea) were retrieved from Protein Data Bank (entry 1VJ5) (Gomez et al., 2004). TCC was docked into the ligand-binding pocket manually by superposition with the parent molecule (CIU) and minimized on MM geometry (MM2). The image was produced using freewares VMD

**Table 1**  
Potency against the murine and human sEHs.

Inhibitors structure	Name	Inhibition against the		
		Murine sEH	Human sEH	
		IC <sub>50</sub> (nM)	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)
	TCC	370 ± 40	13 ± 1	96 ± 5
	<i>t</i> -AUCB	4 ± 1	2 ± 1	1.5 ± 0.2
	TPAU	44 ± 3	12 ± 3	57 ± 6

Data are presented as mean ± SD (N = 3). IC<sub>50</sub> values were determined with the fluorescent substrate CMNPC. The data of *t*-AUCB and TPAU are from (Liu et al., 2009a). The K<sub>i</sub> was determined using *t*-DPPO assay. TCC, trilocarban; *t*-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid; TPAU, 1-trifluoromethoxyphenyl-3-(1-acetyl piperidin-4-yl) urea.

1.8.6 ([www.ks.uiuc.edu/Research/vmd](http://www.ks.uiuc.edu/Research/vmd)) and POV-Ray 3.6 ([www.povray.org](http://www.povray.org)).

**Anti-hypotensive studies.** LPS (4 mg) was dissolved in saline (2 mL) to give an opaque solution after strong mixing for 1 min. TCC (2 mg) was dissolved in oleic acid rich triglycerides containing 10% PEG400 (v/v) and 10% ethanol (v/v) (2 mL) to give a clear solution after strong mixing for 3 h. Specifically, 16 male Swiss–Webster mice (8 weeks) were randomly assigned to 4 equal groups (N=4). Mice in group 1 were injected intraperitoneally (ip) with LPS saline solution at a dose of 10 mg/kg and immediately treated with the vehicle solution per oral (po) as a positive control. Mice in group 2 were ip injected with LPS saline solution at the same dose as group 1 and immediately administered with TCC (po) at a dose of 5 mg/kg. Mice in group 3 were ip injected with saline and treated with vehicle solution (po) as a negative control. The mice in group 4 received saline (ip) and TCC (20 mg/kg, po) to investigate the possible toxicity of TCC at high dose. The systolic blood pressure was measured with the method described previously (Liu et al., 2009a) to characterize the anti-hypotensive effect of TCC. Systolic blood pressure was determined before, 8- and 24-h after treatment with non-invasive tail cuff methods using a CODA™-6 system (Kent Scientific Co., CT). The instrument CODA™-6 was set up in the animal room where the mice were housed to measure blood pressure. For each measurement set, 3 acclimation cycles and 25 data cycles were used. The reported systolic blood is presented as a mean of at least 5 cycles of the measurement set. If the systolic blood pressure was under the detection limit, we recorded it as the detection limit (60 mm Hg) for further analysis. The heart rate and tail volume were also recorded.

**Anti-inflammatory studies.** Swiss–Webster mice (8 weeks old, 36 males) were assigned randomly to 6 equal groups (N=6) for the treatments described in Table 2. LPS and TCC were formulated and treated as same as those in the anti-hypotensive studies. Animals were euthanized at 8- or 24-h after the treatments indicated in the Table 2. Blood was collected and the plasma was separated as in the previously described method (Liu et al., 2009b). Plasma was stored in 3 separate Eppendorf tubes for analysis of cytokine, drug level, and oxylipin. Tissue was removed and immediately frozen with liquid nitrogen. All the samples were stored at  $-80^{\circ}\text{C}$  until analysis.

Plasma cytokine levels were analyzed using a CBA mouse inflammation kit. Briefly, thawed plasma samples (30  $\mu\text{L}$  each) were mixed for 2 h at room temperature with fluorescence-labeled capture beads with the PE detection reagents to measure the concentrations of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon-gamma (IFN- $\gamma$ ). Samples were then washed with washing buffer and analyzed on a FACScan flow cytometer (BD Immunocytometry Systems). Data were analyzed using BD CBA Analysis software (BD Immunocytometry Systems).

**Measurement of oxylipins in plasma.** Thawed plasma (200  $\mu\text{L}$ ) was extracted for oxylipin analysis as previously described (Liu et al., 2009b) and was analyzed by LC/MS/MS as described (Yang et al., 2009). Epoxides such as epoxyeicosatrienoic acids (EETs) and their corresponding sEH-mediated metabolites diols such as dihydroxyeicosatrienoic acids (DHETs) were quantified to evaluate the *in vivo* action of sEH.

**Measurement of plasma level of TCC.** TCC was extracted from the plasma samples by a small modification of the previous method (Liu et al., 2009b). Specifically, surrogate solution (10  $\mu\text{L}$  of 500 nM TCC- $^{13}\text{C}_6$  in MeOH) was added to each thawed plasma sample. After mixing on a Vortex mixer for 30 s, ethyl acetate (200  $\mu\text{L}$ ) was added to each sample. Samples were mixed on a Vortex mixer for 1 min followed by centrifugation at  $20,817\times g$  for 5 min. The supernatant was transferred to a new tube and the residue was extracted with a second 200  $\mu\text{L}$  of ethyl acetate. The supernatants were then combined and the mixture was evaporated to dryness using a centrifugal vacuum concentrator. Finally, 50  $\mu\text{L}$  of internal standard solution [200 nM of 12-(3-cyclohexan-1-yl-ureido)-dodecanoic acid (CUDA) in methanol] was used to reconstitute the residue for high-performance liquid chromatography-tandem mass analysis.

**Analysis of data.** Data are presented as mean  $\pm$  SD unless noted. Statistic analyses were performed by ANOVA followed with Tukey's (variance homogeneity) or Games-Howell's (variance heterogeneity) test using the software SPSS 10.0 (SPSS Inc., Chicago, IL) with  $P<0.05$  as the significant level.

**Table 2**  
Murine plasma level of key oxylipin mediators, cytokines and chemokine (MCP-1) in current study.

Group	1	2	3	4	5	6
LPS (mg/kg)	–	10	10	10	10	–
TCC (mg/kg)	–	–	–	5	5	20
Treatment time (h)	24	8	24	8	24	24
6-keto-PGF <sub>1a</sub> (nM)	2.2 $\pm$ 0.9	12.6 $\pm$ 2.8 <sup>*a</sup>	6.6 $\pm$ 2.6 <sup>*a</sup>	11.9 $\pm$ 2.9 <sup>*a</sup>	4.4 $\pm$ 1.2 <sup>a</sup>	3.6 $\pm$ 1.1 <sup>a</sup>
TXB <sub>2</sub> (nM)	0.8 $\pm$ 0.9	2.7 $\pm$ 1.0 <sup>b</sup>	3.9 $\pm$ 2.0 <sup>*a</sup>	5.1 $\pm$ 2.9 <sup>*b</sup>	3.0 $\pm$ 2.6 <sup>a</sup>	2.0 $\pm$ 1.7 <sup>a</sup>
PGE <sub>2</sub> (nM)	0.05 $\pm$ 0.02	0.20 $\pm$ 0.08 <sup>*a</sup>	0.16 $\pm$ 0.02 <sup>*a</sup>	0.20 $\pm$ 0.08 <sup>*a</sup>	0.11 $\pm$ 0.04 <sup>‡a</sup>	0.08 $\pm$ 0.06 <sup>a</sup>
PGD <sub>2</sub> (nM)	0.25 $\pm$ 0.07	1.0 $\pm$ 0.2 <sup>*a</sup>	0.79 $\pm$ 0.24 <sup>*b</sup>	0.77 $\pm$ 0.18 <sup>*a</sup>	0.52 $\pm$ 0.13 <sup>*b</sup>	0.29 $\pm$ 0.07 <sup>a</sup>
14,15-DHET (nM)	2.8 $\pm$ 0.5	4.3 $\pm$ 2.4 <sup>b</sup>	1.0 $\pm$ 0.1 <sup>*a</sup>	3.0 $\pm$ 0.6 <sup>b</sup>	1.0 $\pm$ 0.1 <sup>*a</sup>	1.9 $\pm$ 0.6 <sup>a</sup>
11,12-DHET (nM)	1.8 $\pm$ 0.4	2.0 $\pm$ 1.4 <sup>a</sup>	0.5 $\pm$ 0.1 <sup>*a</sup>	1.9 $\pm$ 0.3 <sup>a</sup>	0.5 $\pm$ 0.1 <sup>*a</sup>	1.1 $\pm$ 0.4 <sup>a</sup>
8,9-DHET (nM)	1.4 $\pm$ 0.3	1.5 $\pm$ 0.8 <sup>a</sup>	0.5 $\pm$ 0.1 <sup>*a</sup>	1.4 $\pm$ 0.2 <sup>a</sup>	0.6 $\pm$ 0.1 <sup>*a</sup>	1.1 $\pm$ 0.3 <sup>a</sup>
5,6-DHET (nM)	1.4 $\pm$ 0.2	1.0 $\pm$ 0.2 <sup>a</sup>	0.5 $\pm$ 0.1 <sup>*b</sup>	1.3 $\pm$ 0.2 <sup>a</sup>	0.7 $\pm$ 0.1 <sup>*b</sup>	1.2 $\pm$ 0.2 <sup>a</sup>
15-HETE (nM)	5.6 $\pm$ 2.4	4.7 $\pm$ 1.6 <sup>a</sup>	3.1 $\pm$ 0.9 <sup>a</sup>	5.6 $\pm$ 1.0 <sup>a</sup>	2.9 $\pm$ 0.8 <sup>a</sup>	5.0 $\pm$ 2.0 <sup>a</sup>
11-HETE (nM)	2.5 $\pm$ 0.8	2.7 $\pm$ 1.1 <sup>a</sup>	1.8 $\pm$ 0.6 <sup>a</sup>	3.0 $\pm$ 0.6 <sup>a</sup>	1.7 $\pm$ 0.4 <sup>a</sup>	2.0 $\pm$ 0.5 <sup>a</sup>
12-HETE (nM)	300 $\pm$ 110	410 $\pm$ 175 <sup>b</sup>	435 $\pm$ 165 <sup>a</sup>	615 $\pm$ 280 <sup>b</sup>	205 $\pm$ 145 <sup>a</sup>	250 $\pm$ 190 <sup>a</sup>
8-HETE (nM)	6.0 $\pm$ 4.1	4.5 $\pm$ 1.4 <sup>b</sup>	3.6 $\pm$ 0.5 <sup>a</sup>	6.4 $\pm$ 1.4 <sup>b</sup>	3.4 $\pm$ 1.2 <sup>a</sup>	5.0 $\pm$ 3.2 <sup>a</sup>
9-HETE (nM)	6.0 $\pm$ 4.5	6.8 $\pm$ 2.3 <sup>a</sup>	3.4 $\pm$ 0.6 <sup>a</sup>	5.6 $\pm$ 2.3 <sup>a</sup>	5.4 $\pm$ 2.2 <sup>a</sup>	5.5 $\pm$ 1.9 <sup>a</sup>
5-HETE (nM)	5.3 $\pm$ 1.0	3.8 $\pm$ 1.4 <sup>a</sup>	2.4 $\pm$ 0.3 <sup>*a</sup>	4.6 $\pm$ 1.0 <sup>a</sup>	3.1 $\pm$ 1.1 <sup>*a</sup>	1.1 $\pm$ 1.9 <sup>*a</sup>
14,15-EET (nM)	3.2 $\pm$ 1.5	1.3 $\pm$ 0.3 <sup>*a</sup>	0.7 $\pm$ 0.1 <sup>*b</sup>	1.8 $\pm$ 0.4 <sup>a</sup>	1.0 $\pm$ 0.3 <sup>*b</sup>	1.9 $\pm$ 0.5 <sup>a</sup>
11,12-EET (nM)	2.1 $\pm$ 1.0	1.1 $\pm$ 0.5 <sup>b</sup>	0.9 $\pm$ 0.1 <sup>*a</sup>	1.7 $\pm$ 0.2 <sup>b</sup>	1.1 $\pm$ 0.2 <sup>a</sup>	1.7 $\pm$ 0.5 <sup>a</sup>
8,9-EET (nM)	1.6 $\pm$ 1.0	0.4 $\pm$ 0.2 <sup>*b</sup>	0.1 $\pm$ 0.0 <sup>*a</sup>	0.9 $\pm$ 0.2 <sup>†b</sup>	0.5 $\pm$ 0.1 <sup>†a</sup>	1.1 $\pm$ 0.4 <sup>a</sup>
5,6-EET (nM)	8.3 $\pm$ 3.9	3.7 $\pm$ 2.6 <sup>a</sup>	2.9 $\pm$ 0.6 <sup>*a</sup>	6.6 $\pm$ 1.1 <sup>a</sup>	4.0 $\pm$ 1.2 <sup>a</sup>	7.1 $\pm$ 2.9 <sup>a</sup>
TNF $\alpha$ (pg/mL)	16 $\pm$ 2	200 $\pm$ 40 <sup>*b</sup>	60 $\pm$ 10 <sup>*,†a</sup>	175 $\pm$ 75 <sup>*b</sup>	30 $\pm$ 10 <sup>*,‡,§a</sup>	17 $\pm$ 5 <sup>a</sup>
IL-6 (pg/mL)	11 $\pm$ 3	4210 $\pm$ 775 <sup>*b</sup>	190 $\pm$ 45 <sup>*,†a</sup>	4090 $\pm$ 2460 <sup>*b</sup>	65 $\pm$ 30 <sup>*,‡,§a</sup>	9 $\pm$ 3 <sup>a</sup>
MCP-1 (pg/mL)	80 $\pm$ 15	11,900 $\pm$ 1330 <sup>*b</sup>	3240 $\pm$ 715 <sup>*,†a</sup>	12,400 $\pm$ 1285 <sup>*b</sup>	2080 $\pm$ 425 <sup>*,§a</sup>	85 $\pm$ 35 <sup>a</sup>
IFN- $\gamma$ (pg/mL)	6 $\pm$ 2	2805 $\pm$ 755 <sup>*a</sup>	9 $\pm$ 2 <sup>†a</sup>	1820 $\pm$ 540 <sup>*a</sup>	10 $\pm$ 5 <sup>§a</sup>	6 $\pm$ 2 <sup>a</sup>

Data are presented as mean  $\pm$  SD (N=6). \* Significantly different ( $P<0.05$ ) from group 1, † significantly different ( $P<0.05$ ) from group 2, ‡ significantly different from Group 3, and § significantly different ( $P<0.05$ ) from Group 4 were determined by ANOVA followed with Tukey's <sup>a</sup> or Games-Howell's <sup>b</sup> test.

## Results

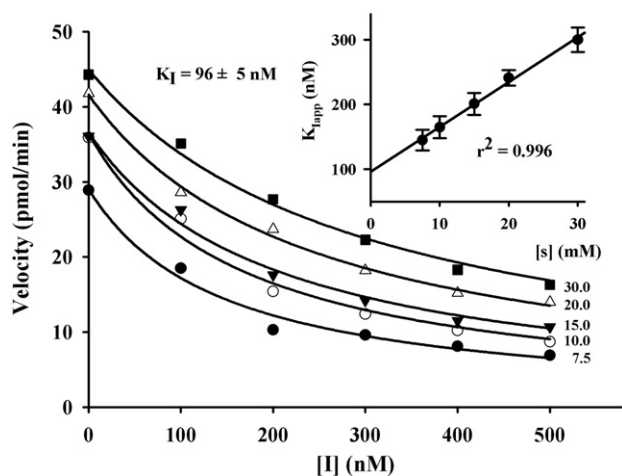
### *In vitro* inhibitory activity of TCC against sEH

As shown in Table 1 for the murine sEH, TCC is roughly 100-fold less potent than *trans*-4-(4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid (*t*-AUCB) and 10-fold less potent than 1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea (TPAU). However, for the human sEH the difference in potency is greatly reduced; TCC has the same potency as TPAU and is only about 6-fold less potent than *t*-AUCB. In addition, to better define the potency of TCC against human sEH, we determined its  $K_i$  (Fig. 1) using a radioactivity-based assay. TCC has a  $K_i$  of 96 nM, which is 60-fold less potent than that of *t*-AUCB, but close to that of TPAU (Liu et al., 2009a).

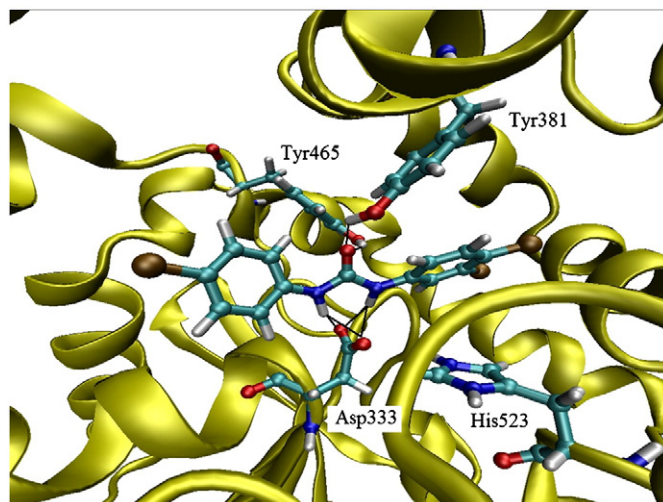
In order to understand how TCC binds to this enzyme, the structure of TCC was manually docked at the active site of the human sEH using an X-ray crystal structure of human sEH complex with the urea-based ligand (CIU) (Gomez et al., 2004; Gomez et al., 2006). All plausible binding modes for TCC bound well at the active site of the enzyme. Among the plausible binding modes for TCC, the orientation given in Fig. 2 was the most stable ( $\Delta E$  ranging from 0.04 to 6.74 kcal mol<sup>-1</sup>), and thus it is the most probable. As seen in Fig. 2, TCC was modeled to bind to sEH primarily through the interactions of its urea pharmacophore with Tyr<sup>381</sup>, Tyr<sup>465</sup> and Asp<sup>333</sup> as expected from a potent sEHI.

### TCC reverses LPS-challenged hypotension

Hypotension resulting from LPS-challenge is a hallmark of septic shock (Weinberg et al., 1992). The anti-hypotensive effect of TCC is illustrated in Fig. 3. Specifically, the systolic blood pressure dramatically fell below the detection limit of the equipment (60 mm Hg) 8 h and 24 h after LPS dosing. Oral administration of TCC at a dose of 5 mg/kg significantly reversed LPS-induced hypotension at 24 h after treatment while it did not show a detectable effect on blood pressure 8 h after treatment. On the other hand, TCC (20 mg/kg, po) did not change the systolic blood pressure of control mice either 8 or 24 h after treatment (Liu et al., 2009b). In addition to the significant alteration in systolic blood pressure, LPS administration slightly increased the heartbeat of mice (790 ± 80 vs. 880 ± 120 bpm,  $p = 0.22$ ) and significantly decreased the tail volume (65 ± 20  $\mu$ L vs. 30 ± 12  $\mu$ L,  $p = 0.01$ ), and administration of TCC reversed the decreased tail volume from LPS-challenged towards normal level at 24 h after treatment. Tail volume is the total volume going in and out



**Fig. 1.** Determination of the  $K_i$  of TCC with the human sEH (1 nM) using [<sup>3</sup>H]-*trans*-1,3-diphenyl-*trans*-propene oxide ([<sup>3</sup>H]-*t*-DPPPO) as substrate (7.5 ≤ [S]<sub>final</sub> ≤ 30  $\mu$ M). For [S] = 0, a  $K_i$  value of 96 nM was found.



**Fig. 2.** Docking TCC with the human sEH enzyme. TCC was manually docked into the active site of human sEH. The amino acids are shown as ball and stick structures on the backbone ribbon diagram.

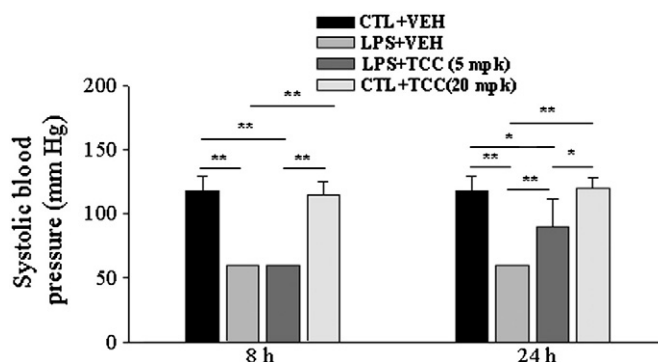
of the tail, essentially based on the amount of blood moving through the tail at each measurement cycle.

### TCC suppresses the production of cytokines and a chemokine

Table 2 presents the plasma levels of the cytokines (TNF- $\alpha$ , IL-6, and IFN- $\gamma$ ) and a chemokine (MCP-1). Table 2 shows the effects of TCC on the release of TNF- $\alpha$  and IL-6, respectively. After LPS challenge, the plasma levels of TNF- $\alpha$  and IL-6 dramatically increased 8 and 24 h after treatment. TCC (5 mg/kg) significantly suppressed the plasma levels of TNF- $\alpha$  and IL-6 at 24 h after treatment while it had poor effects at 8 h after treatment, which correlates with the anti-inflammatory effect of TCC. On the other hand, TCC (20 mg/kg, po) did not alter the release of detected cytokines and a chemokine in control mice 24 h after treatment (Liu et al., 2009b).

### Plasma level of TCC

The plasma concentration of TCC at 8 and 24 h after treatment was 400 ± 100 nM and 200 ± 15 nM, respectively, in the LPS-challenged

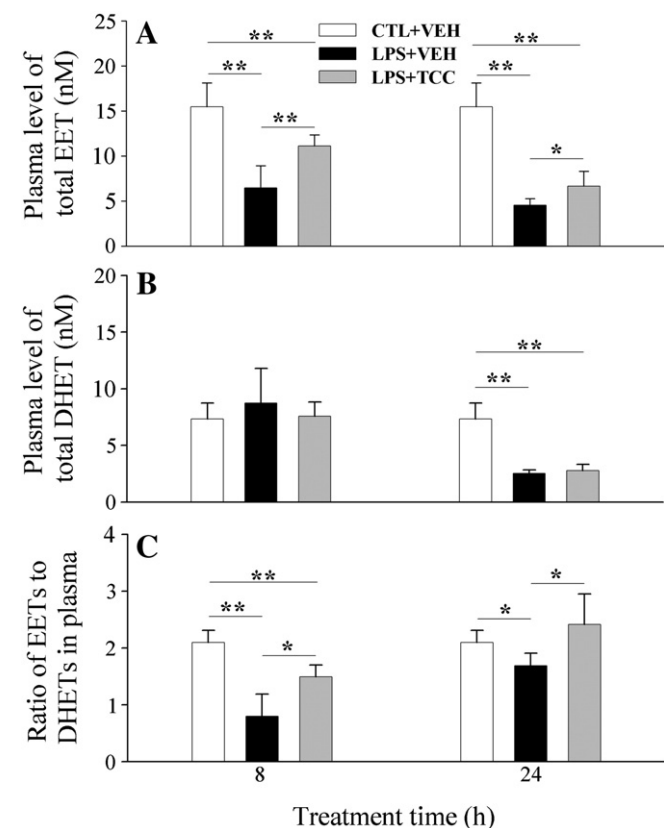


**Fig. 3.** The anti-hypotensive effect of TCC in a LPS-challenged murine model. LPS (10 mg/kg) and TCC (5 mg/kg) were administered to male Swiss-Webster mice (8-week old) with i.p. injection and oral gavage, respectively. Systolic blood pressure was recorded 8 h and 24 h after treatment with a non-invasive tail cuff method. Data represent mean ± SD of 4 mice. If the systolic blood pressure was under the detection limit of instrument (60 mm Hg), the value was recorded as 60 mm Hg. Data represent mean ± SD of 6 mice. Animals were euthanized 8 and 24 h after treatment, respectively. Control (CTL) was saline, and vehicle (VEH) was oleic acid rich triglycerides containing 10% PEG400 (v/v) and 10% ethanol (v/v); mpk: mg/kg. Statistically significant differences ( $*P < 0.05$ ,  $**P < 0.01$ ) were determined by ANOVA followed with Tukey's.

mice orally treated with 5 mg/kg of TCC. The plasma TCC level at 8 h after treatment is significantly higher than those at 24 h after treatment. The plasma levels of TCC at 24 h after treatment were  $540 \pm 180$  nM in the mice orally treated with 20 mg/kg of TCC.

#### TCC shifts oxylipin profile like other sEHs *in vivo*

The efficacy of TCC *in vivo* as a sEH or target engagement was evaluated by the plasma levels of total EETs and DHETs, as well as the plasma ratio of EETs to DHETs (Fig. 4). Specifically, upon LPS challenge, plasma levels of EETs dramatically decreased after 8 and 24 h compared to the normal control. The plasma level of DHETs dramatically decreased 24 h after LPS treatment only in comparison with the normal control. These changes resulted in a significant decrease in the plasma ratio of EETs to DHETs at 8 and 24 h after treatment in comparison with the vehicle control. When compared with the LPS group, treatment with TCC (5 mg/kg) significantly increased the plasma levels of EETs at 8 and 24 h after treatment, while it had little effect on plasma level of DHETs 8 and 24 h after treatment, resulting in significant increase in the ratio of EETs to DHETs at 8 h and 24 h after treatment. This reversal of the expected increase in the ratio of EETs to DHETs has been observed in the same animal model for several sEHs (Liu et al., 2009b). However, without LPS challenge TCC (20 mg/kg) only slightly changes the plasma levels of EETs and DHETs, as well as the plasma ratio of EETs to DHETs (Table 2).



**Fig. 4.** Administration of TCC alters the plasma oxylipin levels of EETs (A) and DHETs (B) *in vivo*, resulting in an alteration in the ratio of EETs to DHETs (C). LPS and TCC were administered to male Swiss-Webster mice (8-week old) with ip injection and oral gavage, respectively. Data represent mean  $\pm$  SD of 6 mice. The total EETs column sums 14, 15-, 11, 12-, 8, 9- and 5, 6-EETs and their corresponding diols compose the total DHETs column. Control (CTL) and vehicle (VEH) are the same as those in Fig. 3. The full oxylipin data are presented in Table 2. Statistically significant differences (\* $P < 0.05$ , \*\* $P < 0.01$ ) were determined by ANOVA followed with Tukey's test.

## Discussion

TCC can inhibit human and murine recombinant sEH *in vitro* (Morisseau et al., 1999; McElroy et al., 2003; Morisseau et al., 2009). Such sEHs have therapeutic utility for the treatment of inflammation, cardiovascular diseases, and nephrotoxicity in multiple animal models (Jung et al., 2005; Larsen et al., 2006b; Morisseau et al., 2006; Schmelzer et al., 2006; Xu et al., 2006; Parrish et al., 2008; Liu et al., 2010). In addition, sEHs have been moved into clinical trials for treatment of diabetes and hypertension (Whitcomb et al., 2009). As shown in Table 1, *in vitro* potency of TCC approaches the one of some of the more potent sEHs known for the latter enzyme. In addition, the  $K_i$ s obtained support the  $IC_{50}$ s results that TCC has a similar potency to TPAU for the human sEH but it is less potent than *t*-AUCB. Thus it seems pivotal to investigate the potential *in vivo* effects of TCC as a sEH. Because TCC is 10-fold less potent than TPAU for the mouse sEH, this antimicrobial could be less effective in perturbing sEH activity in a murine model than in human. Thus, the effects of TCC on human health through sEH inhibition could be expected to be more severe than ones observed in mice.

Here, we reported the *in vivo* effect of TCC in a LPS-challenged murine model of inflammation. TCC at the dose of 5 mg/kg showed a significant anti-hypotensive effect 24 h after treatment. This suggests TCC reverses the LPS-induced hypotension in a time-dependent manner. In addition, the more potent sEH *t*-AUCB (1 mg/kg, po) showed similarly significant anti-hypotensive effects in the same murine model at 24 h post treatment. These observations suggest that anti-hypotensive effect of sEHs is complex and time dependent. In addition to the significant alteration in systolic blood pressure, while LPS administration did not significantly alter the heartbeat of mice, it significantly deteriorated the vascular function (tail volume dropped significantly). The administration of TCC reversed the decreased blood output from LPS-challenged towards normal level at 24 h after treatment. These data indicated that LPS-challenge is involved in the physiological process of vascular function, and inhibition of sEH plays a beneficial role in modulation of vascular function. However, in absence of LPS challenge treatment with either TCC (20 mg/kg) or *t*-AUCB (1 mg/kg) result in no significant change in the systolic blood pressure.

The plasma levels of pro-inflammatory TNF- $\alpha$  (Dinarello, 2000), MCP-1 (Melgarejo et al., 2009), anti-inflammatory IFN- $\gamma$  (Schroder et al., 2004) and pleiotropic IL-6 (Ludat et al., 1994; Xing et al., 1998) at 8 h after LPS treatment are dramatically higher than those of the control mice and those at 24 h after LPS treatment. The plasma cytokine levels at 24 h after LPS treatment are significantly higher than control mice levels. This suggests that the LPS-challenge elicited inflammation in a time-dependent process that is more intense at 8 h than at 24 h post treatment. In this LPS-challenged murine model, TCC significantly decreased the release of cytokines and a chemokine 24 h after treatment while it had no significant effect at 8 h after treatment, also showing a time-dependence for the anti-inflammatory effect of TCC. In addition, in a separate study, *t*-AUCB (1 mg/kg) also significantly reversed the LPS-induced increased plasma levels of TNF- $\alpha$  and IL-6 to  $20 \pm 6$  and  $90 \pm 40$  pg/mL, respectively at 24 h after treatment. Thus a 1 mg/kg dose of *t*-AUCB altered cytokines to a similar degree to the 5 mg/kg dose of TCC. This indicates that *t*-AUCB is more potent than TCC in this anti-inflammatory animal model, reflecting their respective *in vitro* potency (Table 1). On the other hand, in absence of LPS challenge, neither TCC (20 mg/kg) nor *t*-AUCB (1 mg/kg) dramatically altered the plasma cytokines and chemokines tested (Table 2). This is consistent with the observations from the anti-hypotensive studies (Fig. 3).

LPS-challenge significantly decreased the production of EETs in plasma, resulting in a significant decrease in the plasma ratio of EETs to DHETs, a biomarker of sEH action. TCC reversed the LPS-induced decreases in both the level of EETs and the ratio of EETs to DHETs

(Fig. 4). These effects on blood pressure, release of cytokines and chemokines, EETs level and plasma ratio of EETs to DHETs from TCC administration are similar to those from the treatment of previous sEH inhibitor *t*-AUCB (1 mg/kg) in the same model (Liu et al., 2009a). These data also suggest that inhibition of sEH by TCC does contribute to the anti-hypotensive and anti-inflammatory effect observed. Furthermore, TCC at 8 h after treatment with the oral dose of 5 mg/kg reversed the significant decrease in EET levels and the plasma ratio of EETs to DHETs caused by LPS administration while no significant beneficial effect was observed in hypotension and cytokine release. However, all the beneficial effects expected from a sEH inhibitor could be seen at 24 h after treatment. These data also suggest that the TCC acts on sEH enzyme resulting in the alteration in EETs earlier than its regulation of blood pressure and the release of cytokines and chemokine. An alternate hypothesis which might be expected given the physical properties of TCC is that blood levels do not reflect the tissue levels at the sites of action. In addition, when administered to normal mice, TCC (20 mg/kg) did not significantly affect the plasma levels of EETs, DHETs and the ratio of EETs to DHETs, while *t*-AUCB (1 mg/kg) significantly decreased the plasma level of DHETs, did not change the plasma levels of EETs, resulting in a significantly increases the ratio of EET/DHET. This is consistent with the greater potency of *t*-AUCB on sEH. However, in addition to sEH inhibition, other characteristics of TCC may also contribute to the *in vivo* anti-inflammatory effect of TCC. This possibility needs further study.

The plasma levels of TCC are above its murine  $IC_{50}$  value at 8 h but below its murine  $IC_{50}$  value at 24 h after treatment, however the dramatic reverse of LPS induced symptoms was far greater at 24 h after treatment. These observations indicate that the TCC action in the current model is a time-dependent process. This suggests that the drug action is not simply correlated with the drug level in circulation however adequate blood levels of TCC are needed to trigger the cascade of events leading to the reduction of sepsis-induced hypotension.

A previous percutaneous study in human showed that blood levels of TCC were below the detection limit of 80 nM (25 ppb) in the test samples (Howes and Black, 1976), however, this detection limit is much higher than its *in vitro*  $IC_{50}$  against human sEH. In another human study, oral administration of TCC at a low dose of 2.2  $\mu$ mol/kg (~0.7 mg/kg) resulted in a plasma level of 3.7 nmol/g, almost 280 fold higher than its *in vitro*  $IC_{50}$  at 2.8 h after treatment (Hiles and Birch, 1978). A recent study showed that percutaneous use of TCC-containing soap bar resulted in a blood level of TCC higher than its *in vitro*  $IC_{50}$  for over 30 h (Fig. S1). This underlines the urgency of investigation of TCC exposure. In this study, a significant beneficial effects of TCC expected from its sEH property was observed at 24 h after treatment, which provides a direct linkage between use of TCC-containing PCPs and its effects expected as a sEH inhibitor. These data indicate the possibility that endogenous EET concentrations can be elevated in humans through use of TCC in PCPs. Generally, increased blood levels of EETs seem beneficial to reduce inflammation, and other deleterious symptoms due to the beneficial function of the endogenous EETs. These levels are more likely to have a local dermal effect than a general systemic effect. However, caution should be paid to the unanticipated side effects due to the increase in EETs and other epoxy fatty acids. For example, EETs has been found to promote endothelial cell proliferation, migration, and angiogenesis (Michaelis et al., 2005; Pozzi et al., 2005; Wang et al., 2005; Michaelis and Fleming, 2006; Webler et al., 2008; Yan et al., 2008), which have been associated with various cardiovascular diseases and cancers.

In summary, both *in vitro* and *in vivo* studies demonstrated the properties expected from a sEH inhibitor for the antimicrobial TCC, and this inhibition contributes to the anti-inflammatory effect of TCC. This study not only confirms the pharmacological effect of a sEH inhibitor *in vivo*, but also discloses a novel pharmacological action of the common antimicrobial agent TCC that is present at high concentration in many

PCPs. Based on our study and literature data on TCC exposure from environmental contamination, we conclude that systemic inhibition of the sEH by TCC at levels which could lead to significant alterations in the immune system in a normal human is unlikely. However, the use of PCPs with TCC added as an anti-microbial could lead to significant sEH inhibition and the resulting downstream biological effects. These effects are anticipated to be greatest near the site of TCC application. Some individuals (e. g. dental and dermal inflammation, as well as other inflammatory diseases) may benefit from use of TCC-containing PCPs due to its sEH inhibitory potency. Although local reduction of inflammation is often beneficial to human health, use of TCC in PCPs could result in unwanted local effects. Given the environmental persistence of TCC, possibly the benefits and risks of the use of this common anti-microbial agent should be re-evaluated with its use moving to more high value and limited specific applications where it is beneficial to reduce both microbial density and inflammation.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2011.06.017.

### Conflict of interest

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute of Health, National Institute of Environmental Health Sciences, or American Asthma Foundation. CM, SHH and BDH are inventors of composition of matter and use patents in the area of soluble epoxide hydrolase inhibitors.

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