Final report of scientific progress

Dimethyl trisulfide: a promising drug candidate against experimental acute pancreatitis

(Dimetil triszulfid: egy ígéretes hatóanyag kísérletes akut pancreatitisben)

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Background and aims

Dimethyl trisulfide (DMTS) is a member of naturally occurring organosulfurs. It has several biological effects, it reacts with haemoglobin and forms methaemoglobin[1], it activates the TRPA1 ion channel, modulates pain sensation[2] and it has great potential to reduce cyanide poisoning[3]. However, there is no literature on their effect in inflammation. We aimed to investigate how DMTS affects the severity of acute pancreatitis (AP), a potentially lethal gastrointestinal disease, and to reveal its mechanisms of action in both *in vivo* and *in vitro* studies.

References:

1. Dong, X., Kiss, L., Petrikovics, I. & Thompson, D. E. Reaction of Dimethyl Trisulfide with Hemoglobin. *Chem Res Toxicol* 30, 1661–1663 (2017).
2. Pozsgai, G. *et al.* Analgesic effect of dimethyl trisulfide in mice is mediated by TRPA1 and sst4 receptors. *Nato Sci S A Lif Sci* 65, 10–21 (2017).
3. Petrikovics, I. *et al.* Antidotal efficacies of the cyanide antidote candidate dimethyl trisulfide alone and in combination with cobinamide derivatives. *Toxicol Mech Method* 29, 1–24 (2019).

Results

1.1.) Determining the best vehicle for dimethyl trisulfide (DMTS) solution

The DMTS is a lipophilic molecule and insoluble in aqueous-based buffers, therefore a vehicle was needed to keep it in solution. We tested dimethyl sulfoxide (DMSO), Polysorbate 80 (Poly80) and Cremophore RH40 solvents or surfactants in an aqueous buffer as a vehicle to dissolve DMTS.
The Poly80 solution was the best to dissolve DMTS. The 30 mg/ml Poly80 was proved to be the smallest concentration which can safely dissolve 10 mg/ml DMTS, and it was used for our further experiments. DMSO in 1-5% and Cremophore RH40 were also able to dissolve DMTS but only at a higher concentration range, and they resulted in a smaller amount of dissolved DMTS.

1.2.) *In vivo* experiments

1.2.1.) Comparing the effect of subcutaneous and intramuscular DMTS injections in AP

Acute pancreatitis (AP) was induced in mice by 10 times 50 μg/kg hourly intraperitoneal (ip.) caerulein (Caer) injections. DMTS was applied subcutaneously (sc.) and intramuscularly (im.) with 2x100 mg/kg dose. No difference was observed in the effect of DMTS on AP severity in different injection routes. However, mice with im. injection could stand less on injected legs, which was a temporary effect of DMTS treatment. Therefore, the subcutaneous injection was chosen to be used in further experiments.

1.2.2.) DMTS reduces the severity of acute pancreatitis in both mice and rats

Mice: *In vivo* AP model of mice was used to investigate the effect of sc. administered DMTS. The pancreata of the control group had normal morphology (Fig. 1A) and DMTS treatment alone did not induce any visible changes in the tissue. Supramaximal doses of Caer caused AP, which resulted in increased pancreatic oedema, intensive leukocyte infiltration and tissue MPO activity, as well as 30-40% tissue damage (Fig. 1). The administration of 2x50 mg/kg DMTS did not affect the disease. The 2x75 mg/kg DMTS treatment significantly reduced pancreatic injury in AP mice (Fig. 1F), but this dose had no significant effect on other measured parameters of the disease. However, the highest dose of DMTS (2x100 mg/kg) significantly decreased pancreatic leukocyte infiltration, MPO activity, and tissue damage as well. Overall, DMTS showed a dose-dependent protective effect.

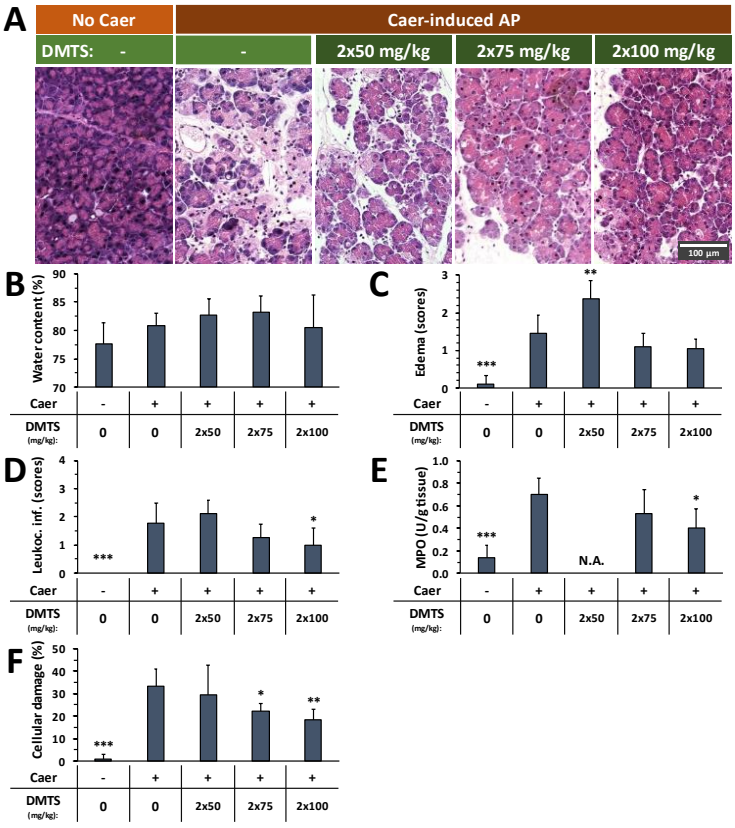

*Figure 1. DMTS administration reduces the severity of caerulein (Caer)-induced necrotizing acute pancreatitis (AP). Mice were treated subcutaneously (s.c.) with 2x50, 2x75 or 2x100 mg/kg DMTS, whereas intraperitoneal (i.p.) injection with 10x50 µg/kg Caer was used to induce AP. Control animals received physiological saline instead of Caer, or vehicle instead of DMTS. Animals were sacrificed at 12 h after the first Caer or physiological saline injection. (A) Representative histopathological images of pancreatic tissues of the treatment groups. Bar charts show the extent of pancreatic (B) water content (as measured by the dry-wet weight ratio), (C) oedema (evaluation of histological sections), (D) leukocyte infiltration, (E) myeloperoxidase (MPO) activity, and (F) cellular damage. Values represent means with standard deviation, n=6-8. (B-E) One-way ANOVA was performed followed by the Dunnett's post hoc test where all of the groups were compared to Caer only group, *p<0.05; **p<0.01; ***p<0.001. (F) Kruskal-Wallis test was performed followed by the Dunn's post hoc test, the groups were compared to Caer only group, *p<0.05; **p<0.01; ***p<0.001. Abbreviation: Leukoc. inf., leukocyte infiltration.*

Beyond the two-time DMTS administration, DMTS injections were performed four times in mice during AP as well. The highest dose could reduce the tissue injury, but it seemed less effective than the two-times doses.

Rats: The Caer model of AP in mice was complemented with another necrotizing AP model. L-ornithine-HCl (LO) was used to induce the disease in rats. The LO-induced AP resulted in a dramatic increase in pancreatic water content, leukocyte infiltration, and tissue damage. 2x50 and 4x25 mg/kg DMTS doses without AP were applied, which also had no effect on the pancreas compared to the control. During AP, only the 4x25 mg/kg DMTS dose could reduce the pancreatic water content and the amount of leukocyte infiltration. DMTS administration did not significantly

affect the very severe tissue damage (approx. 75%) caused by the disease, only a tendency for DMTS protection was observed when AP was compared with AP + 4x25 mg/kg DMTS group (p=0.064).

DMTS induces HSP72 expression during acute pancreatitis

Western blot analysis was performed on homogenized pancreatic tissue to determine the heat shock protein 72 (HSP72) expression, which is the major stress-induced protective chaperone among the HSP70 family in mammalian cells. DMTS alone did not induce HSP72 expression and Caer-induced AP also did not contribute to significant HSP72 expression after 12 h in mice. However, in AP mice 2x75 mg/kg DMTS increased the HSP72 protein production compared to the basal level, and the higher dose (2x100 mg/kg) of DMTS significantly elevated the HSP72 level compared to the control or AP groups.

Pain sensation and behavioral test

In a collaboration with Prof Erika Pintér's group (University of Pécs) the effect of DMTS on pain and behaviour was tested. Pain is one of the most important symptoms of AP, so nociception was assessed using the von Frey test in FVB/N mice. Interestingly, no significant difference in abdominal pain was detected between control and Caer-induced AP groups, consequently these studies were terminated. Since animal behaviour could adequately reflect the extent of pain, open field observations were also carried out. Although AP did not influence movement duration and total distance covered by the FVB/N mice, in AP the highest dose of DMTS (2x100 mg/kg) reduced total distance compared to the AP group without DMTS treatment. The 2x100 mg/kg DMTS dose (in healthy animals) affected neither movement time nor total distance covered compared to the vehicle treated control in mice.

Measurements of cytokines

The concentration of interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) cytokines were determined in pancreatic homogenates by ELISA kit. Unfortunately, we could not measure differences between the control and AP groups at 12 h. Most probably the expression peak of cytokines is in different time point. This peak of expression will be determined in the future.

1.3.) *In vitro* experiments

Pancreas acinar cell isolation

The pancreatic acinar cells are sensitive and in a changed environment or stressful condition they transform to duct-like cells (acinar-to-ductal metaplasia) and lost their original properties. Therefore, the proper cell isolation protocol with an improved culture condition is necessary for this cell type. One of our aim was to improve the earlier isolation protocol.

The following parameters were tested during acinar cell isolation or cell culturing:

- Adding 0.25 mg/ml trypsin inhibitor to all buffer
- Adding 5% fetal bovine serum (FBS) to wash and incubation buffers (HBSS)
- Changing HBSS buffer to M199 medium
- Adding 25 ng/ml recombinant Epidermal Growth Factor (EGF)

- Coating the culture plate with Rat Tail Collagen

The cellular viability was measured after changing the previous parameters, by MTT test or trypan blue exclusion assay.
The use of 0.25 mg/ml trypsin inhibitor, 5% FBS, M199 medium, and 25 ng/ml EGF prolonged the viability of acinar cells for more than 2 days. Without these factors the cells were viable only for 3-4 hours. The collagen coating did not have any effect on cellular viability measured by MTT test. The cells were responsive for Caer treatment showing high intracellular (ic.) Ca^{2+} signals.

Measuring the effect of DMTS on the viability of pancreas acinar cells

Based on the pharmacokinetics of DMTS in mice, its plasma concentration can reach 25-30 µg/ml. Therefore, the 30 µg/ml DMTS concentration was chosen for the *in vitro* measurements, or a concentration range was used between 0-100 or 0-1000 µg/ml.

Cell line experiments: Capan-1 epithelial cell line was treated with 0-1000 µg/ml DMTS for 1 hour and the cellular viability was measured with MTT test, where only the viable cells can convert the yellow MTT dye to blue formazan crystals. The 3-60 µg/ml DMTS treatment significantly increased the cellular viability (mostly the metabolic activity), and until 1000 µg/ml concentration the DMTS did not decreased cellular viability. The vehicle (Poly80 solution between 0-300 µg/ml) did not affect the cellular viability.

Isolated pancreas acinar cells: the effect of 30 µg/ml DMTS and its vehicle, 90 µg/ml Poly80, was tested on mouse primary pancreatic acinar viability by the MTT and PI methods. DMTS or its vehicle did not affect cellular viability and they did not cause toxicity after 4-8h. 500 µM hydrogen peroxide (H_2O_2), a member of ROS, significantly reduced primary acinar viability and evoked toxic effect. However, 30 µg/ml DMTS effectively restored the viability and reduced the H_2O_2-induced toxicity. AP inducing agents, namely Caer, L-arginine-HCl (L-Arg), and sodium chenodeoxycolate (CDC) were tested in acinar cells with or without DMTS. Caer 1 nM and 60 mM L-Arg treatments resulted in 5-19% and 19-33% toxicity between 4-8 h treatments, but 30 µg/ml DMTS significantly reduced their adverse effects, measured by the PI method. CDC was applied at 0.1, 0.3, and 0.5 mM concentrations on acinar cells, and all of these treatments reduced cellular viability from 100% to 73±6.8, 71±7.4, and 11±3.2% respectively in MTT test. The 0.5 mM concentration of CDC caused 19±5.6, 21±5.7, and 25±5.0% toxicity at 4, 6, and 8 h, respectively. 30 µg/ml DMTS significantly reduced the effect of CDC, indicating cytoprotective effects.

Mithocondrial activity measurement

Tetramethylrhodamine methyl ester (TMRM) was used to follow active mitochondria with intact membrane potentials. Isolated acinar cells were loaded with the dye and fluorescent microscope was used to follow the mitochondrial permeability transition pore (MPTP) opening which correlates to membrane potential and the fluorescence of TMRM dye.
During the TMRM measurements the positive control CCCP markedly reduced the mitochondrial potential of pancreatic acinar cells, while the tested concentrations of DMTS (3, 10, 30, 100 µg/ml) did not induce any change in mitochondrial potentials during 10 min treatment period. CDC and

sodium taurocholate from 30 µM concentration reduced the mitochondrial potential, the 10 µM of these bile acids had no effect on mitochondria within 10 minutes. The Caer at supramaximal dose of 100 nM had slight effect on mitochondria of pancreatic acinar cell. Those experiments are ongoing where DMTS treatment is combined with AP inducing agents (Caer, CDC, and sodium taurocholate) and mitochondrial potential is followed.

DMTS reduces the generation of reactive oxygen species in mouse pancreatic acinar cells

The antioxidant effect of DMTS was measured with a general oxidative stress indicator 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) in mouse primary pancreatic acinar cells. DMTS alone in the 1-60 µg/ml concentration range did not induce biologically relevant ROS production. The treatment with 100 µM H_2O_2, elevated the generation of ROS, which was significantly, and dose-dependently reduced by 10-60 µg/ml DMTS treatment. The 10, 30, 100 µM H_2O_2 also dose-dependently increases the presence of ROS, and 30 µg/ml DMTS significantly reduced ROS during those treatments with H_2O_2. Menadione increases intracellular ROS production through a redox cycling mechanism. All applied menadione concentrations (10, 30, 50 µM) increased acinar ROS levels, but the 30 µg/ml DMTS significantly reduced the effect of menadione on ROS generation.

DMTS reduces pathological intracellular Ca^{2+} signals

Intracellular Ca^{2+} concentration (ic[Ca^{2+}]) was followed real-time in mouse isolated pancreatic acinar cells. DMTS alone did not affect ic[Ca^{2+}]. 500 µM H_2O_2 evoked a huge increase inside in ic[Ca^{2+}]. Pre-treatment with 30 µg/ml DMTS significantly ameliorated this H_2O_2-induced increase in ic[Ca^{2+}]. The effect of 0.1 nM Caer on ic[Ca^{2+}] was also measured. The treatment with Caer induced Ca^{2+} oscillation. Ca^{2+} stores were exhausted thereafter and some minutes later carbachol, an acetylcholine (ACh) receptor agonist, could not induce relevant Ca^{2+} signal. However, DMTS pre-treatment modified the effect of Caer, the Ca^{2+} signal spikes were bigger and were more sustained. Furthermore, in this group a remarkable Ca^{2+} signal could be registered in response to carbachol at the end of the experiment. These results showed that DMTS reduces pathological Ca^{2+} signals caused by H_2O_2, it moderates the Caer-evoked Ca^{2+} signals and allowed the cells to remain responsive to carbachol stimuli.

Summary

Based on our results, we observed that DMTS has anti-inflammatory effects in experimental AP. *In vitro* we demonstrated that DMTS is cytoprotective, reduces ROS levels, and alter pathological Ca^{2+} signalling. These effects of DMTS could be caused by upregulation of HSP72 expression, by its antioxidant properties, and by reducing leukocyte infiltration and inhibiting MPO activity

Other important progresses which relate to this research

- Between 26.01.2019 – 05.02.2019 I was visited Prof. Ilona Petrikovics at Sam Houston State University (TX, USA). I participated in several animal studies, where we tested *in vivo* absorption kinetics of DMTS in mice. The results were collected and prepared for publication, and the manuscript related to this project is in writing state (80% is finished).

- A collaboration was formed in 2019 with Prof Erika Pintér's group (University of Pécs). We will publish together the results with DMTS in AP.
- A new collaboration was established this year with Prof. Péter Nagy's group (National Institute of Oncology). They will measure the *in vivo* H_2S production after treatment with or without DMTS.
- A publication was submitted to Scientific Reports journal about this research. This publication also can be found in Research Square preprint server. The DOI number is https://doi.org/10.21203/rs.3.rs-2056740/v1.

Szeged, 29th September 2022.

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