TRC4149 a Novel Advanced Glycation End Product Breaker Improves Hemodynamic Status in Diabetic Spontaneously Hypertensive Rats

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Abstract

Objective: Advanced Glycation Endproducts (AGEs), implicated as one of the major causes of diabetic complications, either directly or via receptor mediated actions, trigger downstream events in the conduit vessels, microvascular bed as well as myocardium leading to microvascular and cardiac dysfunction. The aim of this study was to characterise the activity profile of TRC4149, a novel AGE breaker compound, to determine its ability to reduce the burden of AGEs in vitro and in vivo and to evaluate whether the reduced AGE burden could translate into improvement in hemodynamic function in a model of Streptozotocin induced diabetic Spontaneously Hypertensive Rats (SHR).

Method: AGEs were prepared in vitro by incubating BSA and lysozyme with glucose or ribose while AGE-LDL was generated by copper catalyzed LDL oxidation. TRC4149 was evaluated using in vitro assays to determine its capacity to reduce the burden of AGEs and to test its antioxidant activity.

To study the effect of TRC4149 on hemodynamic function, diabetic SHR implanted with telemetry transmitter were treated with TRC4149 (20mg/kg i.p., b.i.d.) or vehicle for 14 weeks. Losartan was administered once per week and blood pressure was monitored telemetrically throughout the treatment period. Cardiac indices of systolic and diastolic function were assessed terminally using MacLab system. AGE load in aorta was determined immunohistochemically and VCAM expression was quantitated by real time PCR analysis.

Results: TRC4149 was able to break preformed AGEs as well as reduce further AGE accumulation in vitro in a dose dependent manner. It also demonstrated a potent free radical scavenging activity. In diabetic SHR, treatment with TRC4149 retarded the decline in response to losartan over the study period, and also improved cardiac function as evidenced by an improved dP/dtmax/min, left ventricular systolic pressure and decreased left ventricular diastolic pressure as compared to untreated group. AGE load as well as VCAM expression in aorta was also reduced upon treatment.

Conclusions: TRC4149, a novel AGE-breaker compound, by virtue of reducing AGE load preserved endothelial and cardiac function in diabetic SHR, a model that recapitulates the microvascular and cardiac dysfunction associated with hypertension along with long-term diabetes.

Key words: Diabetic complications, Advanced Glycation Endproducts, TRC4149, TRC4186, Spontaneously Hypertensive Rats, Endothelial Dysfunction

Abbreviations: ABTS = 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid), AGEs = Advanced Glycation Endproducts, ALEs = Advanced Lipoxidation Endproducts, BNP = Brain Natriuretic Peptide, BP = Blood Pressure, BSA = Bovine Serum Albumin, cDNA = Complimentary De-Oxyribose Nucleic Acid, CML = Carboxymethylysine, CTGF = Connective Tissue Growth Factor, ECM = Extracellular Matrix, ELISA = Enzyme Linked ImmunoSorbent Assay, H_2O_2 = Hydrogen Peroxide, LDL = Low Density Lipoproteins, LVDP = Left Ventricular Diastolic Pressure, LVSP = Left Ventricular Systolic Pressure, NO = Nitric Oxide, PBS = Phosphate Buffer Saline, RAGE = Receptor for AGE, RNA = Ribose Nucleic Acid, ROS = Reactive Oxygen Species, RT-PCR = Real-Time Reverse Transcription – Polymerase Chain Reaction, STZ = Streptozotocin, SHR = Spontaneously Hypertensive Rats, TNBS = 2,4,6-Trinitrobenzenesulphonic acid, VCAM = Vascular Cell Adhesion Molecule-1

INTRODUCTION

Diabetes is essentially a disorder with chronic hyperglycemia. However, there is a strong link between diabetes and cardiovascular disease, with nearly 80% of the deaths associated with diabetes being due to cardiac complications [1]. In fact, Type 1 and Type 2 diabetes are powerful and independent risk factors for coronary artery disease, stroke, peripheral artery disease [2] and also micro-vascular diseases such as neuropathy [3], nephropathy [4] and retinopathy [5].

There is an increasing body of evidence implicating Advanced Glycation Endproducts (AGEs) as one of the major causes of diabetic complications. AGEs are

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formed by a complex chain of reactions between reducing sugar such as glucose with proteins, resulting in the formation of multimeric complexes that trigger several pathological events [2]. In the extracellular environment, AGE modification of proteins leads to crosslinking of extracellular matrix (ECM) proteins, especially collagen and elastin, leading to increased vascular stiffness and diminished arterial and myocardial compliance [6]. AGEs have been shown to quench nitric oxide (NO), thereby mediating a defective endothelium - dependent vasodilation [7]. Clinical studies have demonstrated increased levels of AGEs on low density lipoproteins (LDL) in diabetics compared to normal individuals. Glycated LDL is more susceptible for oxidative modification, a critical step in the initiation of atherogenesis. Intracellular formation of AGEs leads to functional derangement of macromolecules. Interaction of AGEs with their receptors (RAGE) present on several cell types including endothelial cells, smooth muscle cells and macrophages causes increased oxidative stress and induces the production of pro-inflammatory cytokines and growth factors [1]. Studies show that type 2 diabetes is associated with low grade inflammation and endothelial dysfunction which correlate with an increased risk of cardiovascular mortality [8]. AGEs, thus contribute to the development of a pro-inflammatory and proliferative state with increased oxidative stress resulting in endothelial dysfunction, the common underlying mechanism leading to end organ damage.

Several preclinical studies have established the role of AGEs in the development of microvascular and macrovascular complications of diabetes. Treatment of streptozotocin (STZ) induced diabetic rats with ALT-711, a compound that breaks AGE crosslinks, has been shown to reverse AGE- mediated vascular stiffness, distensibility [9] and attenuate diabetes induced structural changes in the myocardium [10]. ALT-711 treatment also improved arterial compliance in aged humans with vascular stiffening [11]. Aminoguanidine, an inhibitor of AGE formation, has been shown to prevent diabetes induced arterial wall protein crosslinking [12] and also retard the development of diabetic nephropathy in rats. These studies thus provide a rationale for a therapeutic strategy based on reducing the burden of AGE for addressing diabetic complications.

Torrent's discovery program identified TRC4149, a novel AGE breaker compound which was synthesized, screened and evaluated for its activity in long-term studies to study potential benefit in diabetic complications at Torrent Research Centre. The aim of this study was, a) to characterise the activity profile of TRC4149, a novel AGE breaker compound in vitro, to determine its capacity to reduce the burden of AGE across a variety of highly cross-linked proteins and lipid fraction (LDL), and antioxidant activity as well as specificity of its actions, and b) to confirm its activity in vivo by measuring effect on AGE-crosslinks on a long-lived protein and finally, c) to evaluate whether the reduced AGE burden could translate into improvement in hemodynamic function in a rat model of hypertension superimposed with diabetes (STZ induced diabetic SHR). In this model, which simulates the human situation wherein hypertension and diabetes are generally found to coexist, preservation of blood pressure (BP) response to losartan over time was used as a measure of microvascular endothelial and cardiac response to treatment. In addition, invasive measurement of cardiac indices of systolic and diastolic function and estimation of AGE load was undertaken terminally.

MATERIALS AND METHODS

CHEMICAL SYNTHESIS

TRC4149/TRC4186. TRC4149 was synthesized by reacting N-methanesulfonyl nicotinic hydrazide with 2-(Bromoacetyl) thiophene in methanol at reflux temperature. The isolated solid was further purified using water and methanol. Similarly, TRC4186 was prepared by reacting N-methanesulfonyl nicotinic hydrazide with 2-(Chloroacetyl) thiophene instead of 2-(Bromoacetyl) thiophene.

IN VITRO ACTIVITY

Measurement of AGE breaker activity by ELISA. AGE-BSA was prepared in vitro as previously described [13]. Briefly, 160mg/ml of Bovine Serum Albumin (BSA) (Sisco Research Laboratories, India) was incubated with 1.67M glucose (SD fine chemicals, India) at 37 °C for a period of 16 weeks. The preparation was extensively dialyzed to remove excess of glucose and the formation of AGE was confirmed by measurement of the characteristic AGE fluorescence at excitation and emission wavelengths of 355nm and 460nm respectively (Fmax, Molecular devices, U.S.A.) as well as by SDS-PAGE analysis. AGE-BSA aliquots were stored at –20 °C.

AGE breaker activity was determined using the ELISA based method of Vasan et al. with minor modifications [14]. Crosslinking of AGE-BSA to collagen was achieved by incubating it with the collagen coated surface of a 96 well collagen coated plate (BD Biocoat™, BD Biosciences) for 4 hours at 37 °C. The unbound AGE was washed off, and different concentrations of TRC4149 dissolved in Phosphate Buffer Saline (PBS) were added to the wells and the incubation continued further for 18 hours. Wells containing only PBS served as drug free controls. Upon incubation, the wells were once again washed extensively and the amount of BSA crosslinked to collagen was quantitated using anti-BSA antibodies (Sigma, U.S.A.). The AGE breaker activity was calculated using the formula:

% AGE breaker activity = 100 * A450 (PBS control) – A450 (TRC4149) / (A450 (PBS control)).

Measurement of AGE formation by fluorospectrometry. BSA diluted in PBS to a concentration of 10mg/ml was incubated alone, with 100mM D-ribose (Sisco Research Laboratories, India), and with D-ribose and various concentrations of TRC4149. All the samples were incubated at 37°C and fluorescence was measured on day 7, day 15 and day 21 at excitation and emission wavelengths of 355nm and 460nm respectively (Fmax, Molecular Devices, U.S.A.). Appropriate controls were used to determine any non-specific effects of TRC4149 on fluorescence intensity.

Measurement of crosslinked AGE-lysozyme by SDS-PAGE analysis. Lysozyme (Sigma, U.S.A) dissolved in PBS at a concentration of 10mg/ml was incubated alone, with 250mM D-ribose and various concentrations of TRC4149. All the samples were incubated at 37°C for 21days. At the end of the incubation period, the samples were analyzed by SDS-PAGE. The gels were stained with Coomassie blue dye and the extent of formation of lysozyme dimer and trimer in TRC4149 treated samples was quantitated relative to the control (absence of TRC4149) by densitometric analysis (Gel Doc 2000, Biorad).

Measurement of free amino groups. 2,4,6-Trinitrobenzenesulphonic acid (TNBS) binds to the free amino groups of proteins and forms trinitrophenylated lysine which is measured spectrophotometrically. The free amino groups in AGE-lysozyme prepared as above in the presence and absence of TRC4149 were quantitated periodically up to 28 days using the TNBS assay with some modifications [15]. The results were expressed as % free amino groups with the absorbance of lysozyme normalized to 100%.

Measurement of AGE-LDL. LDL (200µg protein/ml, Sigma, St. Louis, MO) was incubated with 10µM $CuCl_2$ at 37°C in phosphate buffered saline for 48 hours with and without TRC4149. The extent of formation of AGE-LDL was determined in terms of fluorescence intensity at excitation and emission wavelengths of 355nm and 460nm respectively.

Determination of antioxidant activity. Incubation of 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS) with peroxidase and hydrogen peroxide (H_2O_2) results in the production of radical cation ABTS•+ with absorbance maxima at 730nm. Antioxidants or free radical scavengers reduce the blue green colour of the ABTS radical by a degree that is proportional to their concentration. ABTS stock solution (2mM) was incubated with horseradish peroxidase (131munits/ml) and H_2O_2 (1.08M) at room temperature for 30 minutes. To the solution so formed, different concentrations of TRC4149 were added and the absorbance was measured at 730nm. Antioxidant activity of TRC4149 was calculated by comparing the absorbance against that of the control (absence of TRC4149) Ascorbic acid was used as a positive control.

Animal Studies. 8-10 weeks old male Wistar rats (NIN, Hyderabad, India) and SHR (Taconic Farms, Germantown, NY, U.S.A.) were used in the study. Rats were housed in standard laboratory conditions and had free access to standard laboratory rat chow and water *ad libitum.* All the procedures used in study were approved by the Institutional Animal Ethics Committee.

Rat Tail tendon collagen solubility assay. Male Wistar rats were randomly divided into non-diabetic control and a diabetic group. Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ 60mg/kg, freshly dissolved in citrate buffer of pH 4.5. Non-diabetic control rats received injection of citrate buffer only. After 12 weeks of diabetes, TRC4149 5mg/kg (n = 5) or 10mg/kg (n = 6) or vehicle were administered twice a day for 8 weeks by i.p. injection.

At the end of treatment period rats were sacrificed, tail tendons were carefully removed and minced, immediately weighed and processed further to determine extent of collagen solubility by hydroxyproline assay according to Edwards and O'Brien [16]. In brief, tail tendons were homogenized and hydrolyzed by heating at 110ºC overnight in HCl (6N). Hydrolyzed rat tail tendons were then dried and remnants of HCl were neutralized. Color development reaction was initiated by adding 1.5ml of 0.05M of Chloramine T and was allowed to continue for 25 minutes following which 1.5ml of aldehyde perchloric acid solution was added. This reaction mixture was heated at 60°C for 15 minutes during which red chromophore develops. After cooling, absorbance was recorded at 550nm within 3 hours.

Hemodynamic measurements. SHR were implanted with radiotelemetry transmitters (TA11PAC40, Data Sciences, St. Paul, MN, U.S.A.) at the age of 8-10 weeks. Conscious rats were anesthetized with isoflurane inhalation and were administered a single dose of Penicillin G (30,000IU/rat, i.m.) and Buprenorphine (0.75 mg/Kg, s.c.). Body temperature was maintained at 37°C. Through a midline abdominal incision, a gel-filled telemetric catheter was introduced into the descending aorta above the iliac bifurcation and pushed just caudal to the renal arteries. The catheter was secured at the entry to the vessel and the transmitter body was sutured to the inner peritoneal wall before the midline incision was closed. After a week's recovery, BP was recorded with help of computer driven data acquisition system (Dataquest A.R.T. 2.2, Data Sciences). The acquisition software was appropriately configured to record BP for 10 seconds every 10 minutes, continuously for 24 hours. On the basis of BP, rats were randomized into diabetic control (n = 25) and diabetic treated (n = 25) group. Diabetes was induced by administering single i.p. injection of STZ at dose of 35mg/kg. TRC4149 (20mg/kg i.p., b.i.d.) or vehicle was administered to rats in treatment and control groups respectively for next 14 weeks.

All the rats were administered the vehicle for losartan on day 3 and losartan (20mg/kg, i.p.) on day 5 of each treatment week and BP was monitored. After 14 weeks of continuous treatment with TRC4149, all the animals underwent a 5 to 7 days treatment free period during which rats were not administered TRC4149 or vehicle but still received losartan challenge to study persistence of response in drug-free recovery period.

Terminal measurement of ventricular function was performed in rats anesthetized with Urethane (1gm/kg, i.p.).

A heparin saline filled catheter was introduced into the right carotid artery and was advanced retrograde into the left ventricle. Ventricular pressure was recorded using MacLab/8s recording system coupled with QUAD Bridge and MLT844 physiological pressure transducer (ADInstruments, Australia). All recording

and analysis were carried out using Chart 4 software for MacLab (ADInstruments, Australia).

Immunohistochemistry. Proximal part of thoracic aorta was fixed in 10% neutral buffer formalin. 4 μm thick paraffin sections were deparafinized and rehydrated with PBS. Antigen retrieval and nonspecific blocking were performed by incubating the sections with 0.05% proteinase-K, followed by 0.35% H_2O_2 in methanol and 1:37 dilution of Horse serum (Vectastain® ABC kit, Burlingame, U.S.A.). Monoclonal mouse anti-CML antibody 6D12 (Research Diagnostic INC, NJ) was applied on the section at dilution of 1:100 in PBS and incubated overnight at 4°C. Sections were rinsed with PBS and incubated with biotinylated anti mouse secondary antibody (1:200, Vectastain® ABC kit) followed by Avidin biotin peroxidase Complex (Vectastain® ABC kit) each for an hour at room temperature. Color reaction was initiated by incubating the section with Diamino benzadimine (Vector laboratories, Burlingame, U.S.A.) in dark for 6 minutes. Sections were counter stained with haematoxylin and were analyzed semi-quantitatively under 1000X magnification in blinded fashion. Staining intensity for Carboxymethylysine (CML) in all the layers of aorta was scored on an arbitrary scale of 0 to 3. Score 0, was given for staining equivalent to negative control, 1 for weak staining, 2 for moderate staining, 3 for strong intense staining. A total of 100 nucleated smooth muscle cells in the media were counted from different fields. Cells showing positive labeling for CML were expressed as a percentage of total number of cells counted.

Real-Time Reverse Transcription – Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from aorta using the Trizol reagent (Gibco BRL) and 1μg was used for cDNA synthesis (Applied Biosystems). VCAM expression in the aorta was measured by Taqman RT-PCR with the ABI 7000 Sequence Detection system (Applied Biosystems). 18s Ribosomal RNA was used as the endogenous control to normalize for variances in the RNA input. The VCAM primer and probe and the other RT-PCR reagents were obtained from Applied Biosystems.
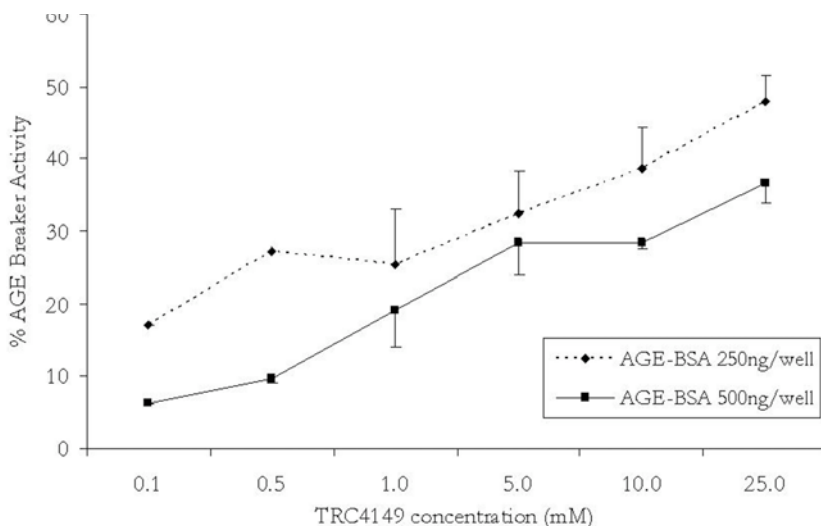
STATISTICAL ANALYSIS

Left ventricular functional data & Graphical representation are expressed as Mean ± SEM. Between group comparison for more than 2 groups has been carried out using ANOVA followed by Post-Hoc Dunnett t-tests (two-sided). Between groups analysis for repeat observations over the period of time has been compared using repeated measures analysis of variance (RMANOVA). Ability of TRC4149 to break preformed AGE crosslinks has been analysed using two way ANOVA. Difference between any two groups has been assessed by t-test for which statistical significance was defined as one-sided 'p' value ≤0.05. Statistical analysis has been performed using SAS (Version-9.1).

RESULTS

TRC4149 and TRC4186 (Fig. 1) belong to the pyridinium class of compounds and are bromide and chloride salts respectively of the parent base.
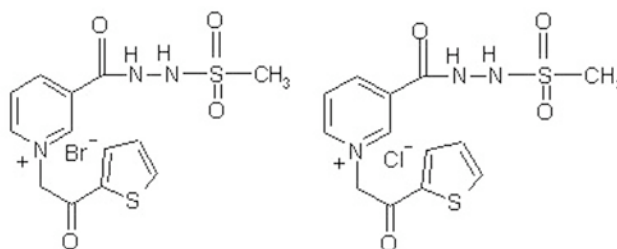

Fig. 1. Structure of TRC4149 and TRC4186.

AGE breaker activity of TRC4149. Treatment with TRC4149 resulted in a concentration dependent decrease in the amount of AGE-BSA crosslinked to collagen as observed by a decrease in absorbance upon ELISA with anti-BSA antibodies. The release of BSA from the preformed crosslinks was linear till a TRC4149 concentration of 25mM (Fig. 2). The results thus indicate that TRC4149 breaks preformed AGEs significantly (p<0.001) as a function of both the AGE load as well as concentration of the compound.

Fig. 2. Ability of TRC4149 to break preformed AGE crosslinks as measured in vitro in the collagen-cross linking assay.
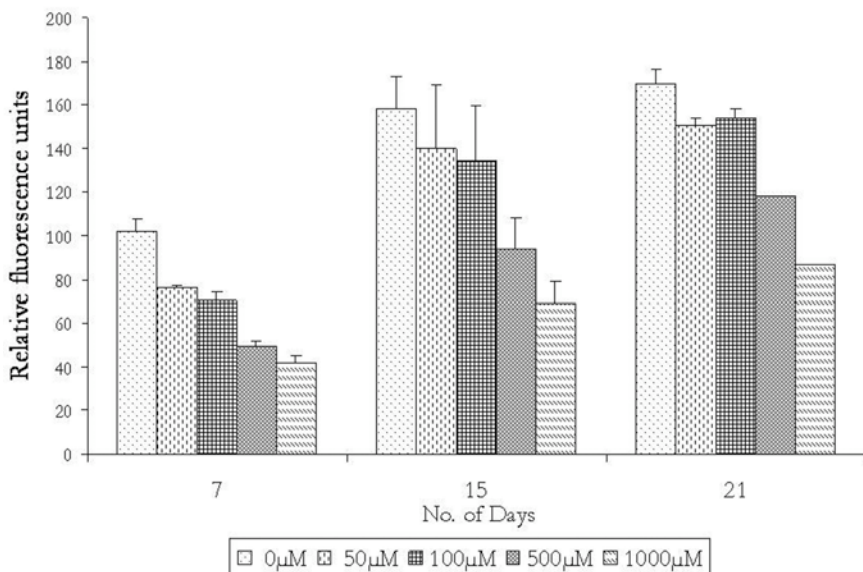
Fig. 3. Ability of TRC4149 to reduce AGE accumulation upon incubation of BSA with ribose for 21 days.

Effect of TRC4149 on AGE accumulation. AGEs have a characteristic fluorescence spectrum and also form protein–protein crosslinks. In vitro assay methods based on these AGE characteristics were used to determine the effect of TRC4149 on AGE accumulation. The use of ribose as the reducing sugar and the supraphysiological concentrations of BSA and ribose accelerated the process of AGE formation.

Incubation of BSA with ribose for one week resulted in a significant AGE accumulation as evidenced by an increase in the AGE specific fluorescence intensity. TRC4149, when incubated along with BSA and ribose retarded the rate of AGE accumulation in a dose dependent manner, with a 60% reduction ($p<0.001$) in AGE accumulation at a concentration of 1mM. A similar trend was observed till day 21 ($p<0.001$) (Fig. 3).

The effect of TRC4149 on lysozyme cross-linking in vitro was evaluated by SDS–PAGE. The gel pattern of lysozyme and ribose incubated together for 21 days showed increased amounts of high molecular weight bands with molecular weights corresponding to that of lysozyme dimer and trimer. Treatment with TRC4149 reduced the formation of crosslinked proteins (Fig. 4).

Effect of TRC4149 on free amino groups. The formation of protein-protein crosslinks, a result of nonenzymatic glycosylation has been postulated to involve the free amino groups of lysine residues. AGE formation leads to an irreversible modification of amino groups thereby reducing the reactivity of the protein with TNBS reagent. Incubation of lysozyme with ribose resulted in a decrease in TNBS reactivity with only 65% of the amino groups remaining free at the end of 28 days. Treatment with TRC4149 prevented the modification of lysine residues with a time and dose response relationship such that 81% and 95% of the amino groups were still free to react with TNBS on day 28 at TRC4149 concentrations of 10mM and 20mM respectively.

Effect of TRC4149 on formation of AGE-LDL. AGE-LDL is formed as a result of adduct formation between reactive aldehydes generated during copper catalysed oxidation of LDL and lysine residues of apoB [17]. Oxidation of LDL with $CuCl_2$ resulted in derivatization of all the lysine residues of apoB within 16 hours. The amount of AGE-LDL formed in the absence of TRC4149 was considered to be 100%.
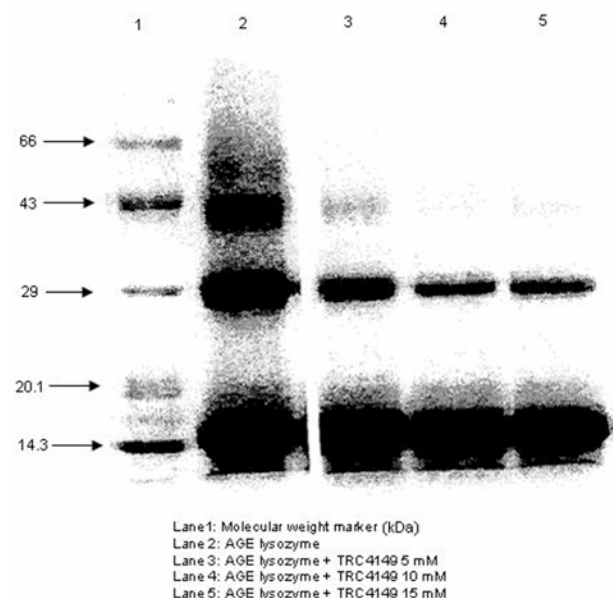

Lane1: Molecular weight marker (kDa)
Lane 2: AGE lysozyme
Lane 3: AGE lysozyme + TRC 4149 5 mM
Lane 4: AGE lysozyme + TRC 4149 10 mM
Lane 5: AGE lysozyme + TRC 4149 15 mM

Fig. 4. Ability of TRC4149 to reduce AGE accumulation upon incubation of lysozyme with ribose for 21 days.

Table 1. Antioxidant activity of TRC4149.

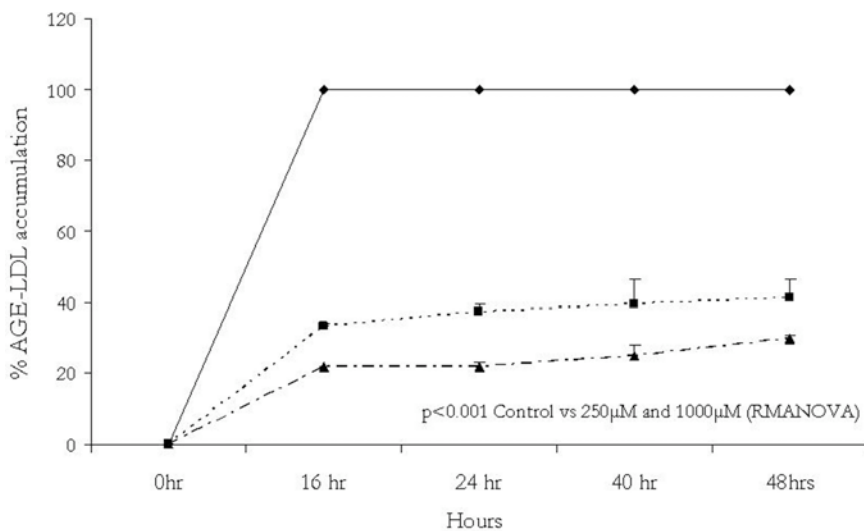| TRC4149 Concn.(µM)	% Antioxidant Activity	
	TRC4149	Ascorbic acid
11.5	47.28	29.51
23.00	65.66	43.81
46.00	83.78	95.41
92.50	100	100

Fig. 5. Effect of TRC4149 treatment on MDA-lysine adduct formation upon incubation with LDL and $CuCl_2$ at 37°C for 48 hours.
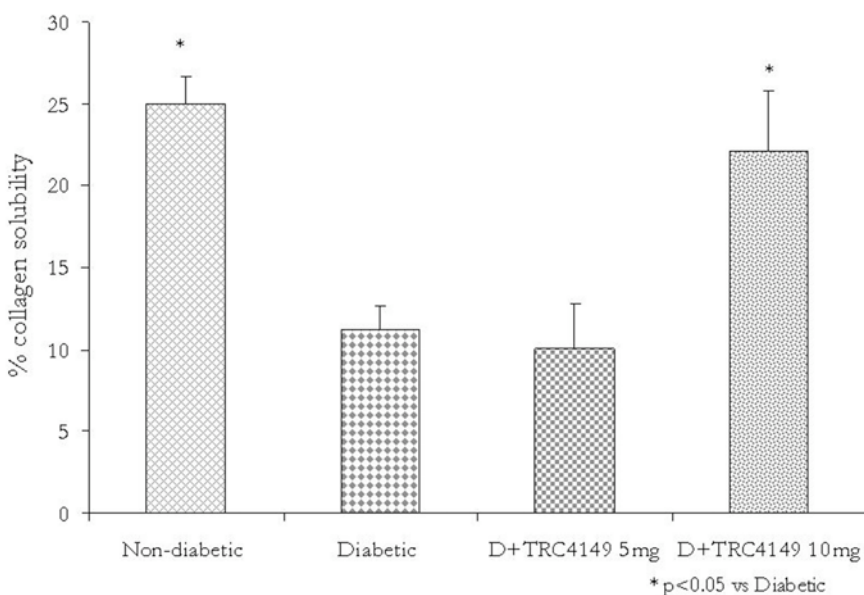

Fig. 6. Percent collagen solubility in non-diabetic, diabetic control, 4149 treated-5mg/kg and 4149 treated-10mg/kg groups.

TRC4149 at concentrations of 0.25mM and 1mM reduced AGE-LDL accumulation significantly by 59% (p<0.001) and 70% (p<0.001) respectively (Fig. 5).

Antioxidant activity of TRC4149. TRC4149 exhibits a dose dependent free radical scavenging activity that is better than that of ascorbic acid at lower concentrations and comparable to ascorbic acid at higher concentration (Table 1).
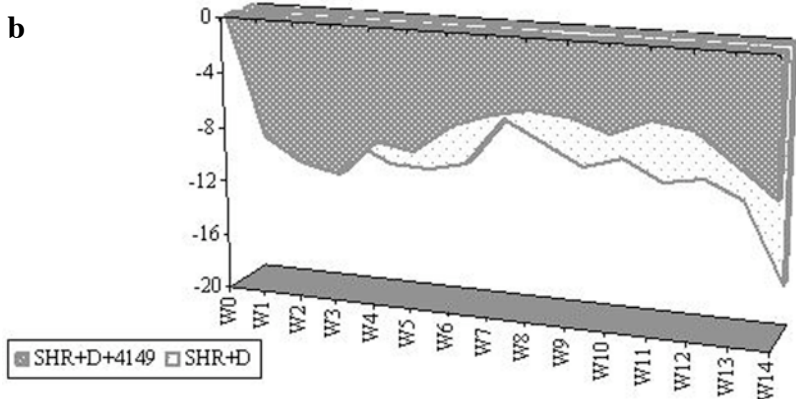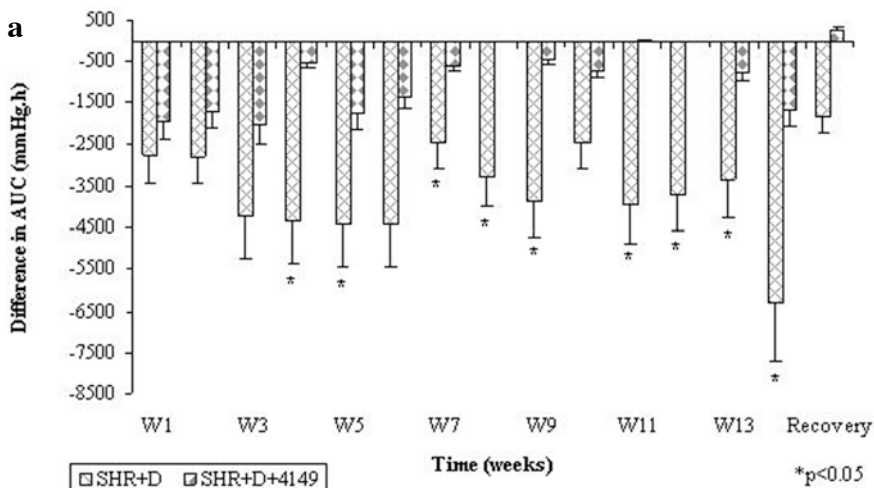
Rat tail tendon collagen solubility. Diabetes caused a significant decrease in the collagen solubility of the rat-tail tendon (p<0.05) as compared with non diabetic control.

A dose of 10mg/kg led to a significant (p<0.05) improvement in collagen solubility as compared to the diabetic controls whereas a dose of 5mg/kg failed to impove collagen solubility (Fig. 6).
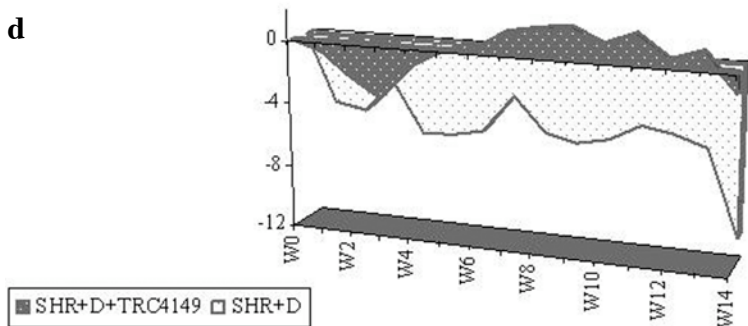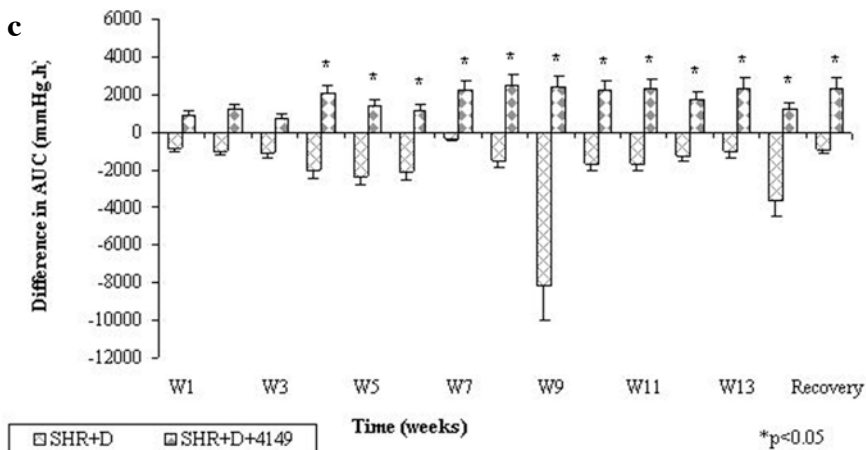
Measurement of hemodynamic parameters. There was a progressive decline in the lowering of blood pressure by losartan over time in diabetic SHR (SHR+D). TRC4149 treatment retarded the decline in response to losartan, as compared to untreated group. The loss

of responsiveness to losartan compared to week 0 was prevented, beginning after 3 weeks of treatment with TRC4149 (Fig. 7) and lasted throughout the study. The improvement in response to losartan was compared as area over curve (AOC) of the difference from baseline in the fall in blood pressure in response to losartan measured each week. This effect was significant (p<0.05) for both diastolic and systolic blood pressure response (Fig. 7). Even after discontinuation of treatment, TRC4149 treated diabetic SHR animals were better able to maintain their response to losartan until the end of recovery period as compared to untreated diabetic SHR group.

Assessment of left ventricular functional parameters. Diabetes caused a fall in left ventricular systolic pressure (LVSP), dP/dtmax and dP/dtmin whereas rise was observed in left ventricular diastolic pressure (LVDP) in comparison to control SHR group (Table 2). Treatment with TRC4149 (20mg/kg) resulted in a significant improvement in all of these 4 parameters (Table 2) as compared with vehicle-treated diabetic rats.

Fig. 7. Difference from baseline in AUC SBP (a) and DBP (c) response to losartan and Area over the curve of the difference from baseline fall in (b) SBP and (d) DBP as a response to losartan.

Table 2. Left ventricular functional parameters.

Groups	Left Ventricular Systolic Pressure	Left Ventricular Diastolic Pressure	dP/dt max	dP/dtmin
SHR+D (n:16)	120.5±8.25	3.5±2.4	3948.7±394.9	-2983.9±320.8
SHR+D+4149 (n:15)	147.6±7.15**	-2.7±2.04*	5157.0±297.1*	-4110.4±269.5**

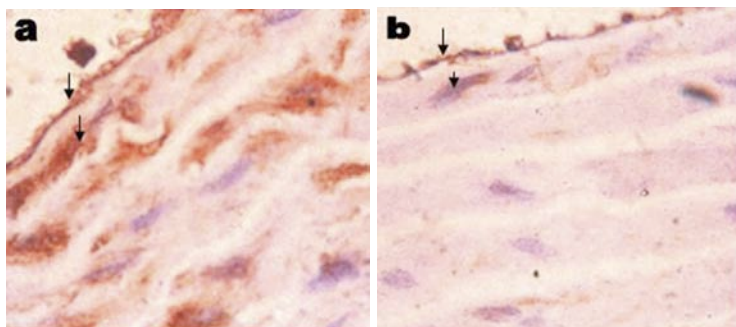* : p<0.05; ** : p<0.01 vs. SHR+D

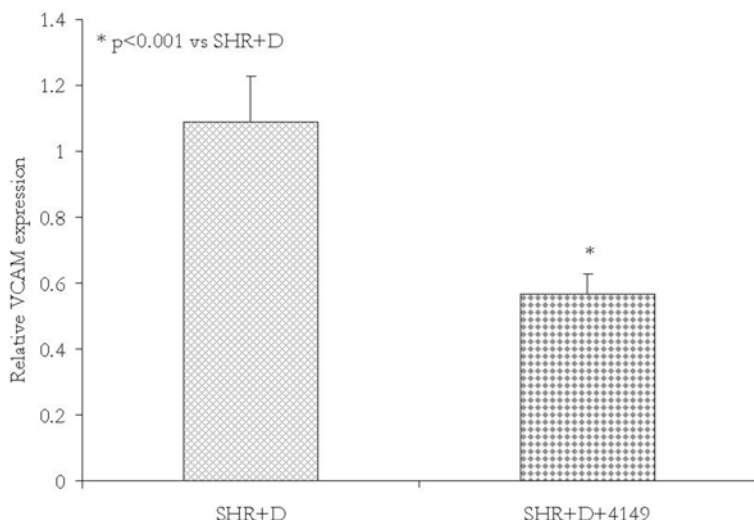*Fig. 8.* Labeling for CML in subendothelial layer in (a). SHR+D and (b). SHR+D+4149.

Fig. 9. Relative VCAM expression in aorta of control versus TRC4149 treated group.

Immunohistochemical detection of CML. Aortic sections stained for detection of CML demonstrated diffuse positive staining in the endothelial layer whereas, intracellular staining was observed in the sub-endothelial layer. Labeling for CML was observed in non diabetic as well as diabetic SHR animals, however intensity of labeling was clearly more intense in diabetic control. Treatment with TRC4149 resulted in a reduction in labeling with significantly more number of animals showing weaker labeling as compared to untreated diabetic group (Fig. 8).

Measurement of VCAM expression. VCAM levels were reduced significantly (p<0.001) upon treatment with TRC4149 in the diabetic SHR group as compared to the respective untreated control group (Fig. 9).

DISCUSSION

In this study, we have shown that TRC4149 breaks AGE cross-links in a variety of heavily cross-linked protein-sugar complexes, and also displays anti-oxidant and anti-inflammatory properties. These actions are clearly shown to translate into prevention of crosslinking of long-lived structural proteins such as collagen, reduction in immunochemically detectable AGE and improving the deleterious effects of AGE, directly or receptor-mediated, as evident from a reduced VCAM mRNA expression in the diabetic animal model. The ultimate result is retardation of development of diabetic microvascular and cardiac complications.

Chronic hyperglycemic state induces AGE accumulation with ensuing triggering of downstream events in the conduit vessels, microvascular bed as well as myocardium. The synergistic deleterious effect of diabetic and hypertensive states on the micro-and macro-vasculature and the myocardium observed clinically is the paradigm sought to be replicated in diabetic SHR animal model.

AGEs are heterogenous in nature and a number of AGEs such as N-methyl carboxymethylysine, pentosi-

dine, pyraline, and crossline, formed during in vitro non-enzymatic glycation of proteins have also been found to be elevated in diabetic tissues [18] and correlate with the development of diabetic complications. Antibodies produced against AGE epitopes formed in vitro, cross-react with in vivo AGEs, thus indicating structural homogeneity [19] between the two. Therefore it can be postulated that a reduction in AGE burden in vitro was reflected as reduced total AGE burden in vivo. Treatment with TRC4149 prevented the progression of AGE-linked modifications of structural and functional proteins at different sites.

In the large conduit vessels, collagen is one of the long-lived structural proteins, which undergoes extensive modifications secondary to AGE formation leading to increased stiffness of the protein matrix resulting into reduced acid solubility and enhanced resistance to proteolytic degradation. The significant improvement in the tail tendon collagen solubility of diabetic rats treated with TRC4149 suggests that the AGE-breaker action demonstrated in vitro was effectively translated in vivo as well, indicating that TRC4149 was able to penetrate and break AGEs from a heavily glycated target like the tail tendon collagen, in vivo.

In the microvascular bed, AGEs contribute to diabetes mediated decrease in the sensitivity to endothelial-dependent relaxation due to reduced availability of NO [20]. AGEs are shown to quench NO, an important mediator of endothelium-derived relaxation [21, 22]. Physiologically relevant concentrations of CML-adducts induce expression of a range of pro-inflammatory cytokines, adhesion molecules by activation of nuclear factor (NF)κB. There is resultant smooth muscle proliferation and accumulation of extracellular matrix proteins which has been reported to occur in the microvasculature in the presence of diabetes in SHR [23]. Reduction in the AGE load with TRC4149 treatment with consequent attenuation of the pro-inflammatory and proliferative events, as evident by a reduced VCAM expression, could have contributed to the maintenance of microvascular response to losartan in this study thereby improving aortic compliance. Similar adaptations would also be responsible for modulating cardiac function in the disease model.

Increased formation of AGEs on myocardial proteins contribute to myocardial stiffness observed in diabetic SHR. AGEs formed on myocardial calcium handling proteins such as Ryanodine receptor (RyR2) and SERCA2a during diabetes are shown to directly compromise intracellular calcium release and consequently alter cardiac contractility on one hand and ineffective removal during diastole leading to inadequate relaxation on the other [24]. Structural and biochemical changes ultimately leading to functional alterations in the vasculature and the myocardium manifesting as reduced left ventricular contractile indexes measured ex vivo [25] and in vivo [26,27] have been widely reported in diabetic models. Activation of presclerotic cytokines and growth factors leading to accumulation of extracellular matrix secondary to AGE-RAGE also adds to the insult [28]. An improvement in AGE-induced impairment of left ventricular compliance has been reported after breaking AGE cross links in aged

monkeys [29]. A significant improvement in cardiac function as evidenced by an improved dP/dtmax/min, LVSP and LVDP upon treatment with TRC4149, suggests that this would be secondary to AGE reduction and curbing of the ensuing chain of events in the cardiac tissue in diabetic SHR. TRC4149 is shown to improve response to losartan, most likely both by direct action on the myocardium and indirectly by correcting endothelial dysfunction. Our findings corroborate studies reported previously in STZ-induced diabetic models wherein ALT-711 was shown to improve left ventricular function, systemic arterial compliance and reduce peripheral resistance [30], and reduction in AGE load was associated with reduced Brain Natriuretic Peptide (BNP) and connective tissue growth factor (CTGF) expression [10].

Contributing to the AGE insult is oxidative stress in various forms. There is general agreement that there are multiple sources and mechanisms of formation of AGEs in vivo [31]. AGEs in turn activate generation of reactive oxygen species (ROS) triggering cell signaling via RAGE, at least in part via activation of NADPH oxidase. Under oxidative stress in diabetes, the polyunsaturated fatty acids of LDL undergo peroxidation reactions and form highly reactive aldehydes such as malondialdehyde, also referred to as Advanced Lipoxidation Endproducts (ALEs), which in turn modify the lysine residues of apoB and also induce protein crosslinking. These mechanisms are similar to those involved in the formation of AGEs and AGE inhibitors such as aminoguanidine and pyridoxamine have been shown to prevent the formation of ALEs [17,32]. On the same lines, we postulated that TRC4149 could also reduce the accumulation of AGE-LDL. Our results demonstrated that TRC4149 prevented LDL protein modification as observed by a reduced AGE induced fluorescence. Although, we did not study the mechanism of its action, considering the fact that TRC4149 is a potent free radical scavenger, it is likely to prevent apoB modification both by its antioxidant activity as well as by a mechanism similar to the one preventing the accumulation of AGEs.

Terminal assessment of cardiac function is a potential limitation of this study which precluded study of progression over time. This study was focused on evaluating microvascular and cardiac components of cardiovascular complications of diabetes, however, benefit accrued could be extended to other functions susceptible to AGE-mediated deterioration. Evaluation of therapeutic benefit in an animal model with more severe form of diabetes making them prone to diabetic heart failure and nephropathy would extend these findings to clinically relevant co-morbidities.

To enunciate the benefits of treatment with TRC4149 on complications arising out of diabetic micro- and macro-vascular dysfunction, preclinical efficacy data points to the fact that by its pharmacological actions, TRC4149 would, by continuous reduction of AGE burden, deal with the underlying pathology at multiple levels. TRC4149 treatment had no effect on the peptide bonds or the disulfide bonds in native proteins and also did not affect enzymatically glycosylated proteins, thus confirming its specificity towards AGE crosslinks (data not shown). One could postulate

therefore that by the combined effect of stabilizing the functional integrity of myocardial proteins on one hand and curtailing the inflammatory process and its deleterious consequences on the endothelial function on the other, TRC4149, would prove to be useful against situations of aggressive vasculopathy as observed in diabetes. TRC4149 and TRC4186 are two different salts of the same active pharmaceutical base. Assuming same therapeutic effects of both salts studies with TRC4186 are expected to recapitulate the findings observed with TRC4149. TRC4186, the chloride salt is presently in clinical development.

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