

PHARMACOKINETICS OF GLUTAPYRONE AND ITS IMPACT ON EXPRESSION OF THE GENES INVOLVED IN DNA REPAIR AND FREE RADICAL PRODUCTION IN INTACT AND DIABETIC RATS

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Modification of expression of several genes encoding enzymes involved in radical production and DNA repair by a 1,4-DHP derivative glutapyrone was studied in intact rats and animals with streptozotocin diabetes mellitus. Glutapyrone stimulated iNos mRNA production in both kidneys and blood. The effect was stronger in kidneys of diabetic animals, however in blood the expression was down-regulated. The compound increased the Parp 1 gene expression in kidneys of both intact and diabetic animals; in blood the effect was adverse. Expression of XDh gene was significantly increased by glutapyrone in kidneys of intact and diabetic animals. Administration of the compound to intact animals triggered significant increase of DNA damage in white blood cells assayed by comet assay; in diabetic animals no effect was produced. To explain discrepancies with the formerly described effects of glutapyrone on cultured cells, metabolism of the compound was studied. Glutapyrone is either oxidised or the residue of glutamic acid is removed, glutapyrone turns into AV-153, and the latter is metabolised to smaller compounds. Formation of AV-153, a DNA binder and genotoxic compound in high concentrations, can explain DNA damage in white blood cells and stimulation of DNA repair.

Keywords: glutapyrone, pharmacokinetics, iNos, Parp 1, XDh, DNA damage, diabetes mellitus.

INTRODUCTION

Diabetes mellitus (DM) remains a serious medical and social problem throughout the world. Oxidative stress and impaired DNA repair are important factors in the development of DM and its complications (Ramani *et al.*, 2020; Novoselova *et al.*, 2021; Kopf *et al.*, 2021). DNA repair enzymes are chosen as targets for antidiabetic therapies (Sarras *et al.*, 2014; Abdelali *et al.*, 2016). Disequilibrium of production of the nitric oxide by different isoforms of the nitric oxide synthases leads to dysfunction of endothelium and other tissues characteristic of DM (Sokolovska *et al.*, 2020; Rostoka *et al.*, 2021). Thus, search for pharmacologi-

cal remedies normalising parameters of free radical production, DNA damage and DNA repair in conditions in DM appears to be a topical research branch. 1,4-dihydropyridine derivatives (1,4-DHPs) appear to be prospective compounds from this point of view. Numerous representatives of this class of compounds are effective antioxidants (Velena *et al.*, 2016; Milković *et al.*, 2018). Moreover, several 1,4-DHPs synthesised in the Latvian Institute of Organic Synthesis manifest some promising effects in experimental DM. For example, metcarbatone and styrylcarbatone mitigated the DNA-damage in conditions of diabetes mellitus (Ošiņa *et al.*, 2017). In rats with streptozotocin-induced DM, several 1,4-DHPs increased expression of the genes involved in

DNA repair, and this could indicate some impact on general pathways of the regulation of transcription (Ošiņa *et al.*, 2016; 2017; Leonova *et al.*, 2016). Down-regulation of the inducible NO synthase protein (iNOS) in diabetes mellitus by carbatonides (Ošiņa *et al.*, 2017), AV-153 (Ošiņa *et al.*, 2016) and both iNOS expression and NO production by etaftorone, fenoftrone, and cerebrocrast (Leonova *et al.*, 2017) seems to be a promising result. In the present article we report data on modification of expression of several genes encoding enzymes involved in radical production and DNA repair by one more 1,4-DHP in rats with streptozotocin diabetes model — glutapyrone. Formerly it was shown that glutapyrone produces genome-protecting effects: it is an antineoplastic and anticlastogenic agent as well as antimutagen (Goncharova *et al.*, 2001). The compound produces DNA-protecting effects in cultured cell, but not in cell-free *in vitro* systems, and it is a weak pro-oxidant (Leonova *et al.*, 2023).

MATERIALS AND METHODS

Wistar male rats weighing 200–250 g were used in this study. Animals were delivered from the Laboratory of Experimental Animals of Riga Stradiņš University (Riga, Latvia).

Type 1 DM (T1 DM) was induced with streptozotocin (STZ) as described previously (Ošiņa *et al.*, 2016; 2017). Rats were divided into the following groups: intact animals, rats with STZ-induced DM, rats treated with glutapyrone (0.05 or 0.5 mg/kg), and diabetic rats treated with glutapyrone in the same doses. There were at least six animals in each group.

For pharmacokinetic studies the rats were given *per os* 100 mg/kg of glutapyrone. The animals were kept in metabolic cages in order to collect urine and faeces. Urine (1 to 5 ml) was diluted with water up to 20 ml, and HCl was added to the mixture to adjust pH 2. The mixture was applied to a kieselguhr column (Exterlut, Merck, Germany). Non-polar metabolites were eluted by ethyl acetate:chloroform (4:1), and polar metabolites – by chloroform:methanol (8:2). Then pH of the column was raised to pH 9 by ammonium and extraction was repeated. Extracts were evaporated in a flow of nitrogen, and metabolites were identified by means of the thin-layer chromatography. Identification was done by comparison of metabolites with the synthesised “witnesses”.

Gene expression levels were determined in kidneys and blood. RNA was isolated with TRI reagent (Sigma-Aldrich, Germany). RNA was purified with a RapidOut DNA Removal Kit (Thermo Scientific, USA). The quality of RNA was determined with 1% agarose gel electrophoresis, and quantity and purity by means of spectrophotometry.

cDNA was synthesised using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) with random hexamer primers from 5 µg of kidney and liver RNA and 2 µg of blood RNA.

The expression levels of *Parp1*, *iNOS*, *eNOS*, and *Xdh*, genes were determined using SYBR® Green PCR Master Mix (Applied Biosystems, USA). Real time polymerase chain reaction was carried out with a StepOne™ Real-Time PCR System (Applied Biosystems, USA). The results were analysed with relative quantification or by the $2^{-\Delta CT}$ method (StepOne software, Applied Biosystems, version 2.1, USA) and shown in relative quantity units (RQ). Primers were designed using NCBI/Primer-BLAST software. RNA polymerase II was used as a reference gene (Sokolovska *et al.*, 2011). Primer sequences for the *Parp1* gene were 5'-CGGAGAGGCTTTACCGAGTG-3' (forward primer) and 5'-GAACATGGGTGACTGCACCA-3' (reverse primer). Primers for the *iNos* gene were 5'-GCTACACTTCCAACGCAACA-3' (forward primer) and 5'-CATGGTGAACACGTTCTTGG-3' (reverse primer). Primers for the *eNos* gene were 5'-GAACCTGAGGGTGCCAG-3' (forward primer) and 5'-TCCGATTCAACAGTGTCTCCT-3' (reverse primer). RNA-polymerase II (5'-GCCAGAGTCTCCCATGTGTT-3' and 5'-GTCGGTGGGACTCTGTTTGT-3') was chosen as a reference gene. Results were analysed by the unpaired *t*-test and two-way ANOVA with Holm-Sidak post-hoc test. Data are presented as means ± SEM.

The single cell electrophoresis (comet assay) was performed as described previously (Leonova *et al.*, 2016)

RESULTS

Glutapyrone stimulated *iNos* mRNA production in both kidneys and blood of intact animals, while induction of diabetes produced a similar, but slightly weaker effect (Fig. 1A). Treatment of diabetic animals with glutapyrone drastically increased the gene expression. Strikingly, the compound decreased elevated *iNos* gene expression in blood of the diabetic animals (Fig. 1B). *eNos* expression in kidneys and blood was not modified by the compound (not shown).

The compound increased the *Parp1* gene expression in kidneys of both intact and diabetic animals, however in blood the effect was adverse — a significant decrease of the gene expression was observed (Fig. 2).

Expression of *XDH* gene was significantly increased by glutapyrone in kidneys of intact and diabetic animals, while a low dose of the compound increased the expression in the control group (Fig. 3)

Administration of the compound to intact animals triggered significant increase of DNA damage in white blood cells assayed by single cell electrophoresis, while induction of diabetes produced a less pronounced effect. However, in diabetic animals almost no effect was produced (Fig. 4).

DISCUSSION

The obtained results appear to contradict formerly published results about glutapyrone. Numerous studies indicate its

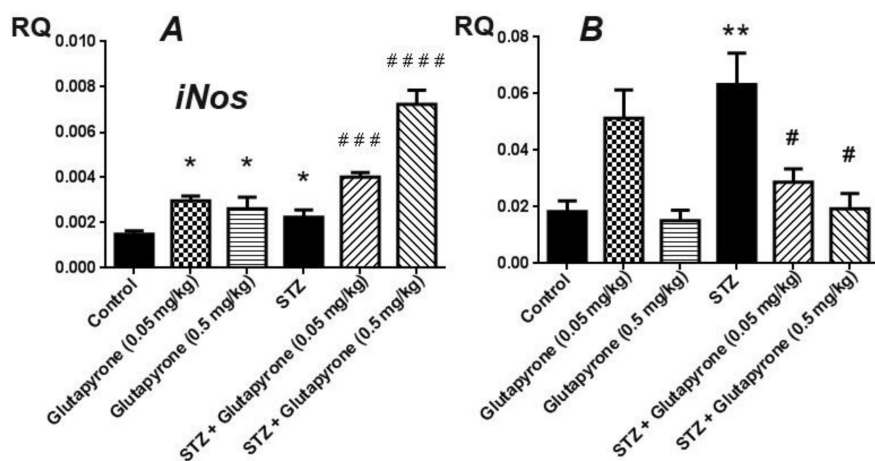


Fig 1. Modification of the *iNos* gene expression in healthy and diabetic rats by glutapyrone in kidneys (A) and blood (B).

* $p = 0.0433$ vs control group

** $p = 0.026$ vs control group

$p < 0.04$ vs streptozotocin (STZ) group

$p = 0.006$ vs STZ group

$p < 0.0001$ vs STZ group

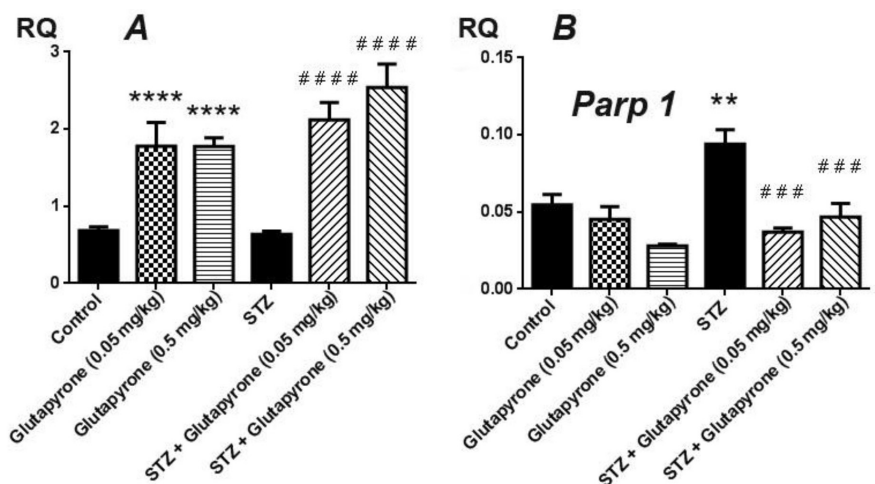


Fig 2. Modification of the *Parp 1* gene expression in healthy and diabetic rats by glutapyrone in kidneys (A) and blood (B).

** $p = 0.046$ vs control group

**** $p < 0.0001$ vs control group

$p < 0.001$ vs streptozotocin (STZ) group

$p < 0.0001$ vs STZ group

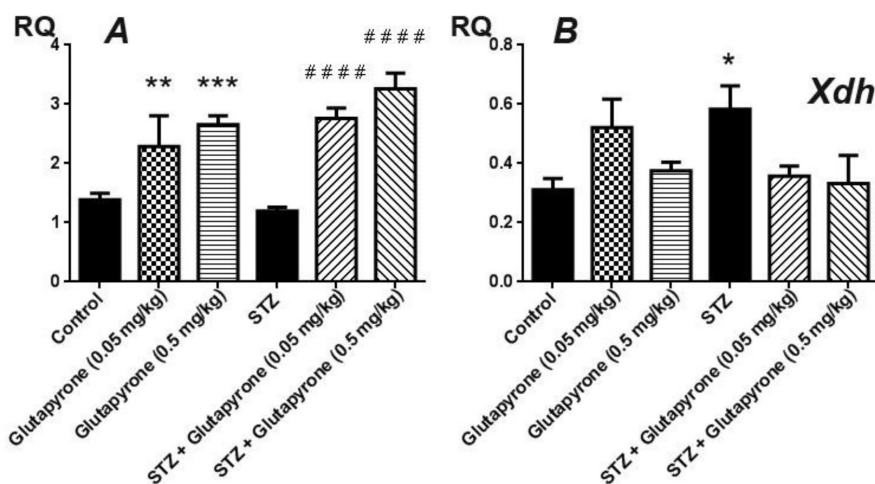


Fig 3. Modification of the *Xdh* gene expression in healthy and diabetic rats by glutapyrone in kidneys (A) and blood (B).

* $p = 0.046$ vs control group

** $p = 0.025$ vs control group

*** $p = 0.001$ vs control group

$p < 0.001$ vs streptozotocin (STZ) group;

$p < 0.0001$ vs STZ group

strong radioprotective effects — the compound reduces frequency of spontaneous tumours in rodents, protects against chemical carcinogens, and alleviates stress effects (reviewed in Ponomareva *et al*, 2018). Our results indicate that glutapyrone up-regulates expression of *Parp1*, *iNOS*, and *Xdh* genes, and this should lead to increase of production of the free radicals and intensification of the DNA repair. Surely, changes in transcription rate do not necessarily result in modification of the protein expression, however the trend is obvious. Moreover, when administered to rats it produced DNA damage in the white blood cells, and formerly performed *in vitro* studies indicate that it does not produce DNA damage in cultured cells, but it protects the

cells against peroxynitrite-produced DNA damage. The compound does not bind DNA *in vitro* (Leonova *et al.*, 2023). We hypothesised that the differences in *in vivo* and *in vitro* effects are due to metabolism of the compound in a living animal. Pharmacokinetic study was performed to test this hypothesis. The results are presented in Figure 5. In rats glutapyrone undergoes metabolism following two pathways: either the compound is oxidised or the residue of glutamic acid is removed, glutapyrone turns into AV-153, and the latter is metabolised to smaller compounds. Formation of AV-153, a DNA binder and genotoxic compound in high concentrations, can explain DNA damage in the white blood cells. AV-153 binds DNA and stimulates DNA re-

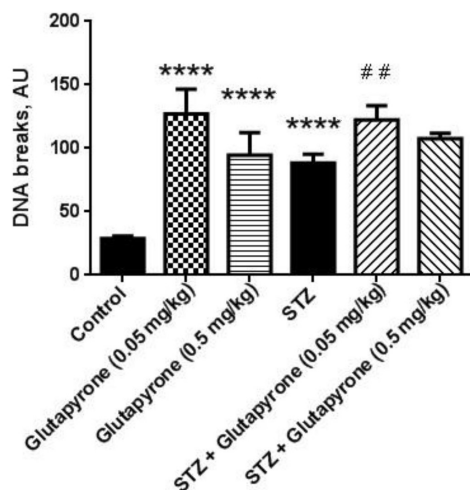


Fig 4. Modification of the DNA break level in nucleated blood cells of healthy and diabetic rats by glutapyrone.

**** $p < 0.0001$ vs control group

$p = 0.0061$ vs STZ group

pair, finally leading to resistance of the animal to other genotoxic agents. DNA lesions can be also produced by oxidative stress due to increase of iNOS and Xdh enzymatic activities. Finally, the compound mobilises DNA repair response, thus preparing the organism to protect itself against

carcinogens and radioactivity. The present results do not indicate any benevolent effects of glutapyrone in *diabetes mellitus*. In any case, the studies should be continued.

ETHICS

All experiments were performed according to Directive 2010/63/EU of the Europe Union and Regulations of the Cabinet of Ministers of 23 October 2001, No. 450. Permission to use animals in experiments was received from the Food and Veterinary Service.

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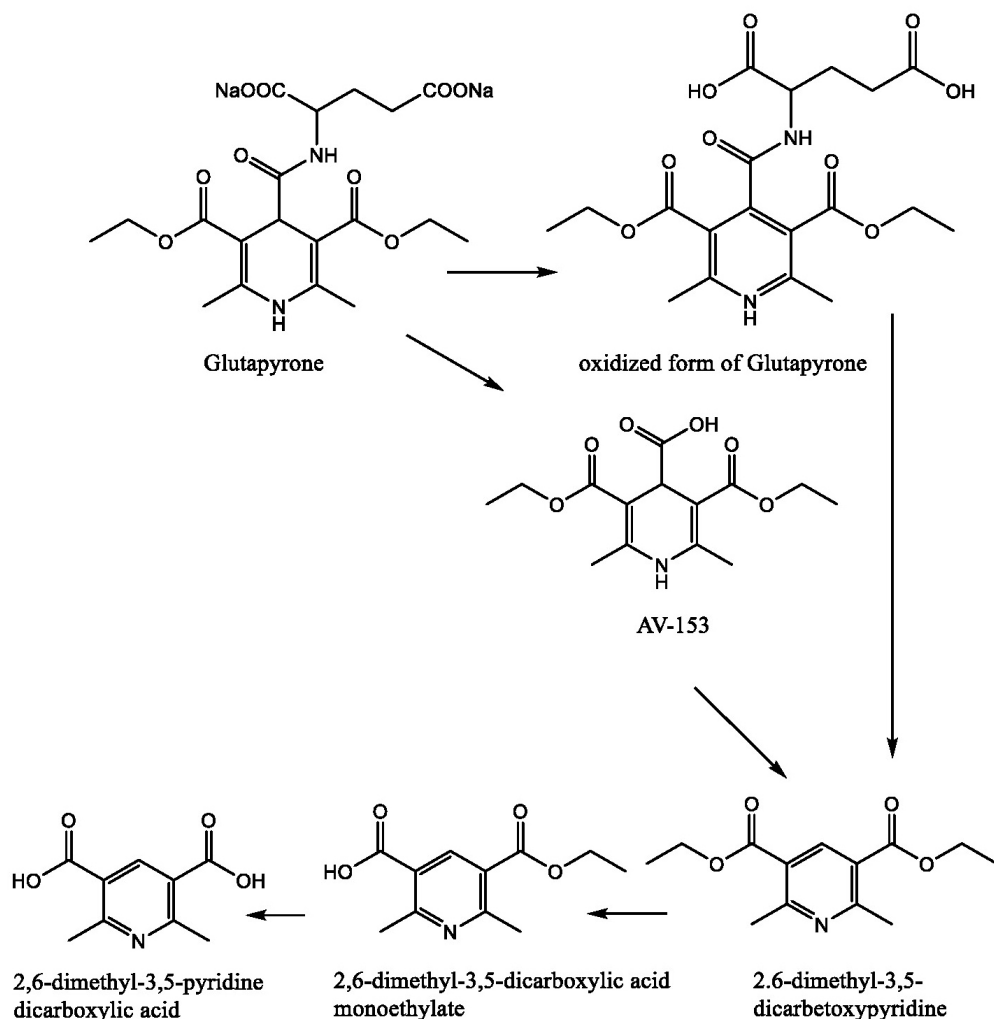


Fig 5. Pathways of glutapyrone metabolism in rats.

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GLUTAPIRONA FARMAKOKINĒTIKA UN TĀ IETEKME UZ DNS REPARĀCIJĀ UN BRĪVO RADIKĀĻU VEIDOŠANĀ IESAISTĪTO GĒNU EKSPRESIJU INTAKTĀM UN DIABĒTISKĀM ŽURKĀM

Izpētita 1,4-DHP atvasinājuma glutapirona ietekme uz DNS reparācijā un brīvo radikāļu veidošanā iesaistīto gēnu ekspresiju veselām žurkām un žurkām ar streptozotocīna izraisītu cukura diabētu. Glutapirons ietekmēja *iNOS* mRNS produkciju nierēs un asinīs. Diabētisko dzīvnieku nierēs efekts bija vairāk izteikts, bet asinīs ekspresija bija samazināta. Viela pastiprināja *Parp1* gēna ekspresiju veselu un diabētisku dzīvnieku nierēs, taču asinīs novēroja pretēju efektu. Glutapirons būtiski pastiprināja *XDh* gēna ekspresiju veselu un diabētisku žurku nierēs. Pēc “komētu metodes” datiem vielas ievadīšana veselām žurkām izsauca DNS bojājumus leukocītos, bet diabētiskiem dzīvniekiem šo efektu nenovēroja. Lai izskaidrotu pretrunas ar agrāk aprakstītiem glutapirona efektiem šūnu kultūrās, tika izpētīts glutapirona metabolisms. Glutapirons vai nu oksidējās, vai, zaudējot glutamīnskābes atlieku, pārvēršas par AV-153, kas savukārt tiek metabolizēts līdz mazākām molekulām. AV-153 saistās ar DNS un ir genotoksisks augstās koncentrācijās. AV-153 veidošanās var būt cēlonis gan DNS bojājumiem, gan DNS reparācijas stimulēšanai.