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ORGANOSULFUR COMPOUNDS AMELIORATE THE SEVERITY OF EXPERIMENTAL ACUTE PANCREATITIS AND EXERT CYTOPROTECTION

Ph.D. Thesis

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PUBLICATIONS

Publications related to the subject of the thesis

Orján EM, Kormányos ES, Fűr GM, Dombi Á, Bálint ER, Balla Z, Balog BA, Dágó Á, Totonji A, Bátai ZI, Jurányi EP, Ditrói T, Al-Omari A, Pozsgai G, Kormos V, Nagy P, Pintér E, Rakonczay Z Jr, Kiss L. The anti-inflammatory effect of dimethyl trisulfide in experimental acute pancreatitis. Sci Rep. 2023; 13(1):16813. doi: 10.1038/s41598-023-43692-9. [IF_{2023}: 3.8], **D1**

Orján EM, Dágó Á, Sándor B, Salamah O, Mihalekné Fűr G, Balog BA, Rakonczay Z Jr, Kiss L. Investigation of organosulfur molecules in experimental acute pancreatitis: Antioxidant and antiferroptotic actions of ATB-346. Biomed Pharmacother. 2026; 196:119089. doi: 10.1016/j.biopha.2026.119089. [IF_{2024}: 7.5**], **D1**
** *On the Norwegian list of journals*

Publications not related to the subject of the thesis

Bálint ER, Fűr G, Kui B, Balla Z, Kormányos ES, **Orján EM**, Tóth B, Horváth G, Szűcs E, Benyhe S, Ducza E, Pallagi P, Maléth J, Venglovecz V, Hegyi P, Kiss L, Rakonczay Z Jr. Fentanyl but Not Morphine or Buprenorphine Improves the Severity of Necrotizing Acute Pancreatitis in Rats. Int J Mol Sci. 2022; 23(3):1192. doi: 10.3390/ijms23031192. [IF_{2022}: 5.6], **Q1**

Balla Z, Kormányos ES, Kui B, Bálint ER, Fűr G, **Orján EM**, Iványi B, Vécsei L, Fülöp F, Varga G, Harazin A, Tubak V, Deli MA, Papp C, Gácser A, Madácsy T, Venglovecz V, Maléth J, Hegyi P, Kiss L, Rakonczay Z Jr. Kynurenic Acid and Its Analogue SZR-72 Ameliorate the Severity of Experimental Acute Necrotizing Pancreatitis. Front Immunol. 2021; 12:702764. doi: 10.3389/fimmu.2021.702764. [IF_{2021}: 8.787], **Q1**

Fűr G, Bálint ER, **Orján EM**, Balla Z, Kormányos ES, Czira B, Szűcs A, Kovács DP, Pallagi P, Maléth J, Venglovecz V, Hegyi P, Kiss L, Rakonczay Z Jr. Mislocalization of CFTR expression in acute pancreatitis and the beneficial effects of VX-661/VX-770 treatment on disease severity. J Physiol. 599, 4955-4971. doi: 10.1113/JP281765. [IF_{2021}: 6.228], **Q1**

Tasi DA, **Orján EM**, Czakó G. Benchmark Ab Initio Mapping of the $F^- + CH_2ClI$ S_N2 and Proton-Abstraction Reactions. J Phys Chem A. 2024; 128(49):10568-10578. doi: 10.1021/acs.jpca.4c06716. [IF_{2024}: 2.8], **Q2**

Orján EM, Nacsa AB, Czakó G. Conformers of dehydrogenated glycine isomers. J Comput Chem. 2020; 41(22):2001-2014. doi: 10.1002/jcc.26375. [IF_{2020}: 3.376], **Q1**

Scientometrics

https://m2.mtmt.hu/gui2/?type=authors&mode=browse&sel=10073212&view=pubTable

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INTRODUCTION

I. The physiology of the pancreas

The pancreas is a retroperitoneal gland of the gastrointestinal tract located in the upper abdomen and possesses both exocrine and endocrine functions. The exocrine pancreas consists primarily of acinar and ductal epithelial cells, and its function is to produce a mixture of digestive enzymes and electrolyte-rich fluid. Acinar cells synthesise, store, and excrete digestive enzymes as inactive precursors (zymogens), which become active in the duodenum. Zymogens are secreted in an isotonic, NaCl- and H^+-rich fluid from which Cl^- is then exchanged for HCO_3^- by ductal cells. The resulting high HCO_3^- concentration ensures that the secretion maintains an alkaline pH, thereby neutralising the acid chyme from the stomach and providing an optimal pH in the intestinal lumen for enzyme activity. In the duodenum, enterokinase catalyses the conversion of trypsinogen to trypsin, initiating an activation cascade. The resulting trypsin then further activates trypsinogen and converts all other proenzymes into their active forms.

II. Acute pancreatitis

II.1. Definition, epidemiology, classification, and aetiology

Acute pancreatitis (AP) is a sudden inflammatory disease of the exocrine pancreas and is a leading cause of non-malignant gastrointestinal-related hospitalisation. The annual incidence of the disease exceeds 30/100,000 population and shows an increasing tendency in developed countries. The severity of the disease varies widely and can be categorised as mild, moderately severe, or severe forms based on the Revised Atlanta Classification system. The diagnosis of AP requires at least two of the following three criteria: (i) characteristic symptoms (epigastric upper abdominal pain); (ii) elevated serum lipase activity (or amylase activity), at least three times greater than the upper limit of normal; (iii) imaging consistent with the diagnosis. Biliary pancreatitis and alcohol-induced AP account for about 60–80 % of AP cases, whereas AP induced by hypertriglyceridemia with serum triglyceride levels above 11.3 mM is less common (approximately 9 %). Less common causes of AP include endoscopic retrograde cholangiopancreatography, hypercalcemia, toxins, and drugs.

II.2. Pathogenesis

The pathomechanism of the disease is complex and not yet fully understood. The disruption of normal intracellular Ca^{2+} signalling triggers the premature activation of digestive enzymes by blocking the secretion of zymogens into the ductal space and by promoting the colocalization of proenzymes with lysosomal enzymes, such as cathepsin B. Independent of trypsinogen activation, pathologic Ca^{2+} signalling also induces the translocation of nuclear factor κB (NF-κB) into the nucleus, and NF-κB activation leads to the release of cytokines (e.g. interleukins 1 and 6, tumour necrosis factor-α), which contribute to leukocyte recruitment and promote the inflammatory response. In addition, the intra-acinar generation of reactive oxygen species (ROS) increases with the decreasing capacity for systemic clearance. Other critical cellular processes during AP include impaired autophagy, endoplasmic reticulum stress, and decreased ductal HCO_3^- and fluid secretion. Although many of the critical pathways of AP have been identified, unfortunately, the disease's management is still based on supportive therapy.

II.3. Ferroptosis and its role in AP

Ferroptosis is an iron-dependent, non-apoptotic form of programmed cell death, characterised by the accumulation of iron and the extensive production of ROS and lipid peroxides. Iron can trigger ROS generation by initiating the Fenton reaction and acts as a cofactor for lipid peroxidase enzymes. Antioxidant systems, such as glutathione peroxidase 4 (GPX4) and its cofactor glutathione (GSH), convert phospholipid peroxides into non-toxic lipid alcohols and provide resistance to ferroptosis. Previous reports have demonstrated that iron accumulation in AP may upregulate ferroptosis-related proteins in the pancreas, and that early trypsin activation can degrade the GPX4 enzyme. Deregulated autophagy was also shown to initiate ferroptosis by degrading ferritin, the primary iron ion-storing protein, leading to oxidative injury.

III. Endogenous hydrogen sulfide

Hydrogen sulfide (H_2S) is recognised as a signalling molecule that appears to regulate diverse cellular physiological processes, and alterations in endogenous H_2S synthesis have been associated with the development of various diseases. In mammalian cells, H_2S is produced mainly from L-cysteine and homocysteine by three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH), and 3-mercaptopyruvate sulfurtransferase. Emerging research indicates that H_2S reduces oxidative stress and exerts cytoprotective effects by scavenging ROS and enhancing antioxidant enzyme activity. Its complex interaction with lipid peroxides, as well as its ability to promote GPX4 expression, also emphasises its role in preventing ferroptotic processes. Although the upregulation of H_2S synthesis or its administration confers protective effects in inflammatory conditions, the overexpression of enzymes responsible for H_2S production may exacerbate inflammation. While the physiological and pathophysiological effects of H_2S have been extensively investigated, many questions and apparent contradictions remain regarding how it acts in living organisms.

IV. Organosulfur molecules

Sulfur-containing organic compounds, known as organosulfurs, are abundantly found in nature, and synthetic ones also exist. Many of them are biologically active and can influence specific signalling pathways (e.g. NF-κB, nuclear factor-erythroid 2 related factor 2 – Nrf2), activate ion channels (K^+, Ca^{2+}, or transient receptor potential ankyrin – TRPA), affect protein function through persulfidation, and exhibit antioxidant properties. Numerous organosulfur compounds are also potential H_2S donors, and their anti-inflammatory effects are most probably linked to H_2S release. Organosulfurs, namely dimethyl trisulfide (DMTS), diallyl trisulfide (DATS), GYY4137, AP39, and ATB-346, have been reported to exhibit anti-inflammatory and antioxidant effects across various disease conditions, but no information exists regarding their impact on AP.

IV.1. DMTS

DMTS is a naturally occurring organosulfur compound and the simplest organic trisulfide. It reacts with haemoglobin, facilitating methaemoglobin formation, and also has a great potential to reduce cyanide poisoning. DMTS was also shown to activate the TRPA1 ion channel, modulate pain sensation, and reduce inflammation in serum-transfer arthritis.

IV.2. DATS

DATS is a natural H_2S donor organosulfur that suppresses the activation of the NF-κB signalling pathway and inhibits lipopolysaccharide binding to Toll-like receptor 4 in macrophages. It also exhibited antioxidant activity by mitigating oxidative stress and augmenting the activity of antioxidant enzymes (e.g. superoxide dismutase).

IV.3. GYY4137

GYY4137 is a synthetic, water-soluble H_2S donor organosulfur molecule, designed to release H_2S in a gradual and sustained manner. Previous research has demonstrated its ability to decrease the levels of pro-inflammatory cytokines (e.g. interleukins 1 and 6, tumour necrosis factor-α) and to reduce the secretion of nitric oxide and prostaglandin E2.

IV.4. AP39

AP39 is a synthetic, slow-releasing H_2S donor organosulfur compound with mitochondria-targeting capabilities. It has been shown to protect endothelial cells during oxidative stress and maintain mitochondrial DNA integrity. AP39 also limited mucosal injury and altered the tissue inflammatory response in necrotising enterocolitis.

IV.5. ATB-346

ATB-346 combines a traditional non-steroidal anti-inflammatory drug (NSAID), naproxen, with a covalently attached H_2S donor chemical moiety, 4-hydroxy-thiobenzamide. It has demonstrated anti-inflammatory effects in various inflammatory diseases by reducing several inflammatory parameters, including pro-inflammatory cytokine expression, leukocyte infiltration, and cyclooxygenase activity. It also reduced inflammation in humans in a model of acute dermal inflammation and provided evidence of significant protection against the development of gastrointestinal ulcers in a Phase 2B clinical study.

AIMS

Given the significant anti-inflammatory potential of organosulfur compounds, our objective was to comprehensively investigate and compare the five most widely studied organosulfur molecules in experimental AP: ATB-346, DMTS, DATS, GYY4137, and AP39. Furthermore, we aimed to provide insights into the mechanisms of action of DMTS and ATB-346 in both *in vivo* and *in vitro* studies. Our detailed aims were the following:

1) to compare the five aforementioned organosulfurs as therapeutic agents against experimental AP both *in vivo* (mice) and *in vitro* (pancreatic acinar cells)
2) to reveal how DMTS influences the severity of AP by investigating its effect on oxidative stress, cellular viability, and intracellular Ca^{2+} signalling, as well as by assessing its impact on serum and tissue sulfide levels
3) to investigate the impact of ATB-346 on AP severity by testing its effect on oxidative stress and ferroptosis

MATERIALS AND METHODS

I. Materials and solutions

All chemicals were obtained from Merck Life Science Kft. (Budapest, Hungary) unless indicated otherwise. Cerulein (Cer) (Glentham Life Sciences, Corsham, UK) was dissolved in dimethyl sulfoxide (DMSO), and the working solution was diluted in physiological saline (PS). Palmitoleic acid (POA) was dissolved in absolute ethanol (EtOH). The organosulfur solutions were prepared in the following manner: ATB-346 (Cayman Chemical, Ann Arbor, MI, USA) and AP39 (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in DMSO; GYY4137 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in PS; DATS (Cayman Chemical, Ann Arbor, MI, USA) and DMTS were prepared in polysorbate 80 (Poly80). Antibodies used for Western blotting, the tissue culture media and the Malondialdehyde (MDA) Colourimetric Assay Kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA)

II. Animals

Eight- to ten-week-old male FVB/N mice weighing 22–30 g were used for the experiments. The experiments were conducted in accordance with the European Union Directive 2010/63/EU and the Hungarian Government Decree 40/2013 (II.14.). Experiments involving animals were approved by both local (University of Szeged) and national ethical committees (permit no. X./1714/2020).

III. *In vivo* experiments: AP induction, treatment with organosulfurs and tissue harvesting

Necrotising AP was induced by intraperitoneal (i.p.) hourly injections of 10 × 50 µg/kg Cer. The different organosulfur treatment doses, administration routes and timing are shown in Table 1. For DMTS and ATB-346, we employed another necrotising AP model in which AP was induced by i.p. hourly injections of 2 × 1.35 g/kg EtOH and 2 × 150 mg/kg POA in mice. Control groups were given PS instead of Cer or EtOH-POA, and Poly80, DMSO or the combination of Poly80 and DMSO instead of the corresponding organosulfur. The animals were sacrificed 12 h after the first Cer or 24 h after the first EtOH-POA injection.

Table 1. Organosulfur treatment doses, administration routes, and timing.

Organosulfur / *AP models*	**DMTS** *Cer AP*	**DMTS** *EtOH-POA AP*	**DATS** *Cer AP*	**GYY4137** *Cer AP*	**AP39** *Cer AP*	**ATB-346** *Cer AP*	**ATB-346** *EtOH-POA AP*
Administration routes	*s.c.*	*s.c.*	*i.p.*	*i.p.*	*i.p.*	*p.o.*	*p.o.*
Doses (µmol/kg)	2 × 400 2 × 600 2 × 800	3 × 600 3 × 800	2 × 25 2 × 50 2 × 100	2 × 25 2 × 50 2 × 100	2 × 0.125 2 × 0.25 2 × 0.5	2 × 20 2 × 40 2 × 80	3 × 40 3 × 80
Timing of the treatments (hours from the first Cer or EtOH-POA injection)	2, 5 or 0, 3	1, 6, 12 or 0, 3 12	2, 5	2, 5	2, 5	2, 5	2, 6, 12

Abbreviations: AP, acute pancreatitis; DMTS, dimethyl trisulfide; DATS, diallyl trisulfide; Cer, cerulein; EtOH-POA, ethanol and palmitoleic acid; s.c., subcutaneous; i.p., intraperitoneal; p.o., per os.

IV. Histological analysis

The formalin-fixed pancreatic samples were dehydrated before being paraffin-embedded and sectioned. These sections were stained with hematoxylin and eosin and were evaluated by two independent, blinded observers. To quantify cellular damage, leukocyte infiltration, and oedema grades, a semiquantitative scoring system was used.

V. Laboratory measurements

To evaluate pancreatic water content, the wet/dry weight ratio was calculated. Serum amylase activity was measured using a commercial amylase activity kit (Diagnosticum Zrt., Budapest, Hungary). Pancreatic myeloperoxidase (MPO) activity was assayed using 3,3`5,5`-tetramethyl-benzidine-H_2O_2, and the absorbance was measured using a microplate reader. MPO activities were normalised to total protein content as measured by the Lowry method.

VI. Western blot

The GPX4 protein expression was measured from pancreatic tissue homogenates using Western blot analysis. Sample protein concentration was determined using the Bradford protein assay, followed by denaturation. The samples were separated using sodium dodecyl sulfate polyacrylamide gel (15 %) electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked in Pierce™ Clear Milk Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) before incubating with rabbit anti-GPX4 antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The immunoreactive protein was visualised using horseradish peroxidase-conjugated anti-rabbit immunoglobulin.

VII. mRNA extraction and reverse transcription

Following the transfer of a small piece of the pancreas to 1 mL of TRI-reagent, the samples were homogenised, and the homogenates were stored at −80°C until use (for a maximum of 1 or 2 days). Total RNA isolation was performed utilising chloroform/isopropanol extraction. After assessing purity using a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA, USA), RNA integrity was determined by agarose gel electrophoresis. Two micrograms of total RNA were used for reverse transcription to cDNA.

VIII. Quantitative real-time PCR

Quantitative real-time PCR reactions were carried out using the Luminaris Colour HiGreen qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with specific primers according to the manufacturer's instructions. The following primers were used for the determination of relative gene expression: *Cth*, *Cbs*, *Hmox1*, *Sod1*, *Sod2*, *Cat*, *Fth1*, *Txnrd1*. The housekeeping gene in mRNA studies was *Gapdh*.

IX. Pancreatic acinar cell isolation

Mouse pancreatic acinar cells were isolated by collagenase digestion. The viability of the acini was rapidly determined using the trypan blue technique, and they were used for *in vitro* measurements if their viability was greater than 90 %.

X. Acinar viability measurements

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Calcein-AM, and propidium iodide (PI) methods were employed to determine cellular viability. Pancreatic acinar cells were treated with treatment solutions or with a specific combination of them. For the MTT assay, the absorbances were measured by a microplate reader. For the Calcein-AM and PI assays, images of pancreatic acinar cells were captured (4 images / well) by an Axio Observer 7 (Carl Zeiss, Oberkochen, Germany) fluorescent microscope. ImageJ software was used to measure the areas of Calcein-AM, PI, and H33342 stainings.

XI. Measurement of ROS

The intracellular ROS was determined by a microfluorescence method. Pancreatic acinar cells were exposed to treatment solutions either individually or in combination. 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), and H33342 were applied to the cells and at the end of the experiment, the cells were placed into a FLUOstar OPTIMA plate reader.

XII. Real-time measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration (ic[Ca^{2+}]) was monitored in real time using an Axio Observer 7 fluorescent microscope. Acinar cells were loaded with FURA-2-AM (Biotium, Fremont, CA, USA). During the measurement, cells were perfused with treatment solutions or with a specific combination of them. The 340/380 fluorescence excitation ratio was measured, and one ic[Ca^{2+}] measurement was obtained per second.

XIII. LC-MS/MS measurement of low molecular weight metabolites from pancreatic tissue and serum samples

The following low-molecular-weight metabolites were determined: cysteine, cysteine persulfide, glutathione, glutathione persulfide, and sulfide. The protein content was measured using a BCA assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements were carried out on a Thermo Q-Exactive Focus Orbitrap mass spectrometer coupled to a Thermo Vanquish UHPLC, and the samples were analysed with two different methods. MS/MS detection was conducted in positive ionisation mode, and higher-energy collisional dissociation was used to detect the analytes.

XIV. Measurement of MDA

The level of MDA, an indicator of oxygen radical-induced lipid peroxidation, was measured in tissue samples using a commercial MDA Colourimetric Assay Kit, as described by the manufacturer.

XV. Statistical analysis

Values represent means with standard deviation (SD). Experiments were evaluated by one- or two-way ANOVA followed by Dunnett's or Tukey's *post-hoc* test (GraphPad Prism, GraphPad Software LLC, version 10.5.0). If necessary, the Kruskal-Wallis test was performed, followed by Dunn's *post-hoc* test. $P < 0.05$ was accepted as statistically significant.

RESULTS

I. Comparison of organosulfurs against experimental AP

I.1. Organosulfur molecules dose-dependently ameliorate the severity of Cer-induced AP in mice

Three different doses of each organosulfur molecule (ATB-346, DMTS, DATS, GYY4137, AP39) were employed to assess their effects on Cer-induced AP in FVB/N mice. AP induced by supramaximal doses of Cer resulted in increased pancreatic water content, intensive leukocyte infiltration, MPO activity, serum amylase activity, and 40–50 % tissue damage. The administration of 2 × 20 μmol/kg ATB-346 did not affect the severity of the disease. In contrast, the 2 × 40 μmol/kg ATB-346 treatment significantly reduced the histological (oedema, leukocyte infiltration, cellular damage) parameters, and the highest dose of ATB-346 (2 × 80 μmol/kg) markedly decreased both histological and laboratory (water content, MPO, serum amylase) parameters as well.

Similar results were observed when investigating the effects of various doses of DMTS and DATS in the same Cer-induced AP model. AP caused damage to the pancreatic tissue, while both DMTS and DATS were able to ameliorate this damage in a dose-dependent manner. DMTS treatments at doses of 2 × 400 and 2 × 600 μmol/kg proved ineffective, whereas the administration of DMTS at 2 × 800 μmol/kg significantly reduced the severity of AP. Administering 2 × 25 μmol/kg DATS did not impact the severity of the disease. However, both 2 × 50 and 2 × 100 μmol/kg DATS treatments significantly reduced the degree of AP-induced increases in pancreatic oedema, leukocyte infiltration, cellular injury, and water content. Only the highest dose of DATS (2 × 100 μmol/kg) could decrease tissue MPO and serum amylase activities.

To examine the effects of GYY4137 and AP39 on pancreatic injury, varying doses were administered in Cer-induced AP. Histological sections revealed severe tissue damage characterised by oedema, inflammatory cell infiltration, and acinar cell necrosis during the disease course. The lowest dose of GYY4137 (2 × 25 μmol/kg) significantly reduced the tissue damage but did not affect other measured parameters during AP. Administering GYY4137 at a 2 × 50 μmol/kg dose lowered the Cer-induced cellular damage and serum amylase activity. However, the treatment with 2 × 100 μmol/kg GYY4137 significantly reduced all measured values, thereby alleviating the severity of AP. AP39 at a 2 × 0.25 μmol/kg dose significantly reduced both oedema and tissue damage, as well as serum amylase activity, while the lowest AP39 dose (2 × 0.125 μmol/kg) was ineffective. Treatment with the highest dose of AP39 (2 × 0.5 μmol/kg) markedly decreased all the AP severity parameters.

I.2. *In vitro* protective effects of organosulfur molecules against AP-inducing agents

To investigate the cytoprotective properties of these organosulfur molecules, cellular viability studies were conducted using the MTT and PI assays. The following organosulfur concentrations could be safely administered and were selected for further experiments: 240 μM DMTS; 100 μM DATS; 10 μM GYY4137; 0.3 μM AP39; 30 μM ATB-346. Primary pancreatic acinar cells were treated with AP-inducing agents, namely Cer, L-arginine-HCl (L-arg), and sodium chenodeoxycholate (CDC). The administration of 60 mM L-arg and 0.5 mM CDC significantly lowered the acinar metabolic activity and

caused toxic effects at 8 h. In contrast, 240 μM DMTS, 0.3 μM AP39, and 30 μM ATB-346 effectively restored the metabolic activity compared to the L-arg-only group, whereas only 240 μM DMTS could mitigate the adverse effect of CDC. All applied organosulfur treatments markedly decreased the L-arg- and CDC-induced toxicity. Administration of 1 nM Cer caused 16.9 ± 4.2 % toxicity at 8 h, which was significantly reduced by all organosulfur treatments, suggesting cytoprotective effects.

II. The anti-inflammatory effect of DMTS in experimental AP

II.1. DMTS reduces the severity of AP in mice when administered with AP induction

Among the five organosulfur molecules, DMTS and ATB-346 were further investigated. An alternative model of necrotising AP induced by EtOH-POA treatment was also employed in FVB/N mice. The treatment doses of DMTS (3 × 600 and 3 × 800 μmol/kg) were given at 1, 6, and 12 h following the onset of the inflammation. The EtOH-POA AP model significantly increased the pancreatic oedema and leukocyte infiltration. The administration of DMTS markedly reduced the leukocyte infiltration and MPO activity at both doses. Cellular damage was detected in the EtOH-POA AP model, reaching ~35 %. DMTS treatments with 3 × 600 and 3 × 800 μmol/kg doses significantly reduced the cellular damage.

II.2. DMTS modulates physiological, but not pathophysiological intracellular Ca^{2+} signalling in acinar cells

The ic[Ca^{2+}] was monitored in real-time in isolated pancreatic acinar cells. To investigate the effect of Cer on ic[Ca^{2+}], cells were perfused with maximal (0.1 nM) and supramaximal (1 nM) concentrations of Cer. Application of 0.1 nM Cer resulted in global, oscillatory increases in ic[Ca^{2+}]. The Ca^{2+} stores were subsequently depleted, and a few minutes later, carbachol, an acetylcholine (ACh) receptor agonist, was unable to elicit a relevant Ca^{2+} signal. The DMTS pre-treatment had no effect on the Cer-evoked oscillation frequency, but there was a significant increase in the average height of Ca^{2+} signals when the Cer treatment was compared to the Cer + DMTS treatment. Additionally, in the Cer + DMTS group, a remarkable increase in ic[Ca^{2+}] could be registered in response to carbachol at the end of the experiment. The supramaximal concentration of Cer (1 nM) evoked an initial peak in the Ca^{2+} signal, followed by a return to the basal levels, and showed no further increase or oscillation during stimulation. The pre-treatment with DMTS did not affect the Cer-induced pathological Ca^{2+} signals.

II.3. DMTS reduces oxidative stress in mouse pancreatic acinar cells

Intracellular concentrations of ROS were measured using the general oxidative stress indicator carboxy-H2DCFDA after 8 h. Via a redox cycling mechanism, menadione promotes intracellular ROS production. All applied menadione concentrations (10, 30, 50 μM) markedly increased the intracellular ROS levels, whereas administering 240 μM DMTS significantly reduced the effect of menadione on ROS formation.

II.4. DMTS increases serum sulfide and persulfide levels

To gain further insights into the protective effects of DMTS against AP, HPLC-MS/MS (high-performance liquid chromatography coupled with tandem mass spectrometry) based sulfur metabolome analyses were carried out. Mouse pancreatic tissue and serum samples were derived from the Cer-AP model (12 h sacrifice time) treated with 2 × 600 and 2 × 800 μmol/kg DMTS doses. DMTS treatments at

doses of 2 × 600 and 2 × 800 μmol/kg increased the persulfide ratio (Cys-SSH/Cys-SH) in AP compared to the Cer-only group. Moreover, both 2 × 600 and 2 × 800 μmol/kg DMTS doses significantly increased the GSSH/GSH ratio. We also observed an elevated level of sulfide (H_2S) in the serum resulting from both DMTS doses in AP compared to the Cer-only group. In pancreatic tissue, the analyses revealed no significant differences in any of the measured metabolite levels between the Cer groups with or without DMTS treatment. Overall, our results showed that DMTS increases serum sulfide and low-molecular-weight persulfide levels and counteracts the effect of Cer on GSH, but it did not alter the levels of any of the measured metabolites in pancreatic tissue.

III. The antioxidant and antiferroptotic actions of ATB-346 in experimental AP

III.1. ATB-346 reduces the severity of EtOH-POA-induced AP

To conduct a more thorough investigation of the impact of ATB-346 on pancreatic injury, it was employed to assess its effects on EtOH-POA-induced AP in FVB/N mice. The treatment doses of ATB-346 (3 × 40 and 3 × 80 μmol/kg) were given at 2, 6, and 12 h following the onset of the inflammation. The EtOH-POA-induced AP elevated the extent of oedema, leukocyte infiltration, and pancreatic injury compared to the control group. Both doses of ATB-346 (3 × 40 and 3 × 80 μmol/kg) significantly reduced all histological parameters, while the highest dose of ATB-346 (3 × 80 μmol/kg) decreased the pancreatic water content during AP. The 3 × 40 and 3 × 80 μmol/kg ATB-346 doses also reduced tissue MPO and serum amylase activities compared to the AP-only group.

III.2. ATB-346 attenuates cellular oxidative stress and promotes cytoprotection

To investigate the antioxidant properties of ATB-346, the mRNA expression of genes involved in oxidative stress defence were determined. The relative gene expression of *Hmox1* mRNA markedly increased in both Cer- and EtOH-POA-induced AP. All tested ATB-346 doses (2 × 20, 2 × 40, and 2 × 80 μmol/kg) further elevated the *Hmox1* mRNA expression compared to the Cer-only group, whilst only the highest dose of ATB-346 (3 × 80 μmol/kg) increased the amount of *Hmox1* mRNA in the EtOH-POA AP model. The expression of the *Sod1* gene was significantly decreased in both employed AP models, and none of the ATB-346 treatments proved effective in restoring the mRNA of *Sod1* to control levels. A markedly reduced *Sod2* mRNA expression was also observed in Cer-induced AP, whereas in the EtOH-POA AP model, there was no significant change in the *Sod2* mRNA compared to the control group. In both AP models, the highest doses of ATB-346 (2 × 80 μmol/kg and 3 × 80 μmol/kg) significantly increased the expression of *Sod2* mRNA. The relative gene expression of *Cat* mRNA decreased in both Cer- and EtOH-POA-induced AP. Treatments with ATB-346 (3 × 40 and 3 × 80 μmol/kg) increased the amount of *Cat* mRNA solely in the EtOH-POA AP model.

Cellular viability measurements were also conducted to assess the protective effect of ATB-346 against oxidative stress-inducing agents using the Calcein-AM and PI methods. Treatment with 0.5 mM hydrogen peroxide (H_2O_2) significantly reduced acinar viability and evoked a toxic effect at 2 h. Conversely, 30 μM ATB-346 lowered the H_2O_2-induced toxicity, yet it was ineffective in increasing the cellular metabolic activity. Administering 50 μM menadione markedly decreased the acinar metabolic activity and resulted in 30 ± 6% toxicity at 2 h. In contrast, 30 μM ATB-346 effectively restored the

metabolic activity compared to the menadione-only group. Intracellular concentrations of ROS were measured using carboxy-H2DCFDA after 8 h. 1 nM Cer, 50 mM EtOH+POA, 60 mM L-arg, 0.5 mM CDC, and 50 μM menadione treatments all promoted intracellular ROS production, whereas 30 μM ATB-346 administration significantly reduced the effects of the aforementioned treatments on ROS formation. Administering 1 μM CCT137690 (CCT) resulted in no change in acinar ROS concentrations when compared to the control group. The treatment with 30 μM ATB-346 significantly decreased the intracellular ROS level, confirming its ability to scavenge ROS.

Cth and *Cbs* are genes involved in the transsulfuration pathway and encode two of the three essential H_2S-producing enzymes. Both applied AP models decreased the expression of *Cth* and *Cbs* mRNA. In Cer-induced AP, the highest dose of ATB-346 (2 × 80 μmol/kg) markedly increased the relative expression of *Cbs* mRNA; however, it proved ineffective in all other instances.

Antioxidant molecules can help combat oxidative stress and guard against lipid peroxidation, thereby offering protection against ferroptosis. Therefore, the effect of ATB-346 on ferroptosis was also investigated by measuring the mRNA expressions of *Fth1* and *Txnrd1*, the presence of MDA and GPX4, and the ability of ATB-346 to reduce the effects of ferroptosis-evoking agents (erastin, 1S,3R-RSL 3 – RSL-3). The relative expression of *Fth1* mRNA decreased in both Cer- and EtOH-POA-induced AP, whereas the highest doses of ATB-346 (2 × 80 μmol/kg and 3 × 80 μmol/kg) significantly increased the amount of *Fth1* mRNA. The mRNA expression of *Txnrd1*, an antioxidant gene, was markedly increased in the Cer AP model, whilst in EtOH-POA-induced AP, there was no significant change in the *Txnrd1* mRNA compared to the control group. ATB-346 treatments with 2 × 80 μmol/kg and 3 × 80 μmol/kg doses significantly increased the expression of *Txnrd1* mRNA. The increased expression of *Fth1* and *Txnrd1* mRNA following ATB-346 treatments indicates enhanced protection against ferroptosis.

MDA is a biomarker of oxidative stress and lipid peroxidation, and the detection of MDA can reflect the level of cellular damage from ferroptosis. Markedly elevated pancreatic MDA levels were observed in both Cer- and EtOH-POA-induced AP, and the highest doses of ATB-346 (2 × 80 μmol/kg and 3 × 80 μmol/kg) effectively reduced the MDA concentration. GPX4 is considered an essential regulatory factor in ferroptosis and a key target to counteract it. The protein expression of GPX4 was decreased in both employed AP models; however, 2 × 80 μmol/kg and 3 × 80 μmol/kg ATB-346 doses significantly increased GPX4 levels compared to the AP group.

Viability assays demonstrated that treatments with 5 μM erastin and 0.5 μM RSL 3, two ferroptosis inducers, significantly reduced the acinar metabolic activity and caused 54 ± 16 and 33 ± 11% toxicity at 8 h, respectively. Administering 30 μM ATB-346 restored the metabolic activity and significantly decreased the erastin- and RSL 3-induced toxicity. The 5 μM erastin and 0.5 μM RSL 3 treatments also increased the intracellular concentration of ROS, while 30 μM ATB-346 markedly reduced the ROS production caused by erastin or RSL 3.

DISCUSSION

I. Comparison of organosulfurs against experimental AP

AP remains a life-threatening inflammatory disease with no specific therapy, making it crucial to explore management options. Organosulfur compounds hold significant potential as drug candidates for various disease conditions, especially those linked to inflammation. In our study, treatments with all five organosulfur compounds (ATB-346, DMTS, DATS, GYY4137, and AP39) dose-dependently reduced the severity of Cer-AP by lowering both histological (oedema, leukocyte infiltration, cellular damage) and laboratory (water content, MPO, serum amylase) parameters. We also demonstrated that AP-inducing agents (L-arg, CDC, and Cer) caused cellular injury, and the administration of organosulfurs significantly reduced the resulting cytotoxic effects. All the organosulfur compounds we tested have been shown to exhibit cytoprotective effects, which can be attributed to various processes. These include the protection of protein function through protein persulfidation, the modulation of specific signalling pathways to mitigate the effects of oxidative stress, and the activation of the Kelch-like ECH-associated protein 1/Nrf2 system. Based on the viability and toxicity measurements, GYY4137 and DATS exhibited less potent cytoprotection in comparison to ATB-346, DMTS, and AP39. Furthermore, we showed that ATB-346 and DMTS exert a more beneficial effect on disease severity relative to DATS, GYY4137 and AP39. Overall, DMTS and ATB-346 demonstrated the strongest cytoprotective effects and had similar impacts on the disease course. Our *in vivo* and *in vitro* results, along with the existing literature, confirm the anti-inflammatory potential of organosulfur compounds and suggest their suitability as drug candidates in AP.

II. The effect of DMTS on the severity of experimental AP

To verify the anti-inflammatory effect of DMTS, it was employed in another AP model induced by EtOH-POA. DMTS administration decreased the pancreatic leukocyte infiltration, MPO activity, and cellular damage, thereby alleviating the severity of AP.

High but physiological concentration of Cer (0.1 nM) caused global ic[Ca^{2+}] oscillations, but these oscillations diminished within 5-7 min, and thereafter, the ACh receptor agonist carbachol could not stimulate further Ca^{2+} release from the intracellular stores. Nevertheless, when the DMTS pre-treatment was applied, the 0.1 nM Cer-induced Ca^{2+} oscillations were sustained for longer periods, and carbachol could evoke a marked increase in ic[Ca^{2+}]. We assume that the intracellular Ca^{2+} stores become depleted in response to maximal Cer (0.1 nM) stimulation, and DMTS can prevent this. However, the DMTS treatment did not affect the cellular ic[Ca^{2+}] response when the supramaximal and pathological Cer (1 nM) concentration was applied. Based on these observations, we can conclude that DMTS can moderate physiological, but not pathophysiological ic[Ca^{2+}].

Sulfur-donor molecules, such as polysulfides, are effective antioxidants, and this property may contribute to their beneficial effects in various diseases. During AP, a significant amount of ROS is generated, and excessive ROS production and ATP depletion promote necrotic cell death rather than apoptosis. DMTS was protective when acinar cells were treated with the oxidative stress inducer, menadione, confirming its antioxidant capability. Via transpersulfidation, DMTS can act as a sulfane sulfur (S^0) donor, generating low-molecular-weight persulfides or persulfidate protein cysteine residues to

conserve their activity by utilising the thioredoxin system. Beyond its direct effect, DMTS, as a similar molecule to DATS, may also induce antioxidant enzymes via the Nrf2 pathway, but this warrants further investigation.

It has been proposed that H_2S signalling occurs via oxidative posttranslational modification of cysteine residues to persulfides. Literature data indicate that, generally, in inflammatory diseases, H_2S production and increased persulfidation levels exert protective roles, but their synthesis is disrupted and decreased. Our sulfur metabolome analysis showed that, in experimental AP, the serum and pancreatic tissue levels of sulfide and protein persulfidations were unchanged. Here, we should note that the detection methods for sulfide and persulfidation significantly affect the outcomes and vary across papers, which could explain the diverse results. In our experiments, DMTS increased the serum levels of sulfide and low-molecular-weight persulfides, and the rise in persulfide levels may also indicate increased H_2S production. Our observations suggest that DMTS acts as an H_2S/S^0-donor, as evidenced by elevated serum H_2S levels and increased protein persulfidation, which might contribute to the observed protective effects against oxidative or inflammatory processes.

III. The effect of ATB-346 on the severity of experimental AP

ATB-346 is a naproxen derivative with a covalently attached H_2S donor moiety, which has shown its anti-inflammatory effect on various inflammatory diseases. To confirm the potent anti-inflammatory effect of ATB-346 on AP severity, it was also utilised in a second necrotising AP model in mice induced by EtOH-POA. In line with the findings in Cer-AP, ATB-346 attenuated the severity of AP by decreasing all histological and laboratory parameters.

Based on literature data, exogenous H_2S exerts its anti-inflammatory effect by activating the antioxidant Nrf2, inhibiting the pro-inflammatory NF-κB, and suppressing the mitogen-activated protein kinase signalling pathways. Given the H_2S-donor potential of ATB-346, the mRNA expression of genes involved in oxidative stress defence were determined in our study. The expression of *HMOX1*, *SOD1*, *SOD2*, and *CAT* reflects the antioxidant defensive ability of the pancreas. ATB-346 treatments markedly increased the relative gene expression of *Hmox1*, *Sod2*, and *Cat* in a dose-dependent manner, which may contribute to its protective effect. The activation of the transsulfuration pathway, induced by genes such as *CBS*, can attenuate oxidative stress by increasing cellular GSH levels. ATB-346 significantly elevated the relative expression of *Cbs* mRNA in Cer-AP, likely due to the exogenous H_2S release. Viability assays demonstrated that ATB-346 protects acinar cells against cytotoxicity caused by H_2O_2 and menadione, confirming its antioxidant properties. During AP, in response to elevated ic[Ca^{2+}], acinar and inflammatory cells generate a significant amount of ROS. Therefore, we tested whether ATB-346 reduces intracellular ROS production and offers cytoprotection against both exogenous and endogenous ROS in primary acinar cells. AP-inducing agents (namely Cer, EtOH+POA, L-arg, and CDC) and menadione increased the intracellular ROS formation, whereas administering the necroptosis inducer aurora kinase inhibitor CCT resulted in no change in acinar ROS concentrations. ATB-346 markedly decreased the ROS production when used in combination with the aforementioned treatments, confirming that ATB-346 likely acts as a scavenger of ambient and induced ROS through H_2S release, thereby reducing oxidative damage.

As an antioxidant, ATB-346 may offer protection against ferroptosis by counteracting oxidative stress and preventing lipid peroxidation, possibly due to its H_2S-donor property and anti-inflammatory effects. Therefore, we investigated the impact of ATB-346 on the mRNA expression of genes involved in ferroptosis. *FTH1* plays a crucial role in maintaining cellular iron balance during ferroptosis and is involved in ferritinophagy, a selective form of autophagy. The administration of ATB-346 significantly elevated the relative gene expression of *Fth1* mRNA, thereby likely limiting free iron and inhibiting the formation of lipid peroxides. *TXNRD1* maintains cellular redox balance by reducing thioredoxin and serves as a key regulator of various antioxidant pathways. ATB-346 markedly increased the relative expression of *Txnrd1*, through which ATB-346 may reduce the accumulation of lipid peroxides. Lipid peroxidation products increase during ferroptosis, and catabolites such as MDA serve as valuable markers for oxidative stress. MDA concentrations were markedly increased in both Cer- and EtOH-POA-induced AP, and ATB-346 effectively decreased the MDA levels, indicating that ATB-346 mitigated the cellular damage caused by ferroptosis. GPX4 neutralises lipid peroxides and protects cells and membranes against peroxidation. It was showen that exogenous H_2S released from NaHS activates the Nrf2/GPX4/GSH pathway and suppresses ferroptosis. ATB-346 significantly increased the GPX4 protein expression, likely through the activation of the Nrf2 pathway triggered by the H_2S-releasing capacity of ATB-346. Inhibiting the GPX4/GSH antioxidant system with the ferroptosis inducer erastin (depleting GSH) or RSL 3 (inhibiting GPX4) led to cell death and increased intracellular ROS formation. ATB-346 administration effectively safeguarded acinar cells, indicating its capacity to support antioxidant defence by enhancing GSH levels and GPX4 activity, as well as independently of this pathway.

IV. Conclusion

This work provides evidence that all five tested organosulfur compounds exert anti-inflammatory and cytoprotective effects. Of the five, DMTS and ATB-346 provided significant protection against experimental AP and were selected for further investigation to gain insights into their mechanisms of action. DMTS effectively alleviated the severity of two necrotising AP models, and we demonstrated that DMTS modulates physiological Ca^{2+} signalling, reduces ROS levels, and elevates serum sulfide and persulfide levels. These effects may result from its antioxidant properties, its role as an H_2S donor, and/or its ability to decrease leukocyte infiltration and inhibit MPO activity. ATB-346 attenuated the severity of two experimental AP models and demonstrated antioxidant properties by increasing the expression of antioxidant enzymes and reducing intracellular ROS concentrations. Additionally, it increased the expression of genes involved in ferroptosis, lowered MDA levels, and protected acinar cells from ferroptosis-inducing treatments. ATB-346 exerted this effect via the enhancement of the GPX4/GSH antioxidant system and also independently from it. The release of H_2S from ATB-346's donor moiety, combined with cyclooxygenase inhibition, may account for the aforementioned effects.

Our results demonstrate that organosulfurs have anti-inflammatory and cytoprotective effects, thus making them promising therapeutic agents in the treatment of AP. Overall, organosulfur compounds are worth further investigation in this potentially lethal disease.

SUMMARIES

Summary of new findings

- All five organosulfurs (ATB-346, DMTS, DATS, GYY4137, AP39) reduced the severity of experimental AP and exerted cytoprotection
- DMTS and ATB-346 emerged as the most potent agents, and they were selected for further testing
- DMTS modulated physiological Ca^{2+} signalling and reduced the menadion-induced ROS levels
- DMTS increased serum H_2S levels and protein persulfidation, indicating H_2S donor properties
- ATB-346 upregulated pancreatic antioxidant genes (*Hmox1*, *Sod2*) and neutralised the produced ROS
- ATB-346 inhibited ferroptosis through the GPX4/GSH system, as well as by increasing the expression of *Fth1* and *Txnrd1*

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"Nothing in life is to be feared; it is only to be understood. Now is the time to understand more, so that we may fear less." — Marie Curie