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ORIGINAL PAPER

3-Mercapto-5*H*-1,2,4-Triazino[5,6-b]Indole-5-Acetic Acid (Cemtirestat) Alleviates Symptoms of Peripheral Diabetic Neuropathy in Zucker Diabetic Fatty (ZDF) Rats: A Role of Aldose Reductase

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Abstract

Peripheral neuropathy is the most prevalent chronic complication of diabetes mellitus. Good glycemic control can delay the appearance of neuropathic symptoms in diabetic patients but it is not sufficient to prevent or cure the disease. Therefore therapeutic approaches should focus on attenuation of pathogenetic mechanisms responsible for the nerve injury. Considering the role of polyol pathway in the etiology of diabetic neuropathy, we evaluated the effect of a novel efficient and selective aldose reductase inhibitor, 3-mercapto-5*H*-1,2,4-triazino[5,6-b]indole-5-acetic acid (cemtirestat), on symptoms of diabetic peripheral neuropathy in Zucker Diabetic Fatty (ZDF) rats. Since the age of 5 months, male ZDF rats were orally administered cemtirestat, 2.5 and 7.5 mg/kg/day, for two following months. Thermal hypoalgesia was evaluated by tail flick and hot plate tests. Tactile allodynia was determined by a von Frey flexible filament test. Two-month treatment of ZDF rats with cemtirestat (i) did not affect physical and glycemic status of the animals; (ii) partially inhibited sorbitol accumulation in red blood cells and the sciatic nerve; (iii) markedly decreased plasma levels of thiobarbituric acid reactive substances; (iv) normalized symptoms of peripheral neuropathy with high significance. The presented findings indicate that inhibition of aldose reductase by cemtirestat is not solely responsible for the recorded improvement of the behavioral responses. In future studies, potential effects of cemtirestat on consequences of diabetes that are not exclusively dependent on glucose metabolism via polyol pathway should be taken into consideration.

Keywords Aldose reductase inhibitor · Cemtirestat · Diabetic peripheral neuropathy · Zucker diabetic fatty rats · Oxidative stress

Abbreviations

AKR1B1 : Human aldose reductase encoded by AKR1B1 gene
AGE : Advanced glycation endproducts
BHT : Butylated hydroxytoluene
CEM : Centre of Experimental Medicine
HbA1c : Glycated hemoglobin
MDA : Malondialdehyde
RAGE : Receptor for AGE
SAS : Slovak Academy of Sciences
TBA : Thiobarbituric acid
TBARS : Thiobarbituric acid reactive substances
TCA : Trichloroacetic acid
ZDF : Zucker diabetic fatty

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Introduction

Peripheral neuropathy is the most prevalent chronic complication of diabetes mellitus [1, 2]. The causal therapeutic strategy approved presently for diabetic neuropathy is limited to strict metabolic compensation. Good glycemic control can delay the appearance of neuropathic symptoms in diabetic patients but it is not sufficient to prevent or cure the disease. Therefore therapeutic approaches should focus on attenuation of pathogenetic mechanisms responsible for the nerve injury [3–5]. Obviously, only deep understanding of these pathogenetic mechanisms may help to identify promising therapeutic targets.

Metabolic imbalances in the peripheral nervous system that are activated in the diabetic milieu of hyperglycemia, dyslipidemia and impaired insulin signaling are considered key players in the development of diabetic neuropathy [6–9]. The crucial pathways comprise multiple mechanisms of glucose toxicity including increased polyol pathway activity, non-enzymatic glycations of proteins, hexosamine pathway and altered protein kinase C activity [10]. Activation of these metabolic pathways may eventually result in oxidative and inflammatory stress in neurons and adjacent microvascular system.

Considering the role of polyol pathway, which is one of the most extensively studied molecular mechanism in the etiology of diabetic neuropathy [11], several aldose reductase inhibitors have been tested as potential remedies to treat the disease, including sorbinil, tolrestat, ranirestat, fidarestat, zenarestat, zopolrestat and epalrestat [7, 9, 12–15]. Despite the early promise of these drugs in pre-clinical rodent models of diabetes, aldose reductase inhibitors did not provide long-term benefit in patients [9, 15–17]. Presently, epalrestat is the only aldose reductase inhibitor used clinically [18].

Failure of new drugs in long term trials probably results from the multiple mechanisms that contribute to neuronal injury in diabetes. The multifactorial nature of diabetic neuropathy thus represents a great challenge in the development of efficient therapy, while targeting just one particular mechanism may have a limited effect.

Recently designed 3-mercapto-5*H*-1,2,4-triazino[5,6-b] indole-5-acetic acid (cemtirestat, Fig. 1) was characterized as a highly selective and efficient aldose reductase inhibitor [19–21] endowed with antioxidant activity [22]. High resolution X-ray crystallographic assay of the human aldose reductase AKR1B1 crystallized with cemtirestat revealed a peculiar mode of cemtirestat binding, leaving the selectivity pocket closed, in contrast to binding of structurally related lidorestat [19]. In the present study, we evaluated the effect of cemtirestat on symptoms of peripheral diabetic neuropathy in male Zucker diabetic fatty (ZDF) rats. The behavioral tests were accompanied by determination of biochemical markers relevant to glucose/lipid metabolism in plasma and sciatic nerves.

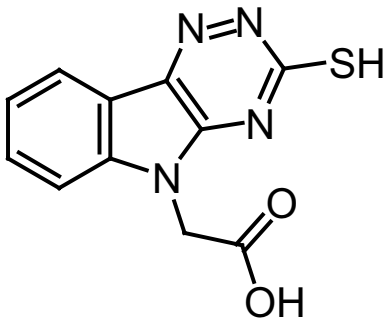

Fig. 1 3-Mercapto-5*H*-1,2,4-triazino[5,6-b]indole-5-acetic acid (cemtirestat)

Materials and Methods

Animals and Drug Treatment

Male ZDF (fa/fa) and lean littermate controls (fa/+) were supplied by our own breeding facility at the Department of Toxicology and Laboratory Animal Breeding, Centre of Experimental Medicine (CEM), Slovak Academy of Sciences (SAS) Dobra Voda. The animals were fed ad libitum standard chow (protein, 19.2%; carbohydrate, 65.1%; fat, 4.0%; fiber, 4.0% and ash, 7.7% by weight).

To characterize the animal model from the point of age-dependent progression of the diabetic state and symptoms of diabetic neuropathy, the rats were divided into the following age groups 1.5, 2.5, 5, 7, and 10 months, as indicated in the result section. At the end of the 5th month, additional groups of animals treated with cemtirestat were created, as given in the result section (Table 1). Yet here was a technical problem regarding randomization of the lean animals by body weights when creating the control groups. The animals were categorized into experimental groups by random choice, primarily based on blood glucose levels to get unbiased groups with well-balanced levels of glycemia. However, this choice resulted into a substantial bias in body weights of the control groups. The drug treatment continued for further 2 months. The drug was administered as an aqueous solution at the dose of either 2.5 or 7.5 mg/kg/day by oral gavage.

Table 1 Initial and final body weights and blood glucose concentrations in male ZDF rats with or without cemtirestat treatment

	Body weight (g)		Blood glucose (mmol/l)	
	Initial	Final	Initial	Final
C (6)	306 ± 16	343 ± 25	9.8 ± 1.5	10.4 ± 2.2
CTII (6)	391 ± 12$^{###}$	417 ± 7$^{###}$	8.6 ± 1.9	12.2 ± 0.6
D (12)	448 ± 54$^{###}$	437 ± 39$^{###}$	27.7 ± 5.4$^{###}$	26.5 ± 4.2$^{###}$
DTI (12)	447 ± 32	437 ± 40	27.6 ± 4.9	26.5 ± 5.3
DTII (12)	439 ± 36	430 ± 38	28.6 ± 5.4	26.8 ± 5.9

Group C, untreated control lean animals; Group CTII, control lean rats treated by cemtirestat 7.5 mg/kg; Group D, untreated fatty rats; Group DTI: fatty rats treated by cemtirestat 2.5 mg/kg/day. Group DTII: fatty rats treated by cemtirestat 7.5 mg/kg/day. Data are mean values ± SD. Number of animals in each group is shown in parentheses

$^{###}p < 0.001$ vs. C; one-way ANOVA followed by the post-hoc Bonferroni multiple comparison test. There is no significant effect of cemtirestat on these parameters

After completion of behavioral testing of each age group, the rats were anesthetized with chloral hydrate (40 mg/100 g i.p.), blood was collected to heparinized tubes by heart puncture and organ samples were collected for biochemical assays. All tissues were rapidly frozen in liquid nitrogen and stored at − 80 °C.

The study was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology, CEM, SAS and the State Veterinary and Food Administration of the Slovak Republic, and it was performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003).

Plasma Assays

Frozen (− 80 °C) samples of plasma were used for analysis of glucose, insulin, cholesterol, triacylglycerides, urea, creatinine and thiobarbituric acid reactive substances (TBARS). Plasma glucose was analyzed by using an enzymatic colorimetric assay for glucose Glucose GOD 1500 (PLIVA-Lachema Diagnostika, Brno, CZ). Determination of plasma insulin level was provided by Rat Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden). Plasma cholesterol, triacylglycerides, urea and creatinine were then assayed by Alpha Medical, Martin, Slovakia. Ketones were measured with the FreeStyleOptium β Ketone Monitoring System (Abbott Diabetes Care Ltd., Witney, Oxfordshire, UK) immediately after blood collection. Plasma levels of TBARS, taken as a marker of oxidative stress, were determined by modification of the method of Buege and Aust [23]. Briefly, plasma sample (150 µl) was combined with TCA-TBA-HCl reagent (300 µl), mixed thoroughly and heated for 15 min at 80°C in a water bath. After cooling the precipitate was removed by centrifugation at 1000*g* for 15 min. The absorbance of the supernatant was determined at 535 nm against a blank that contained distilled water instead of the plasma sample. The content of TBARS in malondialdehyde (MDA) equivalents was determined by calibration curve prepared with 1,1,3,3-tetraethoxypropane standards. Stock TCA-TBA-HCl reagent was obtained by mixing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA), 0.25 N hydrochloric acid and 0.001% butylated hydroxytoluene (BHT).

Glycated Hemoglobin Assay

Blood samples were used to determine glycated hemoglobin (HbA1c) using the rat HbA1c kit of Crystal Chem Inc (Elk Grove Village, IL, USA) according to the manufacturer's instructions.

Sorbitol Assay

The erythrocytes were washed three times with isotonic phosphate buffered saline, pH 7.4. Thereafter ice cold $HClO_4$ (9%, 0.6 mL) was added to an aliquot (0.2 mL) of packed erythrocytes to precipitate proteins. The mixture was kept on ice for 30 min followed by centrifugation at 700 × *g* for 15 min at 4 °C. The supernatant was neutralized with K_2CO_3 (4 mol/L).

The frozen nerves were powdered by crushing under liquid nitrogen. Distilled water (0.4 mL) was added and the suspension was ultra-sounded for 5 min. Thereafter, ice cold $HClO_4$ (9%, 0.4 mL) was added, mixed thoroughly and ultra-sounded again for 5 min. The mixture was kept on ice for 30 min followed by centrifugation at 700 × *g* for 15 min at 4 °C. Aliquot (0.6 mL) was transferred to a clean tube and neutralized with K_2CO_3 (4 mol/L).

The neutralized supernatants, obtained as described above, were used for determination of sorbitol by modified enzymatic analysis according to Mylari et al. [24]. In brief, sorbitol was oxidized to fructose by sorbitol dehydrogenase (SDH) with concomitant reduction of resazurin by diaphorase to the highly fluorescent resorufin. The final concentrations of the assay solutions were: diaphorase (11.5 U/25 mL triethanolamine buffer), NAD^+ (25 mg/25 mL triethanolamine buffer), resazurin (25 µL of 2 mmol/L resazurin solution in 25 mL of triethanolamine buffer), SDH (15.0 U/mL triethanolamine buffer). Reaction mixtures were incubated for 60 min at room temperature with an opaque cover. The sample fluorescence was determined at 544 nm excitation and 590 nm emission. After the appropriate blanks had been subtracted from each sample, the amount of sorbitol was determined in each sample by comparison with a linear regression of sorbitol standards.

Behavioral Tests

On the experimental day the rats were transferred to the experimental room and let to acclimatize for 1 h. To prevent distortion of behavioral responses of rats by their adaptation, repeated testing was avoided and the animals were tested only at the end of the treatment protocol. All behavioral studies were performed 24 h after the last drug treatment to avoid transient effects of the treatment.

Hot Water Immersion Tail-Flick Test

The temperature of the water bath was kept constant at 50 ± 0.5 °C. Rats were gently restrained in a towel with the tail left outside. One-third of the tail was immersed into the water bath in one quick motion. The time between this immersion and the tail-flick reflex was measured using a stopwatch.

Hot Plate Test

A transparent glass cylinder was used to keep the animal on the heated surface of the plate. The temperature of the custom-made hot plate was set to 55 ± 0.5 °C using a thermoregulated water-circulated pump. Rats were gently placed on the hot plate and the time until either licking of the hind paw or brisk stamping to avoid thermal pain was recorded with a stopwatch.

Paw Tactile Responses

For assessment of tactile allodynia, von Frey test was used. Rats were placed in a testing cage with a stainless steel wire mesh bottom and allowed to acclimatize for at least 15 min. A series of calibrated von Frey flexible filaments (range 0.008–300 g; Model: Bio-VF-M, Bioseb, Vitrolles, France) was applied perpendicularly to the plantar surface of a hind paw with sufficient force to bend the filament. Brisk withdrawal of the paw was considered as a positive response. Filaments were presented in order of increasing stiffness, until the paw withdrawal was detected. Positive reaction was paw lifting, shaking or licking.

Statistical Analysis

Age dependent changes of body weights, plasma glucose, tail-flick test response latencies, hot plate test response latencies and tactile response thresholds were statistically analyzed using two-way ANOVA followed by the post-hoc Bonferroni multiple comparison test (GraphPad Prism 6.00 for Windows, GraphPad Software, San Diego, CA). In drug treatment experiments, comparisons between groups were carried out by using one-way ANOVA followed by the post-hoc Bonferroni multiple comparison test (GraphPad Prism 6.00 for Windows, GraphPad Software, San Diego, CA).

Results

Characterization of the Animal Model

The first part of the study was devoted to characterization of the ZDF rat model from the point of view of age dependent development of the diabetic state and concomitant progression of symptoms of diabetic neuropathy. As shown in Fig. 2a, body weights of both lean and fatty animals rose steadily till the 7th month of age, followed by mild decrease of body weights as recorded in the 10th month. Since the age of 2.5 months the body weights of fatty rats were significantly higher in comparison with the lean ones. Hyperglycemia was recorded in fatty animals at 2.5 month of age, then blood glucose of fatty animals increased and reached

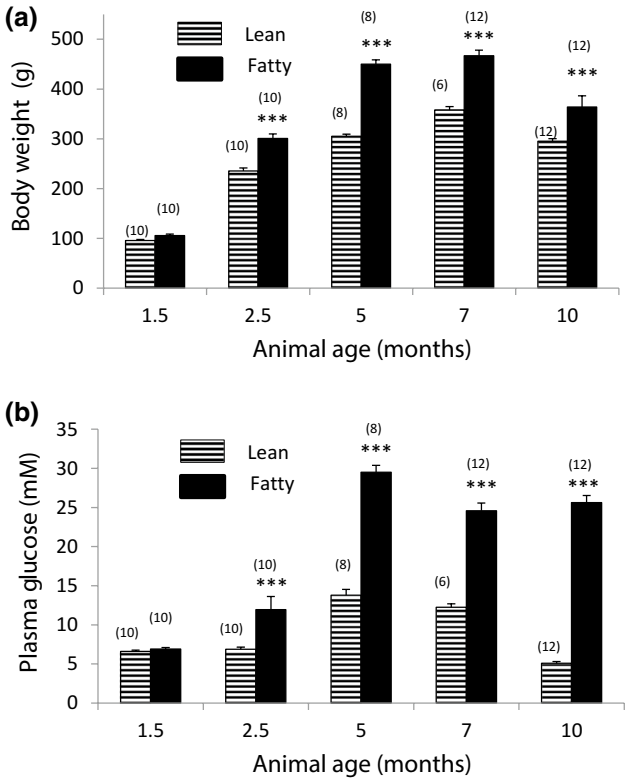

Fig. 2 Body weights (**a**) and plasma glucose levels (**b**) in ZDF male rats on standard diet. Age dependence. Results are mean values ± SEM with number of animals in parentheses. ***p ≤ 0.001 for fatty vs. age-matched lean; two-way ANOVA followed by the post-hoc Bonferroni multiple comparison test

an average value of plasma glucose 29.5 ± 0.9 mM at the age of 5 months. A mild decrease of hyperglycemia was recorded in the 7th and 10th month for both fatty and lean littermates (Fig. 2b).

Figure 3 shows age-dependent development of symptoms of peripheral neuropathy recorded from the 5th through the 7th to the 10th month. Marked thermal hypoalgesia was recorded in the fatty animals at the age of 5 months by measuring response latencies in a tail-flick test. The tail-flick response latencies of the fatty rats further prolonged through the 7th to 10th month (Fig. 3a). Similarly hot-plate response latencies of fatty rats were significantly longer compared to those of lean littermates at the 7th and 10th month (Fig. 3b). Tactile allodynia developed in the fatty animals at the 5th month and even aggravated slightly at the 7th month. Yet at the 10th month mechanical hypoalgesia was recorded in fatty rats (Fig. 3c).

Effect of Drug Treatment

In the second part of the study, treatment of the experimental animals was initiated at 5 months of age. As revealed by the above mentioned preliminary findings, at 5 months of age

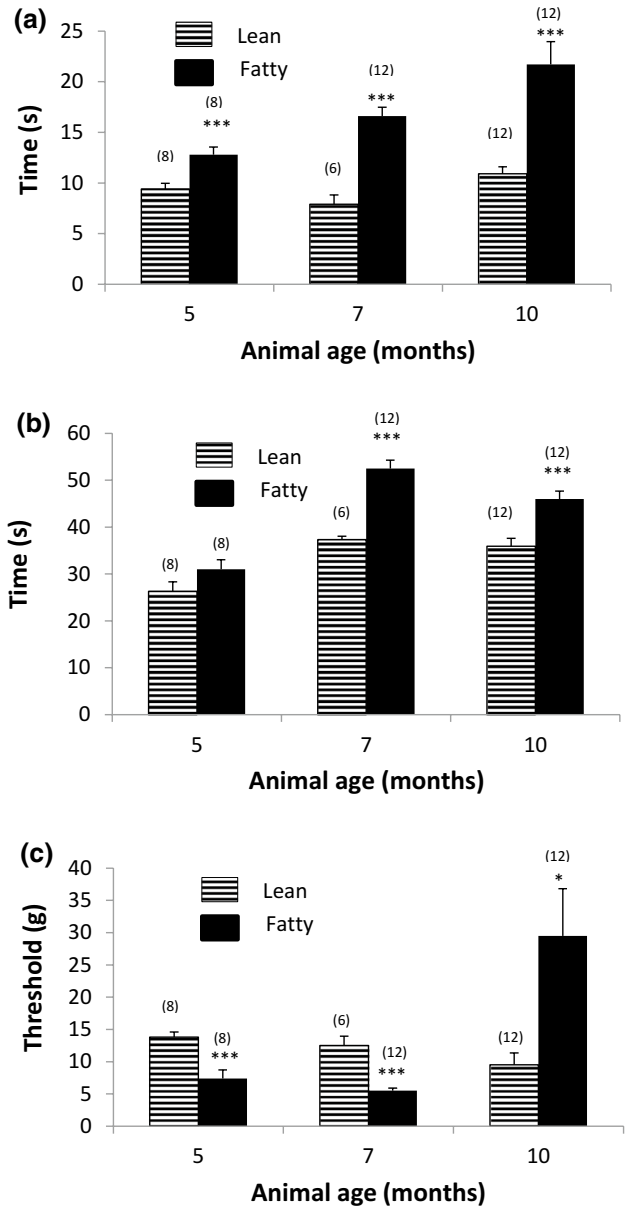

Fig. 3 Symptoms of peripheral neuropathy in ZDF male rats on standard diet. Age dependence. **a** Tail-flick test response latencies (50 °C); **b** hot plate test response latencies (55 °C); **c** tactile response thresholds as a result of stimulation with flexible von Frey filaments. Results are mean values ± SEM with number of animals in parentheses. *p ≤ 0.05; ***p ≤ 0.001 for fatty vs. age-matched lean; two-way ANOVA followed by the post-hoc Bonferroni multiple comparison test

fatty rats were hyperglycemic with symptoms of diabetic neuropathy compared to lean controls. In addition, fatty animals displayed hyperphagia, polydipsia and polyuria.

Two-month treatment of rats with cemtirestat did not affect significantly daily food consumption (data not shown) and body weight gains of both the control and diabetic animals (Table 1). Persistent hyperglycemia over 25 mmol/L was observed in all groups of fatty animals throughout the whole experiment with corresponding levels of glycated hemoglobin HbA1c over 13%. Treatment of animals with cemtirestat did not significantly affect blood levels of glucose (Table 1) and HbA1c (Table 2) in either fatty rats or lean control littermates. Similarly, cemtirestat administration to both control (CTII) and fatty rats (DTI and DTII) did not significantly alter insulin, cholesterol, triglyceride, and creatinine levels. Plasma urea and ketones were not affected either by diabetes or cemtirestat.

In untreated fatty diabetic rats, significant elevation of sorbitol concentration in red blood cells and the sciatic nerve was recorded when compared to control littermates. Cemtirestat administered, i.g. (2.5 and 7.5 mg/kg/day) significantly inhibited sorbitol accumulation both in erythrocytes and in the sciatic nerve of fatty rats (Table 2). In control rats, administration of cemtirestat (7.5 mg/kg/day, i.g.) did not affect significantly sorbitol levels either in the erythrocytes or in the sciatic nerve.

The plasma levels of TBARS were significantly higher in untreated ZDF fatty rats in comparison to untreated control lean littermates. Cemtirestat treatment of the ZDF fatty rats with the daily dose of 2.5 mg/kg significantly decreased this marker while its normalization to the control value was observed at the higher dose of cemtirestat. In control rats, administration of cemtirestat (7.5 mg/kg/day, i.g.) did not affect significantly plasma level of TBARS.

At the end of the 2-month treatment period, the tail-flick response latency was significantly increased ($p < 0.001$) in untreated ZDF diabetic rats compared with control lean littermates (Fig. 4a). Cemtirestat diminished this measure in ZDF diabetic rats to almost control values, without affecting this parameter significantly in control rats. Hot plate response latencies were significantly increased ($p < 0.001$) in untreated diabetic fatty rats when compared with lean controls (Fig. 4b), whereas those of cemtirestat-treated diabetic fatty rats were not significantly different from the controls. This is in agreement with the above-mentioned results of the tail-flick test. At the end of the experiment, tactile withdrawal threshold in response to light touch with flexible von Frey filaments was significantly reduced in diabetic fatty rats compared with lean littermate controls ($p < 0.001$). Cemtirestat, even at the lower dose, restored diabetes-induced decrease in tactile response in diabetic fatty rats ($p < 0.001$ vs. untreated diabetic group), without affecting this marker in the control group (Fig. 4c).

Discussion

In the current study, male ZDF rats were used as an animal model of type 2 diabetes. To characterize the model, the rats were followed for 10 months. As disease progressed to

Table 2 Blood biochemical markers of male ZDF rats recorded at the end of the experiment. Effect of cemtirestat treatment

	C (6)	CTII(6)	D (12)	DTI (12)	DTII (12)
HbA1c (%)	4.65 ± 0.27	5.82 ± 0.17	14.03 ± 0.64$^{\#\#\#}$	13.55 ± 0.75	13.07 ± 0.87
Insulin (µg/l)	0.23 ± 0.01	0.20 ± 0.01	0.52 ± 0.08$^{\#\#}$	0.53 ± 0.08	0.52 ± 0.07
Cholesterol (mM)	2.31 ± 0.04	2.69 ± 0.08	4.30 ± 0.14$^{\#\#\#}$	4.14 ± 0.12	4.49 ± 0.23
Triglycerides (mM)	0.93 ± 0.04	1.87 ± 0.12	4.53 ± 0.32$^{\#\#\#}$	5.10 ± 0.32	4.54 ± 0.35
Urea (mM)	4.61 ± 0.20	4.39 ± 0.12	5.74 ± 0.29§	5.68 ± 0.20§	5.54 ± 0.23
Creatinine (µM)	33.43 ± 0.57	33.03 ± 0.37	23.39 ± 0.75$^{\#\#\#}$	22.93 ± 0.61	23.72 ± 0.81
Ketones (mM)	1.35 ± 0.07	1.02 ± 0.06	1.24 ± 0.08	1.18 ± 0.07	1.27 ± 0.09
Sorbitol in red blood cells (nmol/ml packed RBC)	1.04 ± 0.18	2.23 ± 0.38	18.14 ± 1.38$^{\#\#\#}$	14.75 ± 1.08	12.48 ± 1.44**
Sorbitol in the sciatic nerve (nmol/g nerve)	139.3 ± 28.9	129.6 ± 23.8	1312.0 ± 88.7$^{\#\#\#}$	1107.2 ± 72.9	935.9 ± 51.9**
TBARS in plasma (µM)	7.47 ± 1.16	6.55 ± 1.04	16.15 ± 1.29$^{\#\#\#}$	11.69 ± 1.20*	5.67 ± 0.49***

Group C, untreated control lean animals; Group CTII, control lean rats treated by cemtirestat 7.5 mg/kg; Group D, untreated fatty rats; Group DTI, fatty rats treated by cemtirestat 2.5 mg/kg/day; Group DTII, fatty rats treated by cemtirestat 7.5 mg/kg/day. Data are mean values ± SEM. Number of animals in each group is shown in parentheses

$^{\S}p \le 0.05$ vs. CTII; one-way ANOVA followed by the post-hoc Bonferroni multiple comparison test

$^{\#\#}p < 0.01$ vs. C; $^{\#\#\#}p < 0.001$ vs. C; $^{*}p \le 0.05$ vs. D; $^{**}p \le 0.01$ vs. D; $^{***}p \le 0.001$ vs. D

overt diabetes, fatty ZDF rats demonstrated elevated plasma glucose levels, hyperphagia, polydipsia and polyuria. At the age of 5 months, fatty ZDF rats were hyperglycemic and developed significant symptoms of thermal hypoalgesia as indicated by prolonged response latencies in a tail-flick test in comparison to lean controls. At the same time, the fatty rats revealed symptoms of tactile allodynia as shown by decreased thresholds to flexible von Frey filaments relative to lean rats. With progressing diabetes, the markers of thermal hypoalgesia and tactile allodynia increased at the age of 7 months. Symptoms of thermal hypoalgesia persisted till the 10th month of age in fatty animals. On the other hand, the symptoms of tactile allodynia of fatty ZDF rats turned to mechanical hypoalgesia in the 10th month of age. Literature data on ZDF rats used as a model of diabetic neuropathy are rather ambiguous, nevertheless both in clinics and in animal models of diabetes, it is well documented that hypersensitivity of diabetic individuals to mechanical or heat stimuli observed at the early stages of diabetes would gradually turn to decreased sensitivity. In agreement with our findings, other authors reported the symptoms of hypersensitivity to non-painful mechanical stimuli (tactile allodynia) in fatty ZDF rats recorded in the early stages of diabetes [25–30]. As shown in Fig. 3c, the symptoms of tactile allodynia of fatty ZDF rats, recorded at the age of 5 and 7 months, turned to mechanical hypoalgesia in the 10th month of age. Similarly, other authors reported significant increase in paw withdrawal thresholds of diabetic ZDF rats at the advanced stage of diabetes (38 weeks), compared to the values measured in the same rats at the age of 6 weeks [31]. Likewise, thermal hypersensitivity (thermal hyperalgesia) of STZ-diabetic rats recorded in the 4th week after induction of diabetes was found to turn into thermal hypoalgesia in later stages of diabetes [32].

In our recent study, carboxymethylated mercapto-triazinoindoles were characterized as efficient inhibitors of aldose reductase [19]. Of these, 3-mercapto-5*H*-1,2,4-triazino[5,6-b]indole-5-acetic acid (cemtirestat) was identified as the most efficient inhibitor and patented as a potential remedy to treat diabetic complications [33]. Several advantages of cemtirestat over clinically used epalrestat were reported, namely lower molecular weight, better water solubility, higher inhibition activity recorded both at the level of isolated enzyme and at the organ level of isolated rat eye lenses, and additional antioxidant activity [21, 22, 33].

In our previous short-term study in the streptozotocin-induced model of experimental type 1 diabetes, we reported significant inhibition of sorbitol accumulation in the sciatic nerve after 5-day treatment of the animals with 50 mg/kg/day of cemtirestat via oral gavage [34]. This result pointed to a ready uptake of cemtirestat after its intragastric administration into the central compartment, its supply to the peripheral nerves and inhibition of aldose-reductase-mediated sorbitol accumulation. This finding encouraged us to perform a long-term study in ZDF rats, an animal model of type 2 diabetes.

Two-month treatment with cemtirestat at the daily dose of 2.5 mg/kg resulted in a significant diminution of sorbitol accumulation in the erythrocytes (by 19%) and in the sciatic nerve (by 16%) of the fatty rats. Sorbitol decrease of about 27% in the sciatic nerve of ZDF rats was recorded by Shimoshige et al. [35] after 2-month treatment with the

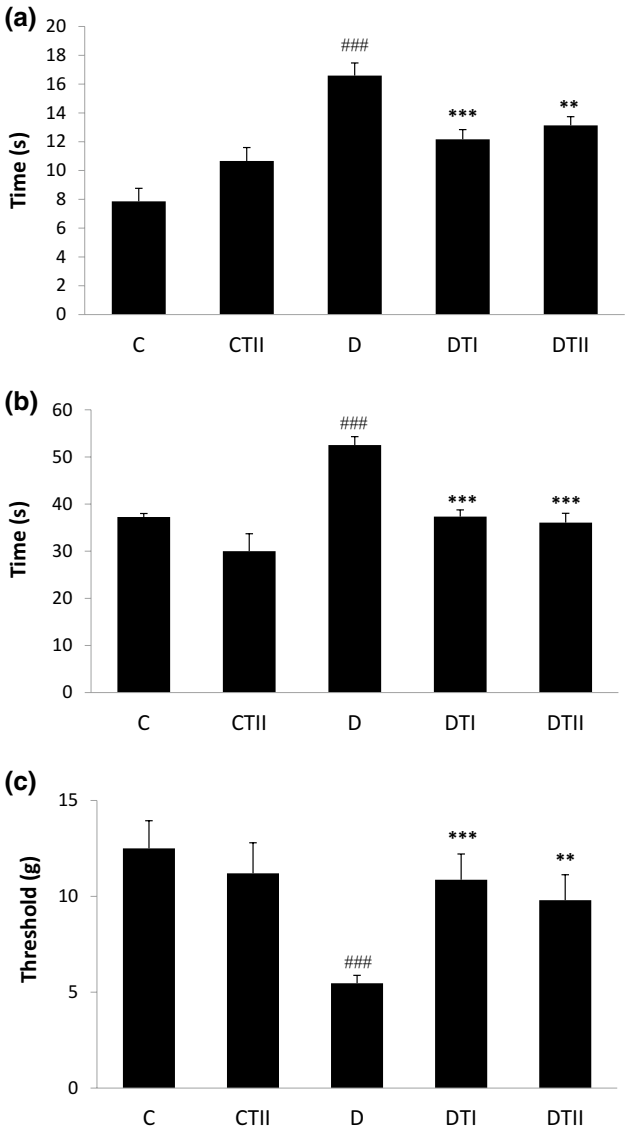

Fig. 4 Symptoms of peripheral neuropathy in ZDF male rats at the end of the 2-month treatment with cemtirestat. Cemtirestat was administered intra-gastrically from the 5th to 7th month according to the following dosage schedule: Group C, untreated control lean animals; Group CTII, control lean rats treated by cemtirestat 7.5 mg/kg; Group D, untreated fatty rats; Group DTI: fatty rats treated by cemtirestat 2.5 mg/kg/day. Group DTII: fatty rats treated by cemtirestat 7.5 mg/kg/day. **a** Tail-flick test response latencies (50 °C); **b** Hot plate test response latencies (55 °C); **c** Tactile response thresholds as a result of stimulation with flexible von Frey filaments, in fatty rats and their lean littermate controls with or without cemtirestat treatment. Results are presented as means ± SEM from 6 (C, CTII) and 12 (D, DTI, DTII) animals. $^{###}p \leq 0.001$ vs. C and CTII, $^{**}p \leq 0.01$ vs. D, $^{***}p \leq 0.001$ vs. D; one-way ANOVA followed by the post-hoc Bonferroni multiple comparison test

aldose reductase inhibitor zenarestat (3.2 mg/kg/day). Yet even at the higher dosage regimen of cemtirestat (7.5 mg/kg/day), the sorbitol levels were not normalized to control values in red blood cells and the sciatic nerve (reduction by 31% and 29%, respectively, Table 2). The recorded drop in sorbitol accumulation most likely reflects passage of cemtirestat into the nerve resulting in inhibition of the flow of glucose through the polyol pathway since the drug did not significantly affect glycemic state of the diabetic animals and other metabolic markers related to hyperglycemia (Table 2).

There is apparent discrepancy between partial reduction of sorbitol accumulation in the sciatic nerve and near normalization of peripheral neuropathy behavioral endpoints of the fatty ZDF rats. Concomitant inhibition of sorbitol dehydrogenase, the second enzyme of the polyol pathway, would increase sorbitol levels in the nerve tissue. Obviously, this is not the case since cemtirestat does not affect activity of sorbitol dehydrogenase as we reported previously [34]. These findings indicate that inhibition of sorbitol accumulation by cemtirestat is not solely responsible for the recorded improvement of the behavioral responses. Osmotic hypothesis, stressing the role of sorbitol intracellular accumulation, widely accepted in the etiology of diabetic cataract, is considered an oversimplification for the diabetic nerve. Metabolic flux hypothesis is used as an alternative to explain the role of the polyol pathway in the etiology of diabetic neuropathy [11]. This notion emphasizes the detrimental role of oxidative rather than osmotic stress linked to the NADPH/$NADP^+$ and NADH/NAD^+ cofactor systems strongly affected by glucose flux through the polyol pathway, with aldose reductase being its first enzyme. Markedly increased plasma levels of TBARS shown in ZDF fatty rats (Table 2) point to the presence of systemic oxidative stress in diabetic animals. Similarly, other authors reported elevated levels of TBARS in plasma of ZDF fatty rats in comparison with their lean littermates [36–38]. The marked diminution of plasma level of TBARS in the fatty animals by cemtirestat is in line with the reported antioxidant action of cemtirestat [21, 22]. This mechanism may contribute to the recorded neuroprotective effect of cemtirestat.

Considering the recently revealed role of aldose reductase in the oxidative stress-induced inflammation in the etiology of diabetic neuropathy [39], another track that should be followed in explaining the neuroprotective action of cemtirestat is a potential inhibition of the proinflammatory actions of aldose reductase in diabetes. In addition, possible effects of cemtirestat on molecular mechanisms independent of the polyol pathway should be taken into consideration, e.g. non-enzymatic glycation followed by AGE-RAGE axis, hexosamine pathway, altered protein kinase C activity. In addition to the above mentioned molecular mechanisms of glucose toxicity, other metabolic imbalances activated in the diabetic milieu, e.g. those related to dyslipidemia and impaired insulin signaling, may represent further therapeutic targets. Moreover, the above mentioned metabolic imbalances activated by hyperglycemia may affect the nervous system at multiple levels of the anatomical hierarchy.

Conclusions

Two-month treatment of ZDF rats by cemtirestat (i) did not affect physical and glycemic status of the animals; (ii) partially inhibited sorbitol accumulation in red blood cells and the sciatic nerve; (iii) markedly decreased plasma levels of TBARS; (iv) normalized symptoms of peripheral neuropathy with high significance. The findings indicate that inhibition of aldose reductase by cemtirestat is not solely responsible for the recorded improvement of the behavioral responses. In future studies, potential effects of cemtirestat on consequences of diabetes that are not exclusively dependent on glucose metabolism via polyol pathway should be taken into consideration.

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