# IMPROVING THE OUTCOME OF EXPERIMENTAL ACUTE PANCREATITIS: THE EFFECTS OF KYNURENIC ACID, SZR-72 AND ANALGESIA

Ph.D. Thesis

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# PUBLICATIONS

## Publications closely related to the subject of the thesis

I. 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$_{2021}$: 8.786]**, Q1

*Authors share co-first authorship

II. 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$_{2021}$: 6.208]**, Q1

## Publications related to the subject of the thesis

I. 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 effect of VX-661 + VX-770 treatment on disease severity. J Physiol. 2021; 599(22):4955-4971. doi: 10.1113/JP281765. **[IF$_{2021}$: 6.228]**, Q1

II. Kui B, Balla Z, Vasas B, Végh ET, Pallagi P, **Kormányos ES**, Venglovecz V, Iványi B, Takács T, Hegyi P, Rakonczay Z Jr. New insights into the methodology of L-arginine-induced acute pancreatitis. PLoS One. 2015; 10(2):e0117588. doi: 10.1371/journal.pone.0117588. **[IF$_{2015}$: 3.057]**, Q1

## Scientometrics

https://m2.mtmt.hu/gui2/?type=authors&mode=browse&sel=10045525&view=pubTable

| | |
|---|---|
| Number of full publications | 4 |
| Publications with first authorship | 1 |
| Cumulative impact factor | 24.279 |
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# INTRODUCTION

## The physiology of the pancreas

The pancreas is a heterocrine gland of the gastrointestinal (GI) tract. The exocrine parenchyma is mostly composed of acinar and ductal epithelial cells. It produces pancreatic juice, which consists of inactive digestive enzymes synthesized by acini and $HCO_3^-$-rich fluid secreted by ductal cells. The 1.5-2 liters of fluid per day, that is excreted into the duodenum, flushes potentially harmful enzymes out of the ductal system, preventing their premature activation. Its high $HCO_3^-$ content provides the enzymes optimal pH in the intestinal lumen. It is essential that the enzymes trypsin, chymotrypsin, amylase, lipase become active only in time, after reaching the duodenum. Once inside the lumen, enteropeptidase cleaves and activates trypsinogen to trypsin, initiating an activation cascade.

## Acute pancreatitis

### *Definition, epidemiology, classification, and etiology*

Acute pancreatitis (AP) is a sudden, sterile inflammation of the pancreas. Its mortality rate is still remarkably high and the annual incidence is approximately 10-45/100,000 population and this is rising slightly in developed countries up to this day.

In 2011, the revised Atlanta classification categorized AP into 3 groups based on severity: mild, moderately severe, and severe. About 75% of the cases are mild, 15% are moderate and 10% are severe. The mortality of severe AP can reach up to 30%.

Etiological factors of AP can be divided into four main categories. 1: toxic and metabolic disorders (alcohol consumption, hyperlipidemia, hypercalcemia). 2: mechanical causes (gallstones, papilla dysfunction, congenital malformations, trauma). 3: genetic causes (mutations in the trypsinogen gene). 4: other (infections, unknown causes). In about 40% of cases, biliary obstruction is observed, whereas ethanol abuse is the cause in 30%.

### *Pathogenesis*

The exact pathogenesis of AP remains to be unclear. Several steps in the early stages of AP have already been described: nuclear factor-kappa B (NFκB) activation caused by toxic intracellular $Ca^{2+}$ overload; mitochondrial dysfunction and damage; impaired autophagy; reactive oxygen species (ROS) release; inhibition of digestive enzyme secretion and drop in intracellular adenosine triphosphate (ATP) levels; premature trypsinogen activation; decreased ductal $HCO_3^-$ and fluid secretion; pancreatic ductal cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction, and reduced GI blood flow. Some of these events lead to the release of tumor necrosis factor-α (TNF-α), cytokines [e.g. interleukin-1β (IL-1 β)] and chemokines, which participate in leukocyte recruitment. Neutrophil garnulocytes are the first immune cells reaching the pancreatic parenchyma. These cells further activate trypsinogen in acinar cells, release inflammatory cytokines and chemokines, secrete myeloperoxidase (MPO) and ROS [e.g. hydrogen peroxide ($H_2O_2$)], all of which contribute to the exacerbation of AP in a vicious circle.

Several protective factors and mechanisms are responsible for keeping digestive enzymes inactive within the pancreas, or washing out those that are already activated: α1-antitrypsin, α2-macroglobulin,

serine protease inhibitor Kazal type 1 (SPINK1), autolysis of prematurely activated trypsin, and so on. However, as soon as the rate of enzyme activation exceeds the capacity of the defense mechanisms, the process shifts towards self-digestion. In addition, trypsin also plays a pathophysiological role in activating the complement system, fibrinolysis, and coagulation, helping the disease to spread beyond the pancreas. The vascular endothelium is also affected leading to impaired microcirculation and increased permeability. This allows the release of free radicals, proinflammatory cytokines, and proteolytic enzymes causing thrombosis, tissue bleeding, and ultimately necrosis. In patients with AP, cytokines and enzymes that are initially present only in the pancreas may enter the circulation and play a critical role in inducing systemic inflammatory response syndrome. Eventually, this allows inflammation of the pancreas to cross the boundaries of its original organ and cause acute respiratory distress syndrome, kidney failure, metabolic disorders, shock, and multiple organ failure. Finally, if that was not enough, damage to the intestinal mucosal barrier creates an opportunity for superinfection of the necrotized pancreas.

But how can it be predicted that a patient's pancreatitis will not remain mild but will become severe? Can we intervene somewhere in this chain of events to save a patient's life? The answer lies in the detailed understanding of the cellular events that take place during AP and the development of an effective cure.

*Treatment and pain management*

Unfortunately, there is still no specific cure for AP, the therapeutic arsenal has not expanded much in recent decades, and predominantly conservative treatment is still used today. Fluid and electrolyte replacement should be started within the first 24 hours. If the AP is not mild, it is recommended to relieve the pancreas with a nasojejunal tube. In this way, the barrier integrity of the intestinal mucosa can be maintained, thus preventing bacterial translocation. The need for further treatments depends on the complications of the disease.

The most common symptom that affects almost all patients is abdominal pain of varying intensity. Non-steroidal anti-inflammatory drugs and opioids can be used intravenously to relieve pain. However, to achieve a continuous effect it is advisable to apply a pump instead of a bolus, and patient-controlled administration is even better. Morphine and its derivatives are often avoided as they can cause sphincter of Oddi spasm. Thoracic epidural anesthesia may be considered in patients with pain that is difficult to tolerate. The advantage of this method is not only to effectively alleviate pain, but also to improve the perfusion of the GI tract. Despite all this knowledge, there is still no widely accepted recommendation for analgesia, and its effect on AP progression is unclear.

Based on our experimental results and literature data, we decided it was worth investigating the effect of analgesia on experimental AP more closely. In our work, we tested different analgesics, including buprenorphine (BQ), hoping to gain a better insight into pancreatitis from the perspective of pain relief.

**Kynurenic acid**

Tryptophan is an essential amino acid that can only be obtained through diet. The majority (95%) of L-tryptophan is metabolized via the kynurenine pathway. As the first step, L-tryptophan is converted to N-formyl-L-kynurenine by indoleamine-deoxygenase or tryptophan-deoxygenase. The expression of these two enzymes is enhanced by inflammation and stress [IL-1β, interferon-γ (IFN-γ), TNF-α, cortisol].

From here, (1) kynurenine aminotransferases convert L-kynurenine to kynurenic acid (KYNA), or (2) L-kynurenine is broken down to 3-hydroxy-L-kynurenine (3-HK) and then in several steps to nicotinamide adenine dinucleotide ($NAD^+$).

KYNA acts as an antagonist at three ionotropic glutamate receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate. The distribution of these receptors in the exocrine pancreas is unknown. Although KYNA is commonly referred to in connection with the central nervous system, it is also abundant in the GI tract, including pancreatic juice. Furthermore, KYNA is a ligand for the G protein-coupled receptor 35 (GPR35), which has been found to be predominantly expressed in the GI tract and immune cells. Although the detailed biochemistry and physiological role of GPR35 are not yet fully understood, it appears to play an important role in immunological processes and in the maintenance of intestinal homeostasis.

**The role of KYNA in pathological conditions**

Tryptophan-kynurenine pathway metabolites are important participants in cellular processes, especially in neuronal cells. They have numerous effects on both innate and adaptive immune responses. KYNA, as an antagonist of the NMDA receptor (NMDAR), has neuroprotective effects. It reduces ischemia/reperfusion-induced retinal ganglion cell death as well. KYNA is also closely associated with intestinal inflammation. Patients with irritable bowel syndrome showed elevated mucosal and decreased plasma KYNA levels. It is important to note that NMDAR antagonists are a separate class of analgesics used in clinical care.

Recently, several studies have investigated SZR-72, the synthetic derivative of KYNA. The blood-brain barrier is impermeable to KYNA but SZR-72 can directly cross it. SZR-72 effectively modulates mitochondrial respiration, while KYNA could restore microcirculation in sepsis. Both KYNA and SZR-72 suppress pro-inflammatory factors released by mononuclear cells and neutrophils e.g. TNF-α, high mobility group box protein 1, and human neutrophil peptide 1–3. KYNA and SZR-72 improve intestinal hypermotility and reduce inflammatory parameters in rat colitis through antagonism of NMDAR.

Metabolites and disturbances of the kynurenine pathway influence the severity of AP. 3-HK generates free radicals and causes cytotoxicity, while KYNA inhibits inflammation, prevents lipid peroxidation and ROS generation. 3-HK concentration is increased during AP in human samples and its plasma levels correlate with the progression of systemic inflammation and the severity of AP. The inhibitors of kynurenine-3-monooxygenase reduce the production of 3-HK. Application of such an inhibitor prevented multiple organ failure in experimental AP in rodents. In contrast to 3-HK, the effect of endogenous KYNA or its synthetic derivative, SZR-72 on AP is unknown.

The GPR35 receptor should also be mentioned, as KYNA is its most potent endogenous ligand and its role has been described in several processes. It is strongly associated with type 2 diabetes, ulcerative colitis, and cardiovascular diseases. Furthermore, KYNA stimulates the energy utilization of adipocytes by activating GPR35 and thus inducing lipid metabolism and anti-inflammatory gene expression.

Moreover, KYNA has an antinociceptive effect via GPR35. As we can see, although we know more and more about the molecules and receptors of the KYN pathway, their role in AP remains to be discovered.

## AIMS

Since there is still no specific cure for AP, the aim of this study was to find a possible starting point for future therapy, and at the same time, get closer to understanding the development and progression of AP.

1. According to the literature, KYNA and its derivative, SZR-72, have demonstrated anti-inflammatory properties in several diseases. It was therefore considered worthwhile to investigate their effect on the outcome of AP.

   Our specific aims were to investigate:

   - the effects of KYNA/SZR-72 on experimental AP and pancreatic acinar cells *in vitro*
   - whether KYNA and SZR-72 act through the NMDAR

2. Pain management is essential in patients with pancreatitis. However, we do not know exactly how pain relief affects the outcome of the disease. Our specific aim was to investigate the effect of the partial opioid agonist buprenorphine on experimental AP.

## MATERIALS AND METHODS

### Materials

All chemicals were purchased from Sigma-Aldrich (Budapest, Hungary) unless indicated otherwise. SZR-72 [2-(2-N, N dimethylaminoethyl-amine-1carbonyl)-1H-quinolin-4-one hydrochloride] was synthesized by the Institute of Pharmaceutical Chemistry (University of Szeged, Hungary). L-ornithine-HCl (LO, 300 mg/ml), KYNA (50 mg/ml), and SZR-72 (50 mg/ml) were dissolved in physiological saline (PS) and the pH of the solutions was adjusted to 7.35-7.4.

### Animals

For the KYNA/SZR-72 experiments, male Sprague-Dawley rats weighing 200-250 g were used, and the analgesia (BQ) was tested on female Wistar rats of the same weight. Our experiments were executed according to the European Union Directive 2010/63/EU and the Hungarian Government Decree 40/2013 (II.14.). Experiments were approved by both local (University of Szeged) and national ethics committees (X/3353/2017.) for investigations involving animals.

### *In vivo* experiments: induction of AP, treatments and tissue harvesting

Necrotizing AP was induced by a single intraperitoneal (i.p.) injection of 3 g/kg LO in the morning. KYNA or SZR-72 was administered 1 h before the induction of AP as a single i.p. injection (75, 150, 300 mg/kg). Control animals were treated with PS instead of LO, KYNA, and/or SZR-72.

Buprenorphine (BQ) was administered via two routes: i.p. and intrathecally (i.t.). For i.t. administration, a catheter was inserted via the cisterna magna into the subarachnoid space 8.5 cm deep

caudally. Thus, the catheter tip was placed between vertebrae Th12 and L2, at the height of the spinal segments that innervate the hind legs. Rats that showed postoperative neurological deficits or whose hind legs were not paralyzed after administration of 100 μg lidocaine were excluded (about 10%). Due to its prolonged analgesic effect, the recommended dose interval for BQ is 8 to 12 h. I.t. injections of 3 and 6 μg/kg BQ were given 1 h before LO and were repeated at 7 and 12 h after AP induction. 10 μL of BQ was infused through the catheter for 120 s, followed by a 10 μL flush of PS. I.p. injections of 0.1, 0.5, and 1 mg/kg BQ were given 1 h before and 12 h after the LO administration.

Animals were sacrificed 24 h after the LO injection by deep anesthesia with 45 mg/kg i.p. pentobarbital (Bimeda MTC, Cambridge, Canada). Blood was collected via cardiac puncture, then the pancreas was rapidly removed. Pancreatic tissue was cleaned from fat and lymph nodes on ice, then cut into pieces. One large piece was immediately frozen in liquid nitrogen and stored at –80 °C until biochemical assays were performed. Another piece of the pancreas was fixed in an 8% neutral formaldehyde solution for histological analysis. The third piece was stored in Eppendorf tubes for dry-wet weight measurement at room temperature. The last piece was frozen in cryomatrix for sectioning and immunofluorescent stainings. Blood samples were centrifuged at 2500 RCF for 15 min at 4 °C, sera were collected and stored at −20 °C until use. After LO administration, animals showed signs of sickness and became sluggish as expected. However, a few of them got depressed and lethargic within 12 h after the LO injection. The core temperature of these animals was monitored with a rectal digital thermometer. Once it decreased to a critical level (27–29 °C), rats were euthanized by pentobarbital overdose (200 mg/kg i.p.) to minimize suffering. The percentage of euthanized rats was 3% in the LO-treated groups.

**Histological analysis**

Formalin-fixed pancreatic tissue samples were sectioned to 3 μm, then stained with hematoxylin and eosin. Samples were analyzed and scored by two experts blinded to the experimental protocol. Semiquantitative scoring method was used to evaluate edema, leukocyte infiltration, and the percentage of acinar cell necrosis.

**Laboratory measurements**

Serum amylase activity was measured with a colorimetric kinetic method. The wet weight (WW) and dry weight (DW) of the pancreas were measured and pancreatic water content was calculated: [(WW−DW)/WW]×100. Pancreatic MPO activity was measured according to Kuebler et al., and was normalized to total protein content evaluated by the Lowry method. Pancreatic IL-1β levels were measured by a commercial ELISA kit from R&D Systems (Minneapolis, MN, USA) as described by the manufacturer. Blood pH, $HCO_3^-$, and partial pressure of $CO_2$ ($pCO_2$) from femoral arterial blood samples were measured with a blood gas analyzer. Pancreatic heat shock protein 72 (HSP72) expression was measured from tissue homogenate using Western blot analysis.

**Measurement of hemodynamics and pancreatic microcirculation**

Animals were anesthetized with sodium pentobarbital (50 mg/kg) i.p. 24 h after the injection of LO. A thermistor-tip catheter was placed into the ascending aorta to measure cardiac output by thermodilution

technique. The left common carotid artery was dissected free and an ultrasonic flow probe was inserted around it to measure carotid artery flow. Carotid artery flow and pressure were measured continuously and registered with a computerized data acquisition system. After median laparotomy, the pancreas was placed on the detector from the abdomen without disturbing the circulation. The microcirculation of the pancreas was visualized with intravital orthogonal polarization spectral imaging technique and represented as red blood cell velocity (RBCV).

**Total RNA isolation and reverse transcription polymerase chain reaction**

Total RNA was isolated from the control rat brain cortex and pancreas and 1 μg RNA from each sample was transcribed to complementary DNA. Gene-specific and exon/exon junction spanning oligonucleotide primer pairs were designed with The Universal Probe Library Assay Design Center (Merck KGaA, Darmstadt, Germany). Primers for hypoxanthine phosphoribosyltransferase (HPRT) gene were used as loading control. PCR was performed with DreamTaq DNA Polymerase (Thermo Fisher) in BioRad C1000 ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA). Products were analyzed on 3% MetaPhor agarose gel (Lonza, Basel, Switzerland), then isolated fragments were sequence-verified by capillary DNA sequencing.

**Immunofluorescent stainings for NMDAR and amylase**

Pancreata embedded in cryomatrix were cut into 7 μm thick slices. Pancreatic sections were incubated overnight with anti-NMDAR1 rabbit monoclonal antibody (1:100, ThermoFisher Scientific, Waltham, USA). The following day, Alexa Fluor 568 goat anti-rabbit secondary antibody was added (1:500). After that, co-immunostaining was performed with anti-amylase mouse monoclonal antibody (1:200) and Alexa Fluor 488 goat anti-mouse secondary antibody (1:500). Nuclei were counterstained with 2.5 μg/ml Hoechst 33342. After mounting, slides were stored at 4°C until visualizing with confocal microscopy (ZEISS LSM 880), and images were processed with ImageJ software (NIH, Bethesda, MD, USA).

**Pancreatic acinar cell isolation and viability measurement**

Rat pancreatic acinar cells were isolated with collagenase digestion technique according to Pandol et al. After digestion, acinar cells were washed three times, then resuspended in Medium 199 solution and placed in 37°C $CO_2$ incubator for 15 min. Acini were used for experiments immediately thereafter.

Isolated pancreatic acinar cells were placed into a 96-well plate and 1 μM propidium-iodide was added to each well. Fluorescence intensity was measured at excitation and emission wavelengths of 540 nm and 620 nm with a Fluorostar Optima plate reader every 5 min. The 300 mg/kg dose of KYNA, used in the *in vivo* experiments, was converted to an equimolar concentration (250 mM). After intensity stabilized, the cells were treated with 20 mM LO, 25-2500 μM KYNA/SZR-72/NMDA according to the experimental protocol. At the end of the experiment, Triton X-100 was added to each well to kill every living cell. The intensity measured at this point was considered to represent 100% toxicity. Data were evaluated by selecting minimum (MIN) and maximum (MAX) intensities in each treatment-group. The percentage of cell death at each time point was calculated using the following formula: [(intensity-MIN)/(MAX-MIN)] ×100.

**Neutrophil granulocyte isolation and measurement of $H_2O_2$ production**

Neutrophils were isolated from rats treated with PS, LO or LO + 300 mg/kg SZR-72 24 h after AP induction using Ficoll-Hypaque density gradient centrifugation. Blood was collected in EDTA coated tubes from each animal and was gently mixed with an equal volume of 3% Dextran solution and left to sediment for 40 min. The leukocyte-rich plasma was carefully added on top of Ficoll-Hypaque. After centrifugation polymorphonuclear and red blood cell pellet was obtained. Erythrocytes were lysed with 0.2% NaCl solution. Granulocytes were resuspended in phosphate-buffered saline containing 10 mM glucose. The cell number was adjusted to $1.5\times10^4$/100 µl. $H_2O_2$ production was measured with Fluorostar Optima plate reader (BMG Labtech, Ortenberg, Germany) using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit described by the manufacturer.

**Measurement of IL-1β production in isolated acinar cells**

Isolated pancreatic acinar cells were placed into 6-well plates and treated with medium, LO (20 mM), KYNA (250 µM), SZR-72 (250 µM) or with the combination of LO and KYNA/SZR-72 for 6 h. After adding 200 µL homogenization buffer to a well, cells were scratched from the bottom. Two wells were pooled into one microcentrifuge tube. After sonication, IL-1β levels were measured from the homogenate by a commercial ELISA kit from R&D Systems as described by the manufacturer.

**Statistical analysis**

Data are presented as means ± SEM. Experiments were evaluated by one-way ANOVA followed by Holm-Sidak post hoc test or two-way ANOVA followed by Bonferroni post hoc test (SPSS, IBM, Armonk, NY, USA). $P < 0.05$ was accepted as statistically significant.

## RESULTS

**Dose-dependent effects of KYNA and SZR-72 on the severity of AP**

Three different doses of KYNA were tested to determine its effects on LO-induced AP. Administration of LO alone induced necrotizing AP, whereas 300 mg/kg KYNA significantly reduced LO-induced pancreatic tissue injury. A marked increase of pancreatic edema was detected in the LO-treated group compared to control, while the highest dose of KYNA (300 mg/kg) significantly reduced it. Leukocyte infiltration into the pancreas and tissue MPO activity also significantly increased in the AP group compared to control. As seen in case of edema, the 300 mg/kg dose of KYNA significantly reduced both leukocyte infiltration and MPO activity during AP, whereas lower doses of KYNA were ineffective. The most important measure of pancreatic inflammation is tissue damage (necrosis), which was highly severe as a result of LO treatment, but was significantly reduced in the 300 mg/kg KYNA group. Serum amylase activity also increased in the LO group, while 150 and 300 mg/kg KYNA significantly reduced the enzyme activity. Overall, the two lower doses (75 and 150 mg/kg) of KYNA did not significantly influence most of the measured values, but 300 mg/kg KYNA reduced the severity of AP.

Similar results to KYNA were obtained when the effect of different doses of its analogue, SZR-72 was examined. The 300 mg/kg dose of SZR-72 was able to significantly reduce the AP-evoked increases in pancreatic edema and leukocyte infiltration. These results were also supported by measurements of pancreatic water content and MPO activity. The score of pancreatic tissue damage was significantly reduced by the highest dose of SZR-72 in AP. Serum amylase activity increased in response to LO injection, which was decreased by all SZR-72 doses. Overall, AP severity parameters were reduced by 300 mg/kg SZR-72 treatment.

**The effects of KYNA and SZR-72 treatment on microcirculation and hemodynamic parameters in AP**

Microcirculatory and hemodynamic changes during AP and KYNA/SZR-72 treatment (300 mg/kg) were measured. AP significantly increased cardiac output and carotid artery flow compared to the control animals. Cardiac output in rats with AP was reduced to control level by both KYNA and SZR-72, whereas the decrease in carotid artery flow was significant only in the case of SZR-72. Mean arterial blood pressure was similar in all experimental groups. Pancreatic microcirculation was quantified by measuring serosal RBCV. Interestingly, microcirculation significantly decreased in LO-induced AP compared to the control group. However, pre-treatment with KYNA or SZR-72 was able to improve microcirculation during AP. AP also caused a significant drop in arterial blood pH and bicarbonate concentration, resulting in metabolic acidosis, which was restored to the control level by KYNA and SZR-72 pre-treatments as well. At the same time, there was no detectable difference in arterial $pCO_2$ between the examined groups.

**Changes in pancreatic IL-1β and HSP72 expression in AP upon KYNA and SZR-72 treatment**

KYNA and SZR-72 alone did not affect the pancreatic IL-1β content. However, the amount of IL-1β was significantly increased in the LO group compared to controls. In animals with AP treated with KYNA or SZR-72, the amount of IL-1β was reduced to the level of control. We examined how KYNA and SZR-72 treatment affects pancreatic HSP72 levels in physiological conditions and during AP. KYNA and SZR-72 alone significantly increased HSP72 expression compared to the control group, and SZR-72 had a more prominent effect on HSP72 protein expression than KYNA. In our experiments, it was clear that the level of HSP72 was elevated in AP compared to the control group. However, when the animals also received KYNA or SZR-72 pre-treatment, the amount of HSP72 significantly increased even compared to the AP group without KYNA or SZR-72.

**The expression of NMDAR1 and GPR35 in the pancreas**

NMDAR1 expression was investigated by RT-PCR and immunohistochemistry, GPR35 expression was examined by RT-PCR. In both methods, brain tissue was used as a positive control. *Gpr35* gene expression was nearly equal in the brain and pancreas, while *nmdar1* expression was much lower in the pancreas than in the brain. This was also confirmed by immunohistochemistry, where NMDAR1 staining of the brain was clearly visible. The image of the control pancreas showed low NMDAR1 expression with well-structured amylase staining. The pancreas was sampled 2 and 24 h after LO administration in order to visualize if there was a difference in NMDAR1 staining depending on how advanced the inflammation was. NMDAR1 staining was found to be more pronounced 2 h after AP induction, however,

the strongest staining was observed after 24 h. In parallel, amylase staining lost its structural integrity as the inflammation progressed.

***In vitro* effect of KYNA, SZR-72, and NMDA on LO-induced acinar toxicity**

The effects of KYNA and SZR-72 were investigated on LO-induced acinar toxicity in *in vitro* experiments. Since both compounds are NMDAR antagonists, NMDA was also applied to reveal whether KYNA or SZR-72 exert their effect on NMDAR. Before testing, the protective properties of KYNA and SZR-72, or their interaction with NMDAR, the safe concentrations of KYNA, SZR-72, and NMDA were determined on isolated pancreatic acinar cells. SZR-72 can be administered safely up to a concentration of 500 μM, and concentrations above this were toxic to acinar cells. As the 300 mg/kg dose of KYNA proved to be effective *in vivo*, the corresponding, equimolar (250 μM) and ten times higher (2500 μM) concentrations of KYNA and NMDA were tested on acini. In case of SZR-72, only the 250 μM concentration was used in further viability studies because the ten times higher concentration has been already proven to be toxic. Neither KYNA nor NMDA affected pancreatic acinar viability even at a concentration of 2500 μM.

Then we measured the effect of LO treatment on cell viability and whether it could be affected by KYNA, SZR-72, or NMDA. LO was shown to be highly toxic to pancreatic acinar cells. However, KYNA prevented the toxic effect of LO at both 250 and 2500 μM concentrations and cell viability was comparable to the control group. Treatment with 250 μM SZR-72 also significantly reduced LO-induced toxicity. NMDA did not affect the toxicity of LO at any concentrations. Last, we examined whether the beneficial effects of KYNA and SZR-72 could be suspended by the addition of NMDA. Besides LO, acinar cells were subjected to 250 μM KYNA or SZR-72 and increasing doses of NMDA (25, 250, 2500 μM). Co-treatment with NMDA had no effect on cell viability. KYNA and SZR-72 were still able to significantly reduce toxicity compared to the LO group. Moreover, KYNA treatment reduced cellular toxicity to a level that was equivalent to the control group.

**SZR-72 reduces $H_2O_2$ production in isolated neutrophil granulocytes but does not affect the IL-1β expression of pancreatic acinar cells**

Neutrophil granulocytes play an important role in the development of AP. Their function is characterized by their $H_2O_2$ production. The effect of SZR-72 was determined on neutrophil granulocyte function. $H_2O_2$ production of granulocytes was examined after cell isolation from control, LO-, and LO + SZR-72-treated animals. In case of control granulocytes, $H_2O_2$ production remained at baseline throughout the experiment. In contrast, neutrophils from AP animals produced increased amounts of $H_2O_2$, the level of which was significantly higher than the control group from as early as 20 min. However, when neutrophils from LO and SZR-72 co-treated animals were examined, a significant decrease was observed in $H_2O_2$ production from 70 min compared to LO treatment alone. The IL-1β protein expression of isolated acinar cells was measured *in vitro* after 6 h treatment with LO, KYNA, and/or SZR-72. LO administration did not induce any change in IL-1β expression compared to the control group in acinar cells. Furthermore, KYNA, SZR-72, or their combinations with LO did not affect the proinflammatory cytokine production.

**BQ administration has no significant effect on the severity of AP**

Two administration routes were investigated for BQ (i.p., i.t.). BQ alone did not cause histological abnormalities in the pancreas. I.p. BQ in the tested doses (2 × 0.1, 2 × 0.5, 2 × 1 mg/kg) did not affect the LO-induced cell damage and leukocyte infiltration in the pancreas, or amylase activity in the serum. However, the water content of the pancreas was slightly increased by the 2 × 1 mg/kg dose of BQ. For the i.t. route of administration, 3 × 3 mg/kg BQ did not affect any of the measured parameters, whereas the higher, 3 × 6 mg/kg dose significantly reduced leukocyte infiltration.

## DISCUSSION

As AP is a disorder without specific therapy, it is important to find possibilities for its management. The pathophysiology of the disease involves multiple cell types and processes. The pathway of tryptophan metabolism is unambiguously disturbed during AP, resulting in overactivation of the kynurenine-3-monooxygenase enzyme and excess production of pro-inflammatory 3-HK. In this study, we tested the possible application of endogenous tryptophan pathway metabolite KYNA, and its synthetic derivative SZR-72 for the treatment of experimental AP. Our novel findings with KYNA or SZR-72 administration in experimental AP are the following: They (1) dose-dependently reduced the severity of the disease; (2) reduced the pro-inflammatory cytokine IL-1β expression *in vivo*; (3) increased the synthesis of HSP72; (4) reduced the extent of metabolic acidosis; (5) restored pancreatic microcirculation; (6) suppressed the function of neutrophil granulocytes. (7) In addition, their effect was likely to be independent of acinar NMDAR. SZR-72 can cross the blood-brain barrier, while KYNA is poorly permeable. Therefore, SZR-72 can exert its effect on the central nervous system as well. As the results with SZR-72 and KYNA were similar, we do not think that the possible central nervous system effects of SZR-72 play part in the protection against AP. We demonstrated that the 300 mg/kg dose of KYNA and SZR-72 exerted strong anti-inflammatory effects. Csáti et al. also used the same dose of KYNA or kynurenic acid amide 2 i.p. in rats, and observed successful suppression of inflammation evoked by trigeminal ganglion activation. Similar results were obtained when SZR-72 was administered in a trigeminal nerve activation model at a dose of 300 mg/kg i.p. in rats, which had an anti-inflammatory effect. In the case of experimental colitis in rats, even doses ten times lower have been shown to be effective. Furthermore, Juhász et al. successfully applied KYNA (2 × 15 mg/kg) and SZR-72 (2 × 23.5 mg/kg) in a sepsis model. Based on these results, it appears that the effective dose of KYNA and SZR-72 is also dependent on the disease model itself.

Our results demonstrate that during AP, in order for an animal to be able to maintain the blood supply to vital organs, i.e., mean arterial pressure, it must significantly increase its cardiac output. The most important means to do this is to increase heart rate, as total peripheral resistance cannot be adequately increased due to the inflammatory milieu caused by pancreatitis. However, when the animals were also treated with KYNA or SZR-72 in addition to LO, mean arterial pressure did not decrease even at normal cardiac output. That is, the autonomic nervous system did not have to intervene to maintain circulation.

This suggests that KYNA and SZR-72 prevent life-threatening hemodynamic imbalance caused by pancreatitis. It is likely to provide protection against the release of such an amount of pro-inflammatory mediator and cytokine that would lead to the severe intravascular volume depletion, vasodilation, and hypoperfusion expected in AP. In addition to restoring total peripheral resistance, KYNA/SZR-72 treatment may play a role in reducing heart rate. Similar findings were found by Badzynska et al. in spontaneously hypertensive rats, where 25 mg/kg/day KYNA treatment reduced heart rate. Pain may serve as an explanation, as pain is an unavoidable symptom of AP and is directly responsible for the increase in heart rate. As NMDA antagonists are a distinct class of analgesics, one obvious explanation for our *in vivo* results may be the analgesic effect of KYNA and SZR-72. GPR35 receptor is considered important for nociceptive transmission, and through this receptor, KYNA could reduce pain, which could contribute to the reduced heart rate.

This is partly what gave us the idea to investigate the effect of analgesia in experimental AP. Opioids are commonly used for pain management in patients with AP, although their effect on AP progression is not yet fully understood. Our experiments examined the effect of the partial opioid receptor agonist BQ in LO-induced necrotizing AP. No substantial adverse complication was observed as an effect of BQ; only the pancreatic tissue water content was increased after the highest i.p. dose. The lower i.t. dose left all the measured parameters unchanged and did not affect the severity of the disease. However, the higher i.t. dose significantly reduced leukocyte infiltration. We first demonstrated the effect of spinally applied BQ on AP. BQ given intravenously in a Na-taurocholate-induced model did not influence the severity of pancreatitis, and when administered subcutaneously in cerulein-induced AP, it reduced the zymogen content and protein synthesis of pancreatic acini. Both our results and literature data support that BQ may be a beneficial analgesic in AP.

AP causes the impairment of both pancreatic and systemic microcirculation, which are among the early signs of AP. We showed significantly decreased RBCV in the pancreas during experimental AP, which was remarkably restored by the administration of KYNA and SZR-72 as well. The reduced organ microcirculation contributes to ischemia and organ failure, not just in the pancreas but in other organs like the kidneys or lungs. Therefore, KYNA or SZR-72 can alleviate the symptoms of multiple and/or persistent organ failure which is present in the severe form of the disease. Furthermore, Zhang et al. found that a decrease in intestinal microcirculation secondary to severe AP can lead to reduced mucosal barrier integrity and immunity, thus increasing the possibility of infection, sepsis, and mortality. Based on this, the beneficial effect of KYNA and SZR-72 on microcirculation is important and should be further investigated. Interestingly, KYNA was found to improve ileal microcirculation in a sepsis model, whereas SZR-72 was ineffective. However, in this model, SZR-72 improved mitochondrial respiration, resulting in better conversion of ADP to ATP. In the present research, the study of mitochondrial function was out of focus. Nevertheless, mitochondrial dysfunction is common in AP and has serious consequences, so further studies are needed to explore how KYNA or its derivatives modulate it.

AP is often accompanied by acid-base disturbance, and there is an association between the severity of AP and metabolic acidosis. Meta-analysis of clinical trials confirmed that the severity of AP is related

to the degree of metabolic acidosis. In addition, experimental AP exacerbated pre-existing acid-base imbalance. Several mechanisms trigger metabolic acidosis during AP: loss of bicarbonate-rich pancreatic juice through pancreatic fistula or drainage; lactic acidosis due to shock or sepsis. An important observation in the present study was that both KYNA and SZR-72 were effective in restoring decreased plasma pH and $HCO_3^-$ concentration. The exact mechanism by which they affect acid-base balance is unknown, but this action may also contribute to reducing the severity of the disease.

HSP72 is an inducible chaperone that is upregulated in the cell under stressful conditions, such as inflammation. It was found earlier that thermal stress-induced HSP72 increase could protect against AP, and pharmacological induction of HSP72 with BRX-220 also significantly improved the outcome of experimental AP. Furthermore, overexpression of HSP72 in transgenic mice enhanced recovery from AP. In our study, we demonstrated not only LO-induced pancreatitis increased greatly HSP72 expression, but also KYNA and SZR-72 treatment alone without AP. However, when animals with pancreatitis were pretreated with KYNA or SZR-72, HSP72 expression was even far more elevated. SZR-72 was a considerably more potent HSP72-inducer than KYNA. The effect of KYNA and SZR-72 on HSP72 may be one of the mechanisms by which they protect against AP.

NMDAR1 was expressed in pancreatic tissue even under physiological conditions. Surprisingly, NMDAR1 protein expression was increased by the progression of AP. This phenomenon could be explained by three reasons: (1) pancreatic cells (e.g. acinar, ductal, beta cells) increased their expression of NMDAR1; (2) invading leukocytes express the receptor; (3) the previous two together. Therefore, we examined whether KYNA or SZR-72 exerts its effect through NMDAR. *In vitro* LO-toxicity measurements of acinar cells showed that the observed protection of KYNA or SZR-72 was unlikely to be related to NMDAR1. The receptor agonist NMDA did not suspend the effects of the receptor antagonist KYNA and SZR-72 even at ten times higher concentrations. Consequently, the protection observed in LO-AP may be a direct effect or an indirect one through another receptor, such as GPR35. KYNA is an endogenous antioxidant, and it can decrease ROS release evoked by AP. GPR35 receptor is also present in macrophages, eosinophil and basophil granulocytes, mast cells, natural killer T cells, and several cells along the digestive tract. GPR35 activation will result in decreased intracellular $Ca^{2+}$ and cyclic adenosine monophosphate signals; inhibition of phosphatidylinositol 3-kinase/protein kinase B and mitogen-activated protein kinase pathways. All these effects of KYNA-GPR35 interaction may contribute to immunosuppression. In our experiments, it was not our aim to investigate the GPR35-mediated effects of KYNA and SZR-72 in AP, but this is an issue worth further research.

KYNA or SZR-72 markedly reduced the pancreatic IL-1β expression *in vivo*. However, when isolated acinar cells were tested *in vitro*, KYNA and SZR-72 did not affect IL-1β levels. Therefore, it appears that the IL-1β reducing effect of KYNA and SZR-72 is independent of acinar cells. Therefore, the tested compounds most probably affect leukocytes, and this can result in decreased cytokine release from the pancreatic tissue. Neutrophil granulocytes are the first inflammatory cells reaching the pancreas during AP. ROS such as $H_2O_2$ is produced in large quantities by neutrophils which reflects the activity of these cells. Our results showed that SZR-72 treatment reduced the $H_2O_2$ production of neutrophil

granulocytes isolated from animals with LO-induced pancreatitis. Since neutrophils contribute to AP by amplifying the inflammatory cascade, the reduced activity of these cells by KYNA and SZR-72 is also beneficial and can contribute to their mechanism of action.

## CONCLUSION

We confirmed the expression of NMDAR in the acini of the exocrine pancreas. We showed that the endogenous tryptophan metabolite, KYNA and its synthetic analogue, SZR-72 reduced the severity of experimental AP. Both provide dose-dependent protection against LO-induced AP and *in vitro* acinar cell toxicity, which action is most likely independent of pancreatic NMDARs. There may be several mechanisms mediating this protective effect. Both KYNA and SZR-72 improved acid-base balance, cardiovascular parameters and pancreatic microcirculation during AP. SZR-72 also reduced the elevated $H_2O_2$ production of neutrophil granulocytes isolated from rats with AP suppressing their activation. KYNA and its synthetic derivative affectes the amount of cytokines (reduced the expression of pancreatic IL-1β), the body's self-defense mechanism (enhanced the expression of pancreatic HSP72), and leukocyte function, demonstrating an immunomodulatory role in experimental AP. Overall, the administration of these molecules could be beneficial in AP.

In addition, we have also shown that BQ treatment can slightly reduce the severity of experimental AP, but its effect is greatly dependent on the dose, timing, and route of administration, suggesting that a well-planned strategy of pain management is crucial.

## SUMMARY OF NEW FINDINGS

- KYNA and SZR-72 administration dose-dependently reduce the severity of LO-induced experimental AP
- KYNA and SZR-72 restored hemodynamic parameters and pancreatis microcirculation during LO-induced experimental AP
- NMDAR1 protein and mRNA expression was detected in pancreatic acinar cells
- KYNA and SZR-72 reduce LO-induced toxicity in isolated pancreatic acinar cells *in vitro*
- SZR-72 reduce *in vitro* $H_2O_2$ production of neutrophil granulocytes isolated from rats with AP
- The beneficial effects of KYNA and SZR-72 are probably independent of pancreatic NMDARs
- I.t. administration of BQ significantly reduced pancreatic leukocyte infiltration during LO-induced experimental AP

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**"If we knew what we were doing, it would not be called research, would it?" –Albert Einstein**