Blood pressure lowering and anti-fibrotic effect of zinc-specific chelator-tetraethyl thiuram disulfide in the rat model of L-NAME-induced hypertension

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ABSTRACT

Background: Tetraethylthiuram disulfide (TTD) is a zinc-specific chelator popularly used to treat chronic alcoholism by targeting zinc-dependent alcohol dehydrogenase enzyme. Interestingly, Angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP) and aminopeptidase N (APN) implicated in hypertension are zinc-dependent enzymes. Objective: This study aimed to evaluate the inhibitory potential of TTD against ACE, NEP, and APN in vitro. Further, the anti-hypertensive and anti-fibrotic efficacy of TTD in vivo were evaluated using N(ω)-nitro-L-arginine-methyl ester (L-NAME)-induced hypertensive rat model. Method: Dose-dependent inhibition of ACE, NEP, and APN by TTD was evaluated in vitro. Further, five groups consisting of 6 rats in each were used for animal studies. Group one served as control, and the second group received L-NAME alone (40mg/kg/day). The third group was TTD control. Fourth and fifth group received L-NAME and simultaneously treated with TTD (10mg/kg/day) and Lisinopril (10mg/kg/day) respectively. Systolic blood pressure, heart and kidney weight, angiotensin, atrial natriuretic peptide, urea, creatinine, aspartate transaminase, alanine transaminase, and nitrite levels were measured in serum of all group of rats. Fibrosis in heart and kidney tissues were evaluated by staining collagen with picrosirius red. Results: TTD effectively inhibited the activities of ACE, NEP, and APN in a dose-dependent manner with IC₅₀ of 10.15 μM, 22.35 μM and 3.032 μM respectively. Further, administration of the TTD effectively decreased systolic blood pressure (140±8 mmHg) when compared to L-NAME group (190±10 mmHg), improved hypertension markers (Ang II and ANP), kidney and liver markers. TTD ameliorated heart and kidney hypertrophy and partially prevented fibrosis. Conclusion: These findings provide evidence that TTD affords efficient anti-hypertensive effect with moderate end-organ protection possibly by cumulative inhibition of the aforementioned enzymes. Key words: TTD, Pro-hypertensive enzymes, Renin-angiotensin system, Natriuretic peptides, Blood pressure, hypertrophy and fibrosis, Zinc chelation

INTRODUCTION

Hypertension or high blood pressure leads to debilitating and life-threatening complications like stroke and myocardial infarction if left untreated. The hormone systems which play a central role in regulating blood pressure and vascular events are the renin-angiotensin system (RAS) and natriuretic peptides system (NPS). The key enzyme of RAS is an angiotensin-converting enzyme (ACE), a zinc-dependent carboxypeptidase which generates vasoressor angiotensin II (Ang II) and results in vasoconstriction. Besides, the key enzyme of NPS is neutral endopeptidase (NEP), which is a zinc-dependent metalloendopeptidase. NEP degrades vasodilatory peptides like bradykinin, substance C and natriuretic peptides such as an atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide resulting in vasoconstriction events. Inhibition of NEP along with ACE has been proven to be a better therapeutic strategy to manage hypertension and related complications. Along with ACE and NEP, one more zinc-dependent exo-peptidase implicated in blood pressure is aminopeptidase N (APN) which generates Ang IV from Ang III by removing N-terminal arginine. The physiological role of APN in modulating vascular status includes degradation of Ang III to form Ang IV, and elevated levels of Ang IV in the brain has been shown to induce hypertension. Hence, inhibition of these enzymes results in increased levels of vasodilators, decreased levels of vasoconstrictors which prevent cardiac and renal hypertension, and also offer protection from adverse effects of hypertension. Currently, some anti-hypertensive drugs which are available in market in-
herit lots of side effects such as nausea, angioedema, headache, fatigue, loss of taste, dizziness, cold hands and feet, dry eyes, mouth and throat and insomnia. These side effects are due to targeting exclusively either RAS or NPS cascades which leads to an imbalance between vasoconstrictors and dilators. Hence targeting ACE, NEP, and APN by a single or cocktail of molecules would be a choice for better management of hypertension and associated risks.

A few studies have shown that chelation therapy with Ethylenediaminetetraacetic acid (EDTA) can be an alternative treatment for cardiovascular diseases (CVDs). However, EDTA being a broad spectrum chelator sequesters all divalent metal ions leading to massive alteration in every regular physiological event along with inhibition of targeted enzymes. Because, all the above-mentioned blood pressure regulating proteins are zinc dependent metallopeptidases, therefore, use of zinc-specific chelator to inhibit them appear to be ideal. Further, in our previous study, we have demonstrated the inhibition of snake venom metallopeptidases using three zinc-specific chelators, viz. N,N,N’,N’-tetrakis (2-pyridylmethyl) ethane-1,2-diamine (TPEN), diethylenetriamine pentaacetic acid (DTPA), and tetraethyl thiuram disulfide (TTD). Though TPEN showed to be a potent inhibitor of snake venom metallopeptidases, toxicity associated such as apoptosis, cytochrome translocation and chelation of membrane zinc limits its use. On the other hand, TTD is an FDA approved drug against zinc-containing alcohol dehydrogenase to treat chronic alcoholism which causes hypotension as a side effect. Hence, in this study, inhibitory potential of TTD against ACE, NEP, and APN was evaluated in vitro. Further, the therapeutic potential of TTD against hypertension was studied in the rat model of L-NAME-induced hypertension.

**MATERIALS AND METHODS**

**Chemicals**

ACE, APN, Tetraethyl thiuram disulfide (TTD), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Hippuryl-histidyl-leucine (HHL), benzyl oxy carbonyl-Gly-Gly-Leu-p-nitroanilide (Z-Gly-Gly-Leu-Nan), HEPES, lisinopril, L-alanine 4-nitroanilide hydrochloride, hippuric acid, N-1-Naphthylethylenediamine dihydrochloride (NED), sulfanilamide and phosphoric acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Ethyl acetate and trichloroacetic acid (TCA) were purchased from Sisco Research Laboratory (Mumbai, India). Human neutral endopeptidase (NEP) was purchased from BioVision, Inc. (San Francisco, USA). Urea, creatinine, AST, ALT assay kits was purchased from AGAPPE (Ermukalam, India). Ang II and ANP ELISA kits were procured from GenxBio Health Sciences Ltd (Delhi, India). All other chemicals and reagents used in this study were of analytical grade.

**ACE activity and Inhibition**

ACE activity was measured using the substrate HHL, by spectrophotometric method, as described previously. In brief, rabbit lung ACE was incubated with 5 mmol/L HHL in HEPES (50 mmol/L; pH 8.3) buffer containing 300 mmol/L NaCl for 30 min at 37 °C, the reaction was stopped by adding 1 mol/L HCl. The absorbance of hippuric acid released by the action of ACE was measured at 228 nm. For inhibition studies, ACE (1 mU) was pre-incubated with various concentrations of TTD for 10 min before addition of substrate and percentage of inhibition was determined by calculating the decrease in ACE activity.

**NEP activity and Inhibition**

NEP activity was measured by the spectrophotometric method. In brief, 5 mmol/L of Z-Gly-Gly-Leu-Nan was incubated with 50 ng of neprilysin in Tris-HCl buffer (50 mmol/L; pH 7.5) for 30 min at 37 °C. The reaction was terminated by adding 0.5 mL of 40% TCA, centrifuged at 5000 g for 5 min and the supernatant was collected. The absorbance of the released end product p-nitro-aniline in the supernatant was monitored at 405 nm. For inhibition studies, 50 ng of enzyme was pre-incubated with various concentrations of TTD for 10 min prior to the addition of substrate and inhibition was expressed as percent activity.

**APN activity and Inhibition**

APN activity was measured by incubating the substrate alanine-p-nitroanilide (250 μmol/L) with APN in Tris-HCl buffer (50 mmol/L; pH 7.5) for 45 min at 21 °C. The reaction was terminated by heating the tubes in a water bath maintained at 80 °C for 10 min. The absorbance of the released end product p-nitroaniline was monitored at 405 nm. For inhibition studies, 50 ng APN was pre-incubated with various concentrations of TTD for 10 min, and the same protocol was followed.

**Animals**

Male Wistar rats (5–6 weeks, weight, 290-310g) were used and each group consisted of 6 animals. Animals were housed separately in polypropylene cages.
and maintained under standard laboratory conditions (28±2 °C, 12 h light/dark cycle) with free access to food (normal pellet diet) and water. Rats were allowed to acclimate for 6 to 7 days, to get used to their surroundings, handling, and measurement of systolic blood pressure (SBP) before the experimental procedures. Animals were treated in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines. The experimental protocol was approved by the Institutional Animal Ethics Committee (approval number UOM/IAEC/01/2017).

**Induction of hypertension by L-NAME**

After acclimatization, the rats were randomly divided into five groups; group I served as control, Group II, III, and IV were rendered hypertensive by administering L-NAME orally at a dose of 40 mg/kg/day for four weeks. At the same time, groups III and IV were orally administered with TTD and lisinopril at a dose of 10mg/kg/day respectively. Group V was applied with TTD alone 10mg/kg/day which served as TTD control. Systolic blood pressure (SBP) and heart rate were measured once a week using non-invasive tail-cuff plethysmography (IITC Life sciences Ltd, Woodland Hills, USA). At the end of the fourth week, the animals were sacrificed by administering pentobarbital (30 mg/kg), followed by cervical dislocation. Blood was drawn by cardiac puncture for serum assays, while heart and kidney tissues were processed for histopathological studies.

**Biochemical analysis**

Ang II and ANP levels were determined in serum of rats by Genxbio ELISA kits, using Thermo Scientific Varioskan Flash multi-mode plate reader. Urea, creatinine, AST, and ALT were estimated in the serum of rats using AGAPPE colorimetric kit following the manufacturer’s protocol using Thermo Scientific UV-Vis spectrophotometer (Biomate-3S).

**Nitric oxide (NO) assay**

The accumulation of nitrite, used as a measure of nitric oxide synthase activity was determined in serum of rats using Griess reagent. Serum was reacted with a Griess solution (4% sulphanilamide in 0.3% NED) under acidic (phosphoric acid) conditions for 20 mins. The absorbance of samples was measured at 540 nm.

**Histology**

Heart and kidney tissues were isolated and fixed in 10% buffered formalin. The tissues were embedded in paraffin blocks, sliced into 4μm-thick sections. The tissue sections were stained with hematoxylin-eosin to observe morphological changes and picro-sirius red dye to study collagen deposition. The images were photographed at 10X magnification using Axioimager bright field microscope.

**Statistical Analysis**

Results are expressed as mean±SEM. One-way analysis of Variance and the Bonferroni test were used for statistical analysis. All groups were compared to L-NAME group and p value of <0.05 was considered to be statistically significant.

**RESULTS**

**Inhibition of ACE, NEP, and APN by TTD**

TTD significantly inhibited ACE, NEP and APN activity in a concentration-dependent manner with lower IC\textsubscript{50} values of 10.15 μM, 22.35 μM and 3.032 μM respectively (Figure 1).

**Effect of TTD on SBP in L-NAME-induced hypertensive rats**

Rats were subjected to daily oral administration of L-NAME (40mg/kg) continuously for four weeks. No significant differences were observed in SBP of control and TTD alone groups (104±10 mmHg) over the four-week experimental period. Animals that received L-NAME showed a considerable increase in the SBP (190±10 mmHg) compared to the control group (104±10 mmHg). L-NAME hypertensive rats treated with TTD (10 mg/kg) were able to substantially overcome hypertension induced by L-NAME with an SBP value of 140±8 mmHg. While lisinopril (positive inhibitor control) treatment showed a significant decrease in SBP (138±8 mmHg) induced by L-NAME (Figure 2).

**Effect of TTD on hypertrophy and hypertensive markers**

Continuous administration of L-NAME (40mg/kg/day) for four weeks engendered cardiac hypertrophy with a marked increase in the heart weight to body weight ratio (HW/BW) compared to control group (3.18±0.3 mg/g vs. 2.15±0.3 mg/g). Whereas, TTD and lisinopril treated groups showed a significant decrease in hypertrophy (3.18±0.3 mg/g vs. 2.95±0.5 mg/g) compared to L-NAME group (Figure 3A). Additionally, L-NAME treatment caused renal hypertrophy, with a significant increase in kidney weight to body weight ratio (KW/BW). TTD treatment was equally effective in decreasing...
Figure 1: Inhibition of ACE, NEP and APN by TTD. ACE, NEP and APN activity was carried out as described in the method section. For inhibition, ACE, NEP and APN were pre-incubated for 10 min with different concentration of TTD (0-100 μM) followed by addition of substrate, percent activity was expressed by measuring the released end product.

Figure 2: Effect of TTD treatment on SBP of L-NAME hypertensive rats. L-NAME was continuously administered orally at 40 mg/kg/day for four weeks and SBP was monitored every week as mentioned in the method section. Treatment groups were administered with either TTD or lisinopril along with L-NAME.
kidney hypertrophy compared to L-NAME group (3.95±0.2 mg/g vs. 4.10±0.4 mg/g), (Figure 3B). Further, serum Ang II was markedly increased in the L-NAME group of rats compared to the control group (65±3.5 pg/ml vs. 40±3.5 pg/ml). Concurrent treatment with TTD decreased Ang II when compared to L-NAME group (59±2.5 pg/ml vs. 65±3.5 pg/ml). Ang II levels were markedly reduced in sera of lisinopril treated group of rats (49±3.7 pg/ml) (Figure 3C). Similarly, there was a significant increase in ANP levels in sera of L-NAME treated group compared to control group (161±4.0 pg/ml vs. 146±3.5 pg/ml) and concurrent treatment of L-NAME with TTD slightly increased ANP compared to L-NAME group (170±4.0 pg/ml vs. 165±4.0 pg/ml), which was comparable to L-NAME group treated with Lisinopril (172±3.5 pg/ml) (Figure 3D).

Effect of TTD on Urea, Creatinine, AST, ALT and NO in the serum of L-NAME hypertensive rats

Serum levels of urea and creatinine were increased significantly in the L-NAME group (50±2.0 mg/dL and 0.81±0.2 mg/dL) compared to the control group (35±3.0 mg/dL and 0.52±0.3 mg/dL). TTD and Lisinopril treatment significantly altered serum levels of urea (40±3.0 mg/dL and 36±3.0 mg/dL) and creatinine (0.75±0.2 mg/dL and 0.64±0.3 mg/dL) as compared to L-NAME group (Figure 4A & Figure 4B). Liver function markers AST and ALT were markedly increased in L-NAME group (170±4.5 U/L and 140±4.2 U/L) compared to the control group (136±3.5 U/L and 55±3.5 U/L). Treatment with TTD and Lisinopril significantly decreased AST (120±2.7 U/L) and ALT (112±5.0 U/L) (Figure 4C & Figure 4D). Chronic administration of L-NAME for four weeks resulted in significant depletion of serum nitrite levels in the L-NAME group when compared to control group (15.47±0.3 μmol/L vs. 22.54±0.8 μmol/L). Treatment with TTD and Lisinopril resulted in elevated serum nitrite levels (18.96±0.6 and 20.78±0.6 μmol/L). Treatment with TTD alone did not show any significant effect on NO (Figure 4E).
Figure 4: Effect of TTD administration on metabolic parameters A: Urea, B: Creatinine, C: AST, D: ALT and E: Nitrite. Serum levels of these parameters were measured using Agappe estimation kits and nitrite was measured by Griess method.

Effects of TTD and L-NAME on heart and kidney tissue morphology

Treatment of L-NAME hypertensive rats with TTD moderately prevented L-NAME-induced morphological changes of heart and kidney tissues as evident by hematoxylin-eosin stained sections. L-NAME group showed significant morphological changes when compared to the control group (Figure 5).

Effect of TTD on fibrosis of heart and kidney

L-NAME group showed significant deposition of collagen when compared with the control group. Treatment of L-NAME group with TTD and Lisinopril revealed a considerable reduction in deposition of collagen when compared to L-NAME group alone (Figure 6). Furthermore, there were differences between L-NAME and L-NAME + TTD and between the control group and TTD alone.

DISCUSSION

Increasing global incidences of CVDs and stroke is highly attributable to hypertension. Hence, management of hypertension remains an important strategy for controlling associated clinical disorders.25 Numerous strategies are available for the management of hypertension but foster side effects. Hence, efforts to find alternative approaches are uninterrupted in research. Apart from binding to the active site, most of the established ACE inhibitors such as lisinopril, captopril, and enalapril also chelate zinc which is essential for ACE activity and inhibit acceleration of high blood pressure and related complications.26 Chelation therapy is a conventional treatment method for cardiovascular diseases (CVDs)12,27. However, multi-specificity of EDTA in chelating all divalent metal ions such as calcium, copper, magnesium in addition to zinc rendered its use minimally. Screening for an FDA approved zinc-specific chelator such as TTD against inhibition of ACE, NEP, and APN to manage hypertension appear feasible. TTD inhibited ACE, NEP and APN dose-dependently with an IC<sub>50</sub> value of 10.15, 22.35, 3.032 μM respectively. Potent inhibition of ACE, NEP and APN activity by TTD indicates its high specificity towards chelation of zinc. RAS and Natriuretic peptide pathway is activated by oxidative stress which operates through a well-known L-arginine nitrous oxide (NO) pathway and plays a significant role in the regulation of blood pressure and cardiovascular system. Chronic administration of L-arginine analog, N<sup>6</sup>-nitro-L-arginine methyl ester (L-NAME) blocks NO synthesis resulting in high
Figure 5: Effects of TTD and L-NAME on heart and kidney tissue morphology. After euthanasia, tissues were harvested and stored in 10% buffered formalin, processed and embedded in paraffin blocks. 4 μm thick sections were cut and stained with haematoxylin-eosin. Heart and kidney sections were photographed at 10X magnification using Axio-imager bright field microscope. Upper panel representing heart tissue sections, A: Control. B: TTD. C: L-NAME. D: L-NAME + TTD. E: L-NAME + Lisinopril. Lower panel represents kidney tissue sections, F: Control. G: TTD. H: L-NAME. I: L-NAME + TTD. J: L-NAME + Lisinopril.

Figure 6: Effect of TTD and L-NAME on fibrosis of heart and kidney. Cross sections of heart and kidney sections (4 μM) were stained with collagen specific picro-sirius red dye. Upper panel representing heart tissue sections, A: Control. B: TTD. C: L-NAME. D: L-NAME + TTD. E: L-NAME + Lisinopril. Lower panel represents kidney tissue sections, F: Control. G: TTD. H: L-NAME. I: L-NAME + TTD. J: L-NAME + Lisinopril.

blood pressure associated with cardiovascular complications such as hypertrophy and fibrosis in rats. Decreased level of NO synthesis leads to persistent activation of RAS which further leads to hypertension, hypertrophy of vascular organs, cardiac remodeling and hence forms a well-established model of hypertension.

In these lines, in vivo efficacy of TTD was evaluated using L-NAME hypertensive rats. Continuous administration of TTD significantly decreased SBP in L-NAME-induced hypertensive rats. One of the reported side effects of TTD treatment in chronic alcoholic subjects is the induction of hypotension. This decrease in SBP may be attributed to inhibition of ACE and NEP activity reflected by Ang II and ANP levels in the serum. Also evident that reduction of SBP was also associated with decreased heart weight to body weight ratio (HW/BW) and kidney weight to body weight ratio (KW/BW) when compared to L-NAME alone, this signifies the anti-hypertrophic property of TTD on vascular organs. Moreover, decreased circulatory levels of Ang II and ANP are probably due to inhibition of ACE and NEP respectively by TTD, which also prevented L-NAME induced fibrosis of vascular organs subsequently reducing HW/BW and KW/BW ratio significantly. Additionally, ANP is known to stimulate the anti-oxidant defense system in cardiomyocytes and vascular cells which in turn prevent oxidative stress-induced cardiovascular complications.

L-NAME-induced pathophysiological modifications lead to alterations of biochemical indices as well. L-NAME increases metabolites such as urea and creatinine levels in serum reflecting L-NAME induced renal injury. TTD significantly reduced serum concentrations of urea and creatinine demonstrating its re-
nal protective effects. Besides, L-NAME treated rats exhibited a marked elevation of liver marker enzymes AST and ALT which signifies hepatic injury.\textsuperscript{36} TTD treated rats showed a marked decrease in the hepatic injury markers signifying hepatoprotective role. There was a two-fold increase in the AST and ALT levels in the L-NAME group, which was markedly decreased in TTD group. L-NAME model is characterized by reduced NO activity, which was reflected by reduced nitrite levels in the L-NAME group.\textsuperscript{37} There was no significant increase in the nitrite levels of TTD group which suggests that SBP lowering is due to inhibition of hypertensive enzymes and not nitric oxide synthase. Whereas, a significant increase in nitrate levels of lisinopril group needs further investigation.\textsuperscript{38}

Chronically elevated levels of Ang II results in deposition of collagen which in turn leads to fibrosis.\textsuperscript{39} Picro-sirus red stained heart and kidney sections show the marked decrease in the collagen deposition, signifying anti-fibrotic effect of TTD. Overall, anti-hypertensive and anti-fibrotic effects of TTD can be exploited as an alternative treatment strategy in hypertensive subjects.

CONCLUSIONS
Administration of TTD prevents the development of hypertension and related complications in L-NAME induced hypertensive rats. The effects of TTD include moderate prevention of myocardial and renal fibrosis and reduction of hepatic and renal markers. The protection may be primarily attributed to the inhibition of pro-hypertensive enzymes and marked the increase of vasodilators such as ANP. In this context, TTD can be considered as an alternative treatment of hypertension and associated cardiovascular complications.

ABBREVIATIONS
RAS: Renin-angiotensin system
ACE: Angiotensin-converting enzyme
Ang II: Angiotensin
ANP: Atrial natriuretic peptide
SBP: Systolic blood pressure

DISCLOSURE
The authors report no conflicts of interest in this work.

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REFERENCES


