Short Thesis for the Degree of Doctor of Philosophy (PhD)

Investigation of Herbal Ingredients in a Mouse Model of Acute Pancreatitis

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INTRODUCTION

The pancreas is dual-function organ, which plays an essential role in sugar metabolism as an ednocrine gland and in the process of digestion as an exocrine gland. The two predominant cell types of the **exocrine pancreas** are the acinar cells, which clusterto form acini and specialise in the synthesis, storage (in zymogen granules) and secretion of digestive enzymes, with an extensive endoplasmic reticulum (ER); and the ductal cells that form ducts to collect the secretion and drain that into the duodenum. Inflammatory diseases of the organ (acute and chronic pancreatitis) are mainly caused by defects in the functionality of these cell types and by obstruction of the pancreatic ductal drainage.

In physiological processes, activation of digestive enzymes secreted mostly as inactive pro-enzyme takes place after their arrival in the duodenum. **Premature activation of enzymes**, whether it happens in the acinar cells, in the intercellular space or in the ductal system, damages the pancreatic tissue. In the process, the enzymes begin to digest the tissue, causing acute pancreatitis. The two most common causes of the disease are gallstones and heavy alcohol consumption. Gallstones can clog the common bile duct, preventing the bile from leaving the pancreas, and can cause bile to flow into the pancreatic ducts. Another common trigger is heavy alcohol consumption. The exact molecular background of the mechanism is still unclear, but complex metabolic disorders (affecting calcium homeostasis, mitochondria, endoplasmic reticulum, zymogen secretion and autophagy) of the acinar cells are largely responsible for the intracellular activation of enzymes. In addition, alcohol may cause blockage of small pancreatic ducts, abnormal blood flow and impaired production of cholecystokinin and secretin, all of which may contribute to the development of inflammation. Over the past 20 years, the incidence of pancreatitis has varied between 30-40 cases/100,000 people/year as a global average, with a slow but increasing trend. In the last 20 years, the incidence of pancreatitis has been 40-45 cases/100,000 people/year in the European Union,, and 60-65 cases/100,000 people/year in Hungary.

As for the molecular etiology of acute pancreatitis, **the disease can develop due to three main functional lesions**. The first is when the emptying of the pancretic juice (from the organ into the duodenum) is impeded and the activation of digestive enzymes accumulated in the ducts occurs in the organ due to lack of drainage. The obstructed ductal drainage may lead to a reflux into the pancreas tissue, where it induces intracellular activation of digestive enzymes via calcium overload mediated by bile acid-sensitive cell surface receptors on acinar cells (or by direct entry into the cells).
The second etiological factor is an abnormality in the ion transport of ductal cells due to an external or internal influence. Various insults (e.g. heavy alcohol consumption) can damage the ion and water transport system of the ductal cells, resulting in changes in the fluidity or pH of the produced gastric juice resulting in intraductal activation of digestive enzymes. The defect may be due to loss of mitochondrial function, damage to the ER, imbalance of intracellular calcium and/or cAMP, or a disturbance in CFTR channel activity, for example due to hereditary causes.

The third etiological factor involves intraacinar activation of the digestive enzymes. The molecular cause may be destabilisation of lysosomal and zymogen granules, mitochondrial dysfunction, ER stress, calcium and/or cAMP imbalance, activation of protein kinase C and NFκB pathways, or direct trypsinogen activation. In addition to intracellular digestive enzyme activation, blockage of apical-site exocytosis due to remodelling of the apical cytoskeleton may also trigger the initiation of inflammatory processes. In this case, the attachment of the granules to the apical cytoskeleton is disrupted and they begin to migrate towards the basal surface, where phosphorylation of a membrane protein by protein kinase C allows basolateral exocytosis of the granules into the interstitium.

The characteristic symptoms of **acute pancreatitis** include sudden onset of severe pain radiating in a belt-like pattern in the upper abdomen, accompanied by bloating, nausea, vomiting, diarrhoea, which worsens after eating. Patients may also experience fever, shortness of breath, rapid or slow heartbeat, kidney problems and jaundice if the bile ducts are blocked. Acinar cell death causes elevated levels of digestive enzymes in the blood, so that, in addition to more complex tests (e.g. CT, abdominal or endoscopic ultrasonography, ERCP), a blood test can easily confirm the presence of the disease. Although drugs are usually overused in pancreatitis, the treatment options for the disease are limited. The mainstay of therapy is sedation through a strict diet provided by intravenous fluids and nutrient supplementation. Targeted, specific therapies are unfortunately not available, despite extensive scientific efforts to identify molecular targets of "AP". Several ongoing clinical trials aim to determine which of these novel therapeutic approaches can be effectively translated into the clinic.

***In vitro* modeling of acute pancreatic inflammation** includes the use of immortalised cell lines (e.g. AR42J - rat acinar cell line) and the use of primary cells (both acinar and ductal) isolated from animals. Isolated primary cells have the obvious advantage of cell identity, but they can only be maintained for a limited period of time and are not capable of dividing. A further disadvantage of isolated acini is that, after about two days, they start to undergo ductal transformation. In various studies, the cells are mostly treated with bile acids (taurocholate, taurochenodeoxycholate), fatty acids, alcohol (or its acetaldehyde or fatty acid- ethylester metabolites) and superphysiological concentrations of secretagogues (e.g. CCK, secretin, VIP) which induce the secretion of zymogenic granules during normal function. Of the latter group, I would highlight the oligopeptide cerulein, an artificial analogue of cholecystokinin with complex digestive effects. At physiological levels (pM) cerulein stimulates zymogen secretion in acinar cells in a manner analogous to CCK, but at supra-maximal doses (10-100nM) it causes intracellular digestive enzyme activation, NFκB activation, vacuole formation and cell death.

For ***in vivo* pancreas studies**, rodents are mainly used, mostly mice or rats. The models used can be classified into invasive and non-invasive groups. The non-invasive models include parenteral treatment with secretagogue analogues (cerulein, mentioned earlier, or carbachol - a cholinergic agonist) by intraperitoneal injection(s). In the case of cerulein, the model includes an i.p. injection of 5-50 μg/kg bw/h, repeated over 4-12 hours. In our studies, we also used a cerulein-induced model of pancreatitis both *in vivo* in mice and on isolated acinar cells.

Although western-type medicine is largely critical of methods for traditional medicine, the active ingredients in the medicinal herbs have significant potential as drug candidates. One such plant is the toothache plant, or also known as paracress, buzz buttons, or tingflowers (*Acmella oleracea, Spilanthes acmella, Spilanthes oleracea*) whose flowers, leaves and stems are chewed from the Amazon basin to India for their mild analgesic properties to cure toothache, stomatitis, gingivitis and sore throats. The plant is also used as a disinfectant, diuretic, anti-parasitic, antiviral and wound-healing agent. The main medicinal compound in the plant is spilanthol (SLT), which has anti-inflammatory, analgesic and other properties.
Several polyphenols that have been studied in recent years for their high biological activity in humans have been shown to have great potential in the fight against pancreatic diseases. The potential protective effect of several members of a subgroup of polyphenols, the flavonoids, in acute inflammatory processes has also been demonstrated. Tricetin (TCT) is a flavonoid found in several plants and has been shown by several studies to have anticancer, anti-inflammatory and antidiabetic activity. Most studies focus on its anticancer potential.

In our study, we aimed to investigate if SLT or TCT have therapeutic potential against acute pancreatitis. Our studies were performed on isolated acinar cell cultures (*in vitro*) and in a mouse model of acute pancreatitis and in contact hypersensitivity of skin as a local inflammation (*in vivo*).

OBJECTIVES

In our work, we investigated the effects of two plant-derived drugs: tricetin and spilanthol, in an *'in vitro'* and *'in vivo'* mouse model of acute pancreatitis.

1. In the case of tricetin, we aimed to investigate its effects on viability and inflammatory mediator production, and how it affects the extent of PARylation induced on cells. In addition, we were interested in its effect on the severity of acute pancreatitis induced in mice and the extent of PARylation that may occur in cells.

2. In case of spilanthol, we set out to investigate how spilanthol affects the severity of acute pancreatitis and and contact dermatitis.

MATERIALS AND METHODS

Isolation and culture of pancreatic acinar cells

The acinar cells used in the experiments were isolated from 8-10 weeks old male and female mice of C57BL/6j strain. After excision, the tissue was placed in HBSS buffer and cut into small pieces . Subsequently, the tissue was subjected to a 20 min collagenase digestion at 37°C, while the pieces were suspended every 5 min. The cells were washed four times with HBSS buffer. Between washes, cells were centrifuged at 450 g. After washing, the clusters were resuspended in HBSS and filtered through a 100 μm filter to eliminate larger clusters that had not been properly digested.

The filtered cluster mass was then sedimented through a BSA solution. This step filtered out the over-digested individual cells and the already over-digested smallclusters of cells. Clusters of 10-30 cells were then washed with HBSS and cultured in DMEM medium containing 10% FBS, 1% antibiotics (penicillin, streptomycin) and 25 ng/ml rhEGF at high glucose concentration.

In vitro treatments

The isolated cells were treated the next day after they were seeded. Tricetin pre-treatment (1 h) at a concentration of 3-10-30-100-300 μM was applied prior to treatments with cerulein, LPS+IFNγ, PMA and hydrogen peroxide. In all experiments, cerulein (100 nM in all cases) treatment was applied for 24 h, except for the caspase assay, when treated cells were examined for 18 h. The 1 h LPS (1 μg/ml) + IFNγ (20 ng/ml) treatment and the 1 h PMA (1 μM) treatments were used for NFκB p65 DNA binding assays. To induce PARylation, we used 7.5 min treatment with hydrogen peroxide. In addition, isolated cells were exposed to hydrogen peroxide for 24 hours for viability studies.

In vivo treatments and tissue processing (pancreatitis model)

Pancreatic inflammation was induced in C57BL/6j mice with cerulein (50 μg/kg body weight) dissolved in physiological saline. During our experiments, the mice (spilanthol - 42 male mice, tricetin - 18 male mice) were randomly divided into 3 groups: CTR - mice receiving only physiological saline, CERU - mice treated with cerulein and N+CERU - animals treated with plant-derived substances in addition to cerulein. The mice were treated with cerulein by 8 intraperitoneal (i.p.) injections per hour. Mice not inoculated with cerulein recieved physiological saline with the same DMSO content at the same time. The effect of spilanthol was investigated only as a pretreatment, and tricetin as a pre- and post-treatment. In the study of tricetin, 10-10 mg/kg bw of tricetin was injected into the mice during two i.p. inoculations, 12 and 1 hour before the first ceruleine injections, respectively. In the post-treatment, tricetin was administered at a dose of 30 mg/kg bw between the 4th and 5th cerulein injection and 10 mg/kg bw after the last injection (also i.p.). In the case of spilanthol, 30 mg/kg bw was administered i.p. to the mice one hour before the first cerulein treatment. Animals not treated with plant-derived substances have received physiological saline containing equimolar DMSO. Animals were bled 24 h after the first cerulein injection in the spilanthol experiment and 10 h after the first cerulein injection in the tricetin experiments, and pancreata were removed for further studies after termination of the experiments.

Blood was drawn from the mice by cardiac puncture was centrifuged at 5.000 g for 10 min. After coagulation, the serum fraction was frozen. When the pancreatic tissue was harvested, the tissue was cut into 3 sections for histological microscopic observation, myeloperoxidase activity assays and RT-qPCR assay. The samples for histology were placed in 10 % formalin (4 % aqueous solution of formaldehyde), the section for enzyme analysis in liquid nitrogen and the section for RT-qPCR in TRIzol extraction reagent and frozen.

***In vivo* treatments and tissue processing (contact hypersensitivity, in case of spilanthol)**

In BALB/c strain mice, contact dermatitis was induced by with phorbol 12-myristate 13-acetate (PMA) administration. During the experiments, the animals (18 males and 18 females) were randomly divided into 3 groups: CTR, dermatitis and spilanthol + dermatitis. Before induction of inflammation, the ear thickness of the mice was measured using a Mitutoyo thickness gauge. Then, mice were treated with DMSO or PMA dissolved in DMSO (20 µl/ear, micropipette application, 10-10 µl on each side of the ear). Sixty minutes later, the control and PMA groups were treated with DMSO and the SLT+PMA group with 10 µM spilanthol (20 µl/ear, micropipette application, 10-10 µl on each side of the ear). Six hours after PMA treatment, ear thickness was measured again and mice were anaesthetised with isoflurane. After extermination, the ears of the animals were removed for histology (H&E staining) and MPO assays. The portion of the ears for histology was placed into 10% formalin and the tissue for enzyme assays were placed into liquid nitrogen, as for pancreatic sections.

***In vitro* experiments** were performed to investigate the radical scavenging (ABTS assay) and PARP inhibitory ability of tricetin (immunofluorescence, WB assay) and its effect on NFκB activation. In addition, we examined the effect of the drug on the viability of cells treated with cerulein or H_2O_2 (Calcein essay, LDH essay, PI essay, Caspase activity assay) and the effect of the drug on inflammatory gene expression by RT-qPCR.

In our ***in vivo* experiments,** we measured the serum α-amylase and lipase levels of mice, and the pancreatic myeloperoxidase (MPO) activity. We analysed the extent of edema, leukocyte infiltration and necrosis induced by the treatment in tissue sections. We also used immunostaining on the sections to detect PARylation.

RESULTS

Effect of tricetin in pancreatitis

Tricetin is an antioxidant

In the literature, several flavonoid compounds have been shown to have antioxidant activity. With ABTS assay, we demonstrated that TCT has similar radical scavenging activity to vitamin C used as a positive control. The IC50 value of vitamin C was 65.15 μM and that of TCT was 49.64 μM.

Tricetin is not toxic to primary acinar cells at low concentrations

We isolated primary acinar cells from mice and used them to investigate the toxicity of TCT. Cell viability was assessed by calcein assay and plasma membrane damage by LDH release assay. We found that TCT is not toxic to primary acinar cells up to a concentration of 30 μM. At three times higher concentrations (100 μM), TCT had a small but significant toxic effect on cells, but this was negligible compared to 1 mM hydrogen peroxide used as a positive control. This was observed in both assays and therefore the 30 μM concentration was chosen for subsequent cell-based experiments.

Tricetin protects cells from cerulein-induced cell death, but not from H_2O_2-induced cell death

The cholecystokinin analogue peptide cerulein is widely used to model overstimulation-induced acinar cell injury in both cell-based experiments and *in vivo*. We found that at a concentration of 100 nM, cerulein reduces the viability of primary acinar cells, as assessed with calcein assay, and this effect was also observed in the LDH release method. Pretreatment of cells with 30 μM tricetin significantly protected acinar cells from cerulein-induced damage in both models. Since necrotic cell death and associated cell membrane damage is a critical feature of AP, we sought to confirm the latter result using an alternative method. To this end, we examined cellular uptake of propidium iodide (PI). Our results showed that the PI uptake data were in agreement with the results of both the calcein assay and the LDH release assay: 10 μM TCT inhibited cerulein-induced necrosis. Moreover, TCT was also found to inhibit cerulein-induced apoptosis.

We then examined whether the findings observed in cerulein treatment also hold for H_2O_2-induced cell injury. H_2O_2 caused concentration-dependent cytotoxicity, but TCT had no effect on the cell-damaging effect of H_2O_2, indicating that the flavonoid is likely to act not simply as an antioxidant but to specifically interfere with the cerulein-induced acinar cell damage pathway.

Tricetin inhibits cerulein-induced inflammatory gene expression in isolated acinar cells

The inflammatory component clearly contributes to the development of AP. We therefore assembled an inflammatory mediator panel and examined the effect of TCT on the expression of these genes. Cerulein treatment induced the expression of IL1β, IL6 and MMP2 mRNAs, whereas it did not induce the expression of TNFα, IFNγ, and the chemokines CCL5 and

CXCL10. Similarly, the expression of TGFβ and IL10 was not altered. Pretreatment of cells with TCT significantly reduced cerulein-induced expression of IL1β, IL6 and MMP2.

Tricetin restrains the translocation of NFκB p65 subunit into the nucleus but does not inhibit its binding to DNA

The NFκB transcription factor is found in an inactivated state in the cytosol at rest but in response to inflammatory triggers,it translocates to the nucleus where it binds to the promoter of inflammatory target genes in its most typical complex with the p50 subunit to form a heterodimer, triggering gene expression. The results of the DNA binding essay of the p65 subunit showed that both LPS (1 uμg/ml) + IFNγ (20 ng/ml) treatment and PMA (1 μM) treatment significantly increased nuclear translocation of the transcription factor. . To clarify whether the reduced rate of binding was due to inhibition of nuclear translocation or to inhibition of DNA binding, the nuclear extracts of cells were subjected to tricetin 'post-treatment', TCT 'post-treatment' experiments suggested that TCT inhibits the nuclear translocation of NFκB, but not the DNA binding.

Tricetin inhibits PARP1

PARP1 activation contributes to tissue injury and inflammation in acute and chronic pancreatitis. Therefore, we investigated whether inhibition of PARylation may play a role in the beneficial effects of TCT. In an enzyme activity assay, TCT showed PARP inhibitory activity similar to that of the first generation, weak PARP inhibitor 3-aminobenzamide (3-AB).

We also examined the effect of tricetin on PARylation in a cellular system. Isolated acinar cells were treated with 250 μM hydrogen peroxide, which induced PARylation as demonstrated in IF and WB assays. One hour pre-treatment with 10 μM or 30 μM TCT significantly reduced PARylation in the cells.

Pretreatment with TCT reduces the severity of acute pancreatitis in mice

Based on the cytoprotective and NFκB inhibitory effects of TCT in primary acinar cells and the suppression of cerulein-induced inflammatory mediators, we hypothesized that TCT would also be beneficial in a cerulein-induced animal model of AP. Our results show that, in agreement with the literature, cerulein caused damage to pancreatic acinar acinar cells, as indicated by elevated serum levels of the digestive enzymes α-amylase and lipase. TCT pretreatment significantly reduced acinar cell damage. H&E-stained sections showed signs of edema and granulocyte infiltration and necrosis in the pancreas of cerulein-treated mice. TCT attenuated both edema formation and the extent of infiltration and necrosis, as confirmed by semi-quantitative analysis of sections by a trained pathologist. Similar protective effects could also be demontrated when TCT was used as a post-treatment,

Tricetin reducesapoptosis in the pancreas

The results of the TUNEL essay correlate well with those observed in the H&E staining previously presented. A large number of apoptotic cells were observed in pancreatic sections after cerulein treatment. The intensity of apoptosis was reduced in the TCT-treated samples. Semiquantitative evaluation by a pathologist showed significant differences between control

(CTR) and cerulein-treated mice, as well as between sections from cerulein-treated mice and mice that received TCT pretreatment.

Tricetin inhibits granulocyte infiltration in a mouse model of acute pancreatitis

One feature of inflammation is the appearance of white blood cells, particularly granulocytes, in the affected tissue. Measurement of tissue myeloperoxidase (MPO) levels is a good indicator of granulocyte content in different tissues. MPO activity measured in pancreatic tissue increased more than sixfold after AP induction. TCT treatment significantly reduced tissue MPO levels, suggesting inhibition of granulocyte migration into the tissue. This results are in line with histological evaluations of H&E-stained sections.

Tricetin reduces inflammatory gene expression *in vivo*

Similar to what was observed in cerulein-treated isolated acinar cells, the mRNA levels of the inflammatory cytokines IL1β and IL6 also increased *in vivo.*and TCT pretreatment reduced their expression. Contrary to the results obtained on acinar cells, MMP2 levels did not change *in vivo* in the AP group and were not affected by TCT. Although the level of TNFα expression in isolated acinar cells was not increased in cerulein-treated samples, in vivo, TNFα mRNA levels were increased in the cerulein-treated group, and TNFα mRNA levels was significantly inhibited by TCT pretreatment.

TCT reduces PARylation in the mouse model of AP

In tissue sections of cerulein-treated animals, we detected the poly(ADP-ribose) (PAR) polymer. In the exocrine pancreas, a large number of nuclei were immunopositive for PAR, suggesting activation of acinar PARP. In contrast, the pancreas of animals that were pretreated with TCT contained hardly any PAR polymers. Analysis of confocal microscopy images demonstrated that TCT pretreatment significantly reduced PAR polymer formation in cerulein-damaged pancreatic tissues.

Effect of spilanthol on inflammation

In vivo, spilanthol reduced the edema and neutrophil infiltration due to contact dermatitis.

Previously we showed that spilanthol inhibited iNOS expression and NO production and disrupted inflammatory transcription factor activation in macrophages. Based on these findings, we found the drug worthy of investigation in an *in vivo* inflammation model. First, we conducted the study in one of the simplest experimental models of local inflammation, irritant contact dermatitis, using an animal model with readily available local treatment options.

Erythema and swelling were assessed at 6 h after application of PMA on the ears of BALB/c mice. Histological sections of the ears stained with hematoxylin-eosin confirmed PMA induced massive inflammation. Spilanthol reduced ear thickness of PMA-treated mice by 40% (from 0.34 mm to 0.26 mm) . We can conclude that spilanthol inhibited the oedematous response, as observed in tissue sections. Leukocyte infiltration induced by PMA treatment was also significantly reduced in the group treated with spilanthol.

Spilanthol reduces neutrophil infiltration in AP

Significant cell damage was induced by cerulein treatment, which was not reduced by the 30 mg/kg bw spilanthol treatment as assessed by serum α-amylase and lipase levels. In contrast, the increased neutrophil infiltration rate was significantly reduced by spilanthol treatment. The results for enzyme assays were confirmed by images of H&E stained histological sections: the extent of cerulein-induced tissue damage was not inhibited by spilanthol, whereas increased neutrophil infiltration was reduced.

DISCUSSION

Pancreatitis is an increasingly common disease in developed societies for which we still have no specific therapy. Several plant alkylamides and flavonoids have been shown to alleviate the inflammatory process. Spilanthol, an alkylamide isolated from Acmella oleracea, and TCT, a flavonoid compound, have been investigated in inflammatory processes, but have yet to be explored in the therapy of acute pancreatitis.

To investigate the effects of TCT in acute pancreatitis, we chose the cerulein-induced model of AP. In our experiments, cerulein treatment induced cell death in primary acinar cells isolated from mice. The decrease in viability of cerulein-treated cells demonstrates the toxic effect of CCK receptor hyperstimulation. The induced cell death exhibited features of both apoptosis and necrosis, as indicated by caspase activation (apoptosis) and LDH release and propidium iodide uptake (necrosis). A significant cytoprotective effect of TCT was observed in both the general viability assay and the apoptosis and necrosis assays.

In addition, TCT treatment also contributed to the maintenance of cell viability in the mouse model of AP, as indicated by reduced amylase and lipase levels measured from the animals' serum. Interestingly, the effect of TCT was more pronounced on serum lipase activity than on amylase activity. This, in our opinion, is probably due to a more robust lipase release response compared to the less pronounced amylase release seen in cerulein-treated animals. A previous study also suggested that lipase is indeed a more sensitive indicator of pancreatitis. Nevertheless, both serum markers confirmed the *in vivo* cytoprotective effect of TCT.

The preserved pancreatic tissue architecture of TCT-treated animals also suggests that cerulein induced less severe tissue damage, and TUNEL staining of sections also shows that TCT prevents apoptotic cell death *in vivo*.

Oxidative stress is a common feature of different forms of inflammation and plays a key role in the pathomechanism of acute pancreatitis. Since several flavonoids possess antioxidant properties, we hypothesized that an antioxidant mechanism may contribute to the protective effect of TCT in AP. Indeed, TCT possesses radical scavenging activity, as demonstrated by the ABTS assay. Interestingly, however, TCT was not protective against hydrogen peroxide-induced acinar cell damage. This may be due to the lack of hydrogen peroxide-specific antioxidant activity at the concentration of TCT used in the study. In addition, antioxidants act primarily by increasing nucleophilic tone (e.g. via Nrf-2 induction) rather than by eliminating free radicals. Whether this is also the case for tricetin, requires further investigation.

Since ROS production has been associated with cerulein-induced acinar cell damage in AP, we hypothesized that TCT may act, at least in part, by inhibiting the PARP1-mediated ROS-induced cell death pathway. Of note, the oxidative stress-induced and PARP1-mediated cell death pathway exhibits features of necrosis (mostly through membrane permeabilization), consistent with the partial necrotic death of acinar cells in AP that we have also shown. Some

flavonoids have been reported to exert PARP inhibitory effects upon DNA alkylation. We have investigated the effect of TCT on PARP enzyme activity and found that TCT inhibits the enzyme with a potency similar to 3-aminobenzamide.

In primary pancreatic acinar acinar cells exposed to oxidative stress, PAR polymer formation was observed, suggesting PARP activation. TCT abolished PAR synthesis *in vitro*, as shown by immunofluorescence staining and Western blot. The acinar cells' nuclei of the cerulein-treated animals also contained PAR polymer, whereas PAR formation was absent in the group pretreated with TCT. PARP1 and PARylation have previously been shown to contribute to the pathogenesis of AP. Our present results confirm that the *in vitro* PARP inhibitory effect of TCT can be translated to *in vivo* conditions, suggesting that PARP inhibition may be a key factor in the protection afforded by TCT in acute pancreatitis. Other studies have suggested that the cytoprotective effect of flavonoids may also be mediated by their activating effect on PI3K-Akt signalling.

Our data clearly show that TCT suppresses inflammation in AP. Reduced edema, restrained inflammatory infiltration and preserved tissue architecture all support this claim. Inhibition of the production of inflammatory mediators underlies the anti-inflammatory effect of TCT. From cellular experiments, we have been able to establish that TCT inhibits the activation of inflammatory mediator genes (IL1β, IL6, MMP2). Our *in vivo* data extended this list with TNFα as a central mediator of cerulein-induced inflammation, whose expression was inhibited by TCT. TNFα was not induced in experiments on isolated acinar cells, consistent with our current understanding that macrophages are the major source of this cytokine in AP.

From the above, we conclude that TCT can inhibit inflammatory gene activation in both acinar cells and macrophages. We hypothesise that inhibition of NFκB may underlie this effect and our results indeed show that TCT inhibits NFκB activation in acinar cells. However, the drug had no direct effect on the binding of the transcription factor to its consensus sequence, suggesting that TCT acts at a proximal step of the NFκB signaling pathway, which reduces the nuclear entry of the transcription factor.

The effect of TCT on NFκB is also thought to be due to its PARP inhibitory effect, as PARP1 acts as a coactivator of NFκB, and PARP inhibition/PARP1 knockout caused suppression of NFκB activation in various model systems.

Early protease activation and NFκB activation are fundamental features of AP; both occur in parallel during disease manifestation and strongly influence each other. However, it is not only protease and NFκB activation that play a critical role, but also the cell type in which their activation occurs.

Although the focus of this work has been on the antioxidant and PARP inhibitory effects of TCT, it should be noted that TCT may have additional effects that contribute to the protection against pancreatitis. Pancreatic ischemia plays a dominant role in the development of AP and in the progression of the disease to severe forms. This claim is supported by observations that

a decrease in pancreatic blood supply increases the severity of AP, whereas an improvement in pancreatic blood supply reduces the severity of acute pancreatitis and accelerates pancreatic regeneration. Previous studies have shown that the flavonoid quercetin increased pancreatic blood flow in AP resulting in tissue protection and faster pancreatic recovery. These observations suggest that the protective effect of TCT in AP may be at least partly related to an improvement in pancreatic blood flow.

In our previous experiments, **spilanthol**, significantly reduced the expression of the iNOS gene and protein and the amount of NO produced by RAW macrophage cells. NO can induce, among other things, protein translational modifications (nitrosation, nitrosylation, nitration), cXMP signalling (e.g. cell proliferation), and form reactive nitrogen derivatives (e.g. peroxynitrite). As a result of these effects, the agent also significantly increased the viability of treated cells.

Based on the results from macrophage studies, spilanthol was also tested in inflammatory processes (contact hypersensitivity and acute pancreatitis) and was shown to be an anti-inflammatory agent in a mouse model of dermatitis, probably in association with its inhibitory effect on iNOS and thus NO production. The compound significantly reduced ear edema and tissue granulocyte infiltration. Our previous studies suggest that reprogramming of inflammatory transcription factor activation pathways is likely to be a crucial mechanism underlying this anti-inflammatory effect. With respect to the inhibitory effect of the agent on granulocyte infiltration, spilanthol inhibits the expression of adhesion molecules and these events may contribute to the reduction of inflammatory cell migration in spilanthol-treated mice.

The activation of iNOS has already been studied in a cerulein-induced acute pancreatitis model, and it was found that elevated levels of the enzyme are also characteristic of acinar cell-induced inflammatory processes. Spilanthol, reduced granulocyte infiltration into the pancreas and suppressed inflammation. However, the cells were not protected from the adverse effects of cerulein treatment, which may suggest that elevated levels of iNOS and thus NO formation do not play a cardinal role in the development of cellular injury. Among other things, the inhibition of the expression of adhesion molecules by spilanthol may be responsible for the decrease in inflammatory cell migration.

In conclusion, of the two herbal compounds tested, spilanthol is the less effective compound in the treatment of AP, whereas tricetin may be a new candidate for reducing the severity of the disease by inhibiting two central pathways of AP pathophysiology: acinar cell death and inflammation. The antioxidant and PARP inhibitory effects of TCT may play a central role in the protective effects of this flavonoid in acute pancreatitis.

SUMMARY

With its growing incidence and potentially life-threatening course and complications, acute pancreatitis (AP) poses a great challenge to the health systems worldwide. Specific targeted therapies are not available prompting efforts to identify new pathways and novel therapeutic approaches.

Alkylamides and flavonoids comprise several groups of biologically active compounds with wide ranging effects. Spilanthol (an alkylamide) and tricetin (a flavonoid) have not yet been investigated in pancreatitis, but reports in the literature have demonstrated a wide range of biological activities, including anti-inflammatory effects. In the present study, we evaluated the potential therapeutic effects of spilanthol (SLT) and tricetin (TCT) in acute pancreatitis.

Tricetin protected isolated primary acinar cells against the cytotoxic effect of the CCK-analogue cerulein. The compound significantly reduced PARylation in the cells exposed to oxidative stress. The effects of TCT were probably not related to its potential antioxidant activity, as it did not protect against H_2O_2-induced acinar cell death despite its radical scavenging activity. The cerulein-induced expression of IL1β, IL6 and matrix metalloproteinase 2 (MMP2) was also significantly reduced by TCT treatment.

In vivo tricetin suppressed pancreatic edema formation and protected pancreatic acinar cells from death. In addition, TCT inhibited the expression of IL1β, IL6 and TNFα in pancreatic tissue and restrained oxidative DNA damage, as indicated by a decrease in the level of PAR, a product of the enzyme poly(ADP-ribose) polymerase 1 (PARP1). *In vivo* spilanthol did not protect the pancreatic cells of cerulein-treated mice from cell death, but significantly attenuated inflammatic edema and granulocyte infiltration.

Our data suggest that tricetin can be considered as a potential treatment option for AP.

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List of publications related to the dissertation

1. **Nagy-Pénzes, M.**, Hajnády, Z., Regdon, Z., Demény, M. Á., Kovács, K., El-Hamoly, T., Maléth, J., Hegyi, P., Hegedűs, C., Virág, L.: Tricetin Reduces Inflammation and Acinar Cell Injury in Cerulein-Induced Acute Pancreatitis: the Role of Oxidative Stress-Induced DNA Damage Signaling.
   Biomedicines. 10 (6), 1-20, 2022.
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   IF: 4.757 (2021)

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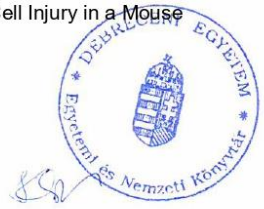

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Total IF of journals (all publications): 29,751
Total IF of journals (publications related to the dissertation): 9,313

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

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