Protecting the gastrointestinal mucosa: novel NSAID conjugates with therapeutic efficacy and reduced ulcerogenic potential

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LIST OF PUBLICATIONS

Full papers related to the subject of the thesis

I. Varga G, Lajkó N, **Ugocsai M**, Érces D, Horváth G, Tóth G, Boros M, Ghyczy M. Reduced mucosal side-effects of acetylsalicylic acid after conjugation with tris-hydroxymethyl-aminomethane. Synthesis and biological of a new anti-inflammatory compound. Eur J Pharmacol. 2016;781:181-9. **Q2; IF: 3.125**

II. Varga G, **Ugocsai M**, Hartmann P, Lajkó N, Molnár R, Szűcs S, Jász DK, Érces D, Ghyczy M, Tóth G, Boros M. Acetylsalicylic acid-tris-hydroxymethyl-aminomethane reduces colon mucosal damage without causing gastric side effects in a rat model of colitis.

Inflammopharmacology 2018;26(1):261-271. **Q2; IF: 2.985**

III. **Ugocsai M**, Bársony A, Varga R A, Gajda Á, Vida N, Lajkó N, Rónaszéki B, Tóth G, Boros M, Érces D, Varga G. Conjugation with Tris decreases the risk of ketoprofen-induced mucosal damage and reduces inflammation-associated methane production in a rat model of colitis. Pharmaceutics. 2023;15(9):2329. **Q1; IF: 5.8**

Abstracts related to the subject of the thesis

I. Lajkó N, Varga G, **Ugocsai M**, Mészáros A, Tuboly E, Érces D, Ghyczy M, Tóth G, Boros M. Acetilszalicilsav-aminoalkohol konjugátum mikrokeringésre kifejtett hatása kísérletes colitisben. Érbetegségek, XX(1): 39. 2014.

II. Varga G, Lajkó N, **Ugocsai M**, Ghyczy M, Tóth G, Boros M. Nem-szteroid gyulladásgátlók és származékaik mikrokeringési hatásai a gasztrointesztinális traktusban. Érbetegségek, XX(1): 46-47. 2014.

III. **Ugocsai M**, Lajkó N, Strifler G, Ghyczy M, Boros M, Varga G. L-α-gliceril-foszforil-kolin védőhatása acetilszalicilsav által okozott gastritisben. Magy. Seb. 68(3): 115-116. 2015.

IV. **Ugocsai M**, Varga G, Lajkó N, Strifler G, Ghyczy M, Boros M. Protective effects of L-alpha-glycerylphosphorylcholine treatment on acetylsalicylic acid induced gastric mucosal injury. European Surgical Research 2015;55(suppl 1):22

Full papers not related to the subject of the thesis

I. Bari G, Szűcs S, Érces D, **Ugocsai M**, Bozso N, Balog D, Boros M, Varga G. A cardiogen sokk modellezese pericardialis tamponaddal [Experimental model for cardiogenic shock with pericardial tamponade]. Magy Seb. 2017. 70:297–302 **Q4; IF: 0**

II. Szűcs S, Bari G, **Ugocsai M**, Lashkarivand RA, Lajkó N, Mohácsi A, Szabó A, Kaszaki J, Boros M, Érces D, Varga G. Detection of intestinal tissue perfusion by real-time breath methane analysis in rat and pig models of mesenteric circulatory distress. Crit Care Med 2019. **Q1; IF: 6.6**

1. INTRODUCTION

1.1. The Progression of Inflammation

Inflammation is a complex biological response initiated by various harmful stimuli, including physical trauma, infections, and chemical agents. The primary goal of inflammation is to eliminate these harmful agents, remove damaged tissues, and restore tissue integrity and function (Kumar et al, 2004). This response is characterized by the release of signaling molecules, particularly arachidonic acid (AA), which is a precursor for the cyclooxygenase (COX) and lipoxygenase (LOX) pathways. The COX pathway is crucial for producing prostaglandins, which are essential for various physiological functions, while the LOX pathway leads to the synthesis of leukotrienes. The upregulation of COX-2 during inflammatory states is associated with increased prostanoid production and has implications in various diseases, including cancers and neurodegenerative disorder (Murakami et al, 2017, Vane et al., 1998).

The acute inflammatory response is characterized by changes in the circulatory system, particularly the microcirculatory-microvascular system. Hyperemia and increased vascular permeability facilitate the migration of leukocytes and the extravasation of plasma proteins and fluid into the surrounding tissues. If the inflammatory state persists, chronic inflammation can develop, which is characterized by the persistent presence of cellular activation, macrophage infiltration, and tissue damage (Treppels et al., 2006, Feletou et al., 2006). Therefore, inflammation can have both beneficial and detrimental effects on the function of tissues and organs, depending on the cause, type, and severity of the inflammation. Acute inflammation, such as wound healing, typically leads to scarring and tissue remodeling, reconstruction, and regeneration (Maher et al., 2011, Murata et al., 2018). In this process, activated macrophages play a key role. In contrast, chronic, long-lasting inflammation can lead to irreversible tissue damage and pathological fibrosis (Oishi et al., 2018). Histotoxicity is driven by various mechanisms, including polymorphonuclear (PMN) leukocyte-endothelin interactions, as well as oxidative-proteolytic pathways (Dallegri et al., 1997).

1.2. Inflammation in the gastrointestinal tract

The selection of appropriate therapy for inflammation in the gastrointestinal (GI) tract poses a significant challenge, as the absorption of orally administered drugs is often impaired, and the chemical properties of the drugs also influence the effectiveness of treating the damaged GI tissues (Barros et al., 2019, Parikh et al., 2019). Gastritis can lead to the development of ulcers, typically starting with superficial mononuclear inflammation, accompanied by acute

polymorphonuclear (PMN) infiltration. The progression of the condition is characterized by the destruction of the normal mucosal glands, and the loss of functional gland and epithelial cells leads to atrophic gastritis (Sipponen et al., 2015). This process is often associated with Helicobacter pylori infection and is a significant risk factor for the development of malignant gastric lesions (Lahner et al., 2014, Lahner et al., 2009, Kaptan et al., 2000).

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, are pathological processes that occur in the GI tract, characterized by immune system dysregulation, tissue damage, and disruption of the mucosal barrier function. In the pathogenesis of IBD, the initial event is an increase in intestinal permeability, leading to a functional disturbance of the mucosal barrier and the development of dysbiosis. The local and systemic immune response (recruitment of effector cells and the release of humoral mediators) further exacerbates the mucosal barrier damage (Appleyard et al. 2002; Hatoum et al. 2003; Hatoum and Binion 2005). Impairment of mitochondrial oxidative phosphorylation (OxPhos) plays a critical role in this pathomechanism, as lower ATP levels and reduced mitochondrial complex II activity have been observed in the mucosa of IBD patients (Parikh et al., 2019; Xavier et al., 2007; Stange et al., 2019; Liu et al., 2021; Kameyama et al., 1984; Santhanam et al., 2012).

1.3. Therapeutic possibilities in GI inflammatory conditions

The treatment of gastritis aims to reduce inflammation, manage symptoms, and maintain remission (e.g., reducing gastric acid production with proton pump inhibitors (PPIs) and H2 receptor antagonists, eradicating H. pylori with antibiotics and PPIs). In the treatment of inflammatory bowel disease (IBD), immunomodulators, anti-inflammatory drugs, and anti-cytokine therapies such as TNF-α blockers are primarily used (Rutgeerts et al., 2004). Non-steroidal anti-inflammatory drugs (NSAIDs) may also be useful for some patients, particularly for extraintestinal muscle pain. Mesalamine (5-ASA) as a first-line therapy (Managlia et al., 2013) inhibits the production of proinflammatory precursors, playing an important role in symptom management and maintaining remission, and has relatively few systemic side effects due to its local action (Bickston and Cominelli, 2003).

1.4. NSAIDs

NSAIDs are a heterogeneous group of drugs that are widely and effectively used to treat inflammation, but GI complications can be severe: they can cause bleeding and the formation of ulcers. NSAIDs inhibit the COX/prostaglandin-endoperoxide synthase (PGHS) enzymes

with varying selectivity (Kumar et al., 2018). The analgesic, anti-inflammatory, antithrombotic, and antipyretic effects of NSAID salicylates are based on the inhibition of COX enzymes in a dose-dependent manner (Rao et al., 2008). Propionic acid derivatives, which act non-selectively, are often used in the treatment of rheumatic and joint disorders (Cathcart et al., 1973). Indomethacin, as the most commonly used acetic acid derivative, can also be used for joint diseases and tocolysis (Baber et al., 1979). However, special attention is required for the severe side effects of these drugs, such as sodium retention, edema, hypertension, tachycardia, and bronchospasm (Donker et al., 1976). Pyrazolone derivatives exert rapidly reversible effects on inflammatory processes, while oxicam derivatives can act as non-selective and selective COX-2 inhibitors, depending on the molecular structure. Selective COX-2 inhibitors cause fewer GI side effects, but their long-term use may increase the risk of coagulation disorders and cardiovascular diseases.

1.4.1. Acetylsalicylic acid and ketoprofen

Acetylsalicylic acid (ASA) and ketoprofen (Ket) are two very commonly used NSAIDs with different mechanisms of action. ASA has analgesic, antipyretic, and anti-inflammatory effects at high doses, while at lower doses, it is effective in the prevention of cardiovascular diseases (Baigent et al., 2009). Ket has a strong anti-inflammatory effect and is used to treat acute and chronic rheumatoid arthritis, osteoarthritis, Bechterew's disease, primary dysmenorrhea, and musculoskeletal injuries (Kantor et al, 1986, Lastra et al, 2002, Jerussi et al, 1998). However, it is important to emphasize that both NSAIDs have severe GI side effects (Sørensen et al., 2000; Delaney et al., 2007).

1.4.2. Mechanisms of GI side effects of NSAIDs

The use of NSAIDs can lead to mucosal damage, ulcers, and perforations due to structural and functional impairment (Appleyard et al., 2002). The causes of bleeding complications are multifactorial, but the main mechanisms include irreversible inhibition of COX-1 and non-prostaglandin-mediated irritation (Hochain et al., 2000). NSAIDs, such as ASA, can influence mitochondrial activity and generate signals that induce apoptosis (Nulton-Persson et al., 2004; Raza et al., 2012; Redlak et al., 2005). Another identified mechanism is the inhibition of the COX pathway, which reduces the level of prostaglandin-E2 (PGE2), which has an anti-inflammatory effect, but increases the risk of bleeding due to the decrease in thromboxane A2 (TXA2) levels. The reduction in prostacyclin (PGI2) production also impairs the protection of the GI mucosa (Bacchi et al., 2012; Henry et al., 1988; Vane et al., 1994). NSAIDs also weaken

the defense mechanisms of the GI system due to the direct harmful effects of the acidic carboxyl groups (Bjarnason et al., 1993; Price et al., 1990). Inhibition of mitochondrial OxPhos can disrupt ATP production, which can lead to increased mucosal permeability through damage to cell-cell connections (tight-junctions) (Chan et al., 2005; Bjarnason et al., 2018). Nephrotoxicity is also a significant side effect, as certain NSAIDs can stimulate angiotensin-II-mediated vasoconstriction, reducing local renal circulation and glomerular filtration rate (Dunn et al., 1984). In summary, although NSAIDs have different effects and COX-2 selectivity, they can all influence the protection of the GI mucosa through the reduction of prostaglandin levels, leading to tissue lesions (Price et al., 1990; Bjarnason et al., 2018; Huang et al., 2011; Rodriguez et al., 2019).

1.4.3. Chemical modification of NSAIDs to develop new NSAID derivatives

For new active substances synthesized from NSAIDs and appropriate precursors, a reduced extent of mucosal damage is expected, but preserving the original biochemical function is a major challenge (Uzzaman et al., 2020, Narsinghani et al., 2014, Rahman et al., 2024). The biochemical application of tris(hydroxymethyl)aminomethane or 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) is already well-known (Gomori et al., 1955), and Tris is often a component of buffer solutions (in the pH range of 7.07-9.07). Tris buffers have a significant effect on arterial pH and base deficit, reducing the partial pressure of carbon dioxide in arterial blood, and are therefore also used to treat patients with mixed acidosis (Hoste et al., 2005; Kallet et al., 2000). Tris can also inhibit the activity of certain enzymes (aminopeptidases and alpha-amylases) (Desmarais et al., 2002; Ghalanbor et al., 2008), and it has been investigated as a cationic salt-forming agent to increase the water solubility of ketorolac-tromethamine (1:1 ratio) (Mroszczak et al., 1987).

2. GOALS

Effective NSAID formulations with reduced side-effects would be of clinical interest and therapeutic importance. Our first goal was to develop novel compounds from representative molecules of this class of drugs; the specific objective of the research and development activity was to obtain formulations that retain the original efficacy of the parent molecule while reducing or eliminating side effects, particularly haemorrhagic complications in the GI tract. Building on this premise, we hypothesized that chemical combination of NSAIDs with Tris could offer therapeutic benefits over existing NSAID. Therefore, the primary aim of the studies in this thesis was to demonstrate a significant reduction in drug-induced mucosal damage and

complication rates when a newly developed NSAID conjugate was used. In line with this, our objectives were:

- to synthesize ASA-Tris and Ket-Tris molecules from ASA, Ket, and Tris precursors;
- compounds derived from ASA and mono- and bis-hydroxymethylaminomethane were also synthesized and tested to obtain comparative information and clues for the possible mode of action and to examine the mucosa-damaging properties of the newly developed modified NSAID derivatives in comparison to the original substances;
- to test the analgesic and anti-inflammatory effectiveness in established preclinical rodent models, specifically in carrageenan-induced paw inflammation and TNBS-induced colitis.
- to assess the effects of newly developed NSAID derivatives on blood coagulation, platelet activation;
- to examine the possible effects of the new conjugates on mitochondrial function.

3. MATERIALS AND METHODS

3.1. Experimental animals

The experiments were performed in male Sprague-Dawley rats (average weight 200 g ± 10 g) housed in plastic cages under a 12-h dark-light cycle, in a thermoneutral environment (21±2 °C). The animals were kept on normal laboratory chow and then for 3 days prior to the experiments were fed with a carbohydrate-rich diet (bread rolls). The experimental protocols were in accordance with EU directive 2010/63 for the protection of animals used for scientific purposes and were approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority) with the licence number V./146/2013. This study also complied with the criteria of the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3.2. Synthesis of NSAID-amino-alcohol conjugates

The synthesis of NSAID-Tris conjugates was carried out at the Department of Medical Chemistry, University of Szeged, using ASA, Ket, ethanolamine (Mono), 2-amino-1,3-propanediol (Bis), and Tris as precursors. The newly developed derivatives were: ASA-Mono, ASA-Bis, ASA-Tris, and Ket-Tris*. *Our co-author, Miklós Ghyczy was until 09.2016 applicant and proprietor of European patent application EP 2889286A1 and International patent

application WO 2015/101501 (PCT/EP2014/078296) entitled “Pharmaceutically active compound for use as anti-inflammatory agent.

3.3. In vivo experimental methods

The in vivo examination of microcirculation was performed using intravital videomicroscopy. The study of gastric and colonic serosal microcirculation was carried out using orthogonal polarization spectral (OPS) imaging (Cytoscan A/R, Cytometrics, Philadelphia, Pennsylvania, USA), which allows the visualization of red blood cells in the capillaries within the superficial 200 μm of the mucosa without the use of a contrast agent. S-VHS video recordings of the microcirculation were made (Panasonic AG-TL 700, Matsushita Electric Ind. Co. Ltd, Osaka, Japan) and analyzed offline (IVM Pictron, Budapest, Hungary) to determine the average red blood cell velocity (RBCV; μm/s).

In vivo assessment of vascular and tissue injury: In our rodent model, the extent of gastric and colonic mucosal damage was determined using in vivo histological examination with fluorescent confocal laser scanning endomicroscopy (CLSEM; Five1, Optiscan Pty. Ltd., Australia). The gastric and colonic lumens were opened, and the mucosa was exposed. The microvascular structure of the mucosa was examined after the administration of fluorescein isothiocyanate-dextran (FITC-dextran; 150 kDa, 20 mg/mL solution in saline, 0.3 mL iv). The device's objective was placed on the colonic mucosa, and confocal images were acquired 5 minutes after the dye administration (1 scan/image, 1024 x 512 pixels and 475 x 475 μm per image). Structural changes in the mucosa were examined in separate areas after the topical application of acriflavine.

To test the analgesic effect of the new ASA-Tris conjugate, we used the carrageenan-induced paw inflammation model in rodents. The analgesic effect was determined using a dynamic plantar aesthesiometer, with measurements performed using a straight metal filament placed under the plantar surface of the right hind paw.

3.4. In vitro experimental methods

Evaluation of inflammatory markers: At the end of the experiments, we collected gastric, colonic, and liver biopsy samples, as well as blood samples from the animals. The tissue samples were stored at -70°C until use. For the measurements, the tissue samples were homogenized and centrifuged at 24,000 g for 20 minutes at 4°C in Tris-HCl buffer (0.1 M, pH=7.4) containing 0.1 mM polymethylsulfonyl fluoride to inhibit tissue proteases. The

myeloperoxidase (MPO) activity in the examined tissue reflects the accumulation of leukocytes and was determined from the sediment of the tissue homogenate according to the method of Kübler et al. (1996). The xanthine oxidoreductase (XOR) enzyme is a significant source of superoxide radical production in the tissues, and its level was measured in the supernatant of the homogenate using a fluorometric kinetic assay according to the method of Beckman et al. (1989). The nitrite/nitrate (NOx) level was measured using the Griess reaction. First, the nitrate content of the sample was converted enzymatically to nitrite, which was then determined spectrophotometrically at 546 nm (Moshage et al. 1995). The plasma TNF-α level was measured by ELISA, while the malondialdehyde (MDA) level was used as an estimate of lipid peroxidation. High-resolution respirometry (Oxygraph-2k respirometer, Oroboros Instruments, Innsbruck, Austria) was used to assess the respiratory activity and reactive oxygen species (ROS) production capacity of rat liver mitochondria to evaluate mitochondrial function. We analyzed the changes in mitochondrial OxPhos respiratory activity and ROS production capacity using the substrate-uncoupler-inhibitor titration (SUIT) protocol.

3.5. Experimental protocols

Study 1 - Evaluation of ASA-Mono, ASA-Bis, and ASA-Tris

To investigate the GI side effects, we used 35 animals divided into 5 groups (n=7 per group). Group 1 served as the control, and the animals received the vehicle (10 mL/kg of buffered 0.11 M potassium hydroxide (KOH) solution, orally three times a day for three consecutive days). Group 2 received ASA treatment (0.55 mmol/kg, 10 mL/kg volume, orally three times a day for three days). Groups 3-5 were treated with the ASA conjugates at equimolar doses to ASA (orally three times a day for three days). Group 3 received ASA-Mono, Group 4 received ASA-Bis, and Group 5 received ASA-Tris. After the treatments, the animals were maintained on a carbohydrate-rich ad libitum diet. On the third day, 2 hours after the last treatment, the animals were anesthetized (sodium pentobarbital; 50 mg/kg i.p.). Following median laparotomy, OPS microcirculatory studies were performed on the gastric serosa. In vivo histological examinations of the gastric mucosa were conducted using CLSEM. At the end of the experiments, gastric and liver biopsies were collected to determine inflammatory parameters (XOR and MPO enzyme activity, NOx, MDA levels, Cyt c oxidase release), and venous blood samples were taken to measure plasma TNF-α levels.

Platelet aggregation measurement: The effect of ASA-Tris on platelet function was investigated in three groups of 15 rats (control, ASA, ASA-Tris; treatments as described above,

n=5 per group). Platelet aggregation was measured using an aggregometer (Multiplate analyzer, Roche, Basel, Switzerland) two hours after the last treatment.

Evaluation of analgesic effect: In the carrageenan-induced paw inflammation model, we tested the analgesic effect of ASA-Tris in three groups of 18 rats (positive control, ASA, ASA-Tris; treatments as described above, n=6 per group). Carrageenan (300 μg/30 μL) was injected into the right hind paw tibiotarsal joint, and ASA or ASA-Tris treatments were started 3 hours later in the 2nd and 3rd groups. The 1st group served as positive controls. The analgesic effect was determined using a dynamic plantar esthesiometer (mod-37450; Ugo Basile, Comerio, Italy).

Study 2 - Evaluation of the anti-inflammatory effect of ASA-Tris

To investigate the anti-inflammatory effect, we used the TNBS-induced rat colitis model. The study was conducted on 30 animals divided into 5 groups (control, Col, Col+ASA, Col+ASA-Tris, Col+Mes, n=6 per group). Colitis was induced by TNBS enema (40 mg/kg) in Groups 2-5. Groups 3 and 4 received ASA and ASA-Tris treatments, respectively, while Group 5 received repeated doses of mesalamine (Col+Mes; 0.77 mmol/kg (118 mg/kg, 10 mL/kg volume; three times a day for three days) 12 hours after colitis induction. Two hours after the last treatment, microcirculatory and in vivo histological examinations were performed, and inflammatory markers (XOR and MPO enzyme activity, NOx, MDA levels, Cyt c oxidase release) were determined from tissue samples, and plasma TNF-α levels were measured from blood samples.

The effects of ASA-Tris on mitochondrial function were investigated in three groups of 15 rats (control, ASA, ASA-Tris; treatments as described above, n=5 per group). On the third day, 2 hours after the last treatment, liver samples were collected from the anesthetized animals for the assessment of mitochondrial function.

Study 3 - Evaluation of the GI side effects of Ket-Tris

The protocol included 18 animals divided into 3 groups (control, Ket, Ket-Tris; n=6 per group). The control animals received the Ket vehicle (10 mL/kg, 0.11 M potassium hydroxide buffered solution). The 2nd group received Ket solution (0.56 mmol/kg, 10 mL/kg volume), and the 3rd group received the Ket-Tris conjugate (0.56 mmol/kg, 10 mL/kg volume). On the second day, the animals were anesthetized, and OPS microcirculatory studies were performed on the gastric serosa, followed by in vivo histological examinations of the gastric mucosa using CLSEM. At the end of the experiments, gastric and liver biopsies were collected to determine inflammatory

parameters (XOR and MPO enzyme activity, NOx, MDA levels, Cyt c oxidase release), and venous blood samples were taken to measure plasma TNF-α levels.

Evaluation of the anti-inflammatory effect of Ket-Tris: This was performed in the TNBS-induced rat colitis model. The study was conducted on 24 animals divided into 4 groups (control, Col, Col+Ket, Col+Ket-Tris, n=6 per group). Colitis was induced by TNBS enema (40 mg/kg) in Groups 2-4. Groups 3 and 4 received Ket (0.08 mmol/kg, 20 mg/kg, 10 mL/kg volume) or Ket-Tris (0.09 mmol/kg, 30 mg/kg, 10 mL/kg volume) treatment, respectively, 12 hours after colitis induction. The control and untreated colitis groups received the Ket vehicle (10 mL/kg, 0.11 M potassium hydroxide buffered solution). On the second day, 24 hours after the Ket or Ket-Tris treatments, the animals were anesthetized, and in vivo microcirculatory and histological examinations were performed in all groups. At the end of the experiments, colonic and liver biopsies were collected to determine inflammatory parameters (XOR and MPO enzyme activity, NOx, MDA levels, Cyt c oxidase release), and venous blood samples were taken to measure plasma TNF-α levels.

3.6. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Within the groups we applied Friedman repeated measures analysis of variance on ranks was applied. Time-dependent differences from the baseline for each group were assessed by Dunn's method. Differences between groups were analysed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures, median values and 75th and 25th percentiles are given; P values < 0.05 were considered significant.

4. RESULTS

Study 1

Evaluation of the GI side effects of ASA-Mono, ASA-Bis, and ASA-Tris: After ASA treatment, we observed significant weight loss, severe microcirculatory destruction, and mucosal damage, accompanied by a significant increase in the levels of inflammatory parameters (MPO, XOR, NOx, MDA, plasma TNF-α, Cyt c). ASA-Mono treatment did not cause weight loss, but moderate microcirculatory and tissue damage was detected in the gastric mucosa. Concurrently, elevated levels were measured in the following parameters: MPO, XOR, NOx, plasma TNF-α, Cyt c. With ASA-Bis treatment, no weight loss was observed, but microcirculatory and tissue

damage was detected in the gastric mucosa, accompanied by an increase in NOx levels. No adverse effects were observed with ASA-Tris treatment. There was no difference in animal weight, and no mucosal damage or increase in the examined markers was detected.

Effect of ASA-Tris on platelet aggregation: After ASA treatment, aggregation was reduced by approximately 80% (ASPI, ADP, and Col tests), while after ASA-Tris treatment, the ASPI test showed a 20% lower platelet aggregation rate compared to the control group.

Evaluation of the analgesic effect of ASA-Tris: In the carrageenan-induced model, pain was confirmed in all three groups at the 3rd hour. After the administration of ASA and ASA-Tris, we demonstrated that ASA treatment temporarily, while the ASA-Tris conjugate, persistently reduced the pain sensation.

Study 2

Evaluation of the anti-inflammatory effect of ASA-Tris: In the Col group, TNBS induction resulted in the appearance of bloody stool, diarrhea, and weight loss. Our in vivo histological examinations of the colon area showed severe tissue damage (edema formation, complete mucosal epithelium loss), while the gastric mucosa showed a normal histological picture. MPO and XOR enzyme activities were elevated in both colon and gastric tissues. The NOx level was significantly higher in the colon tissue, and plasma TNF-α levels also showed an increase compared to the control group. ASA treatment mitigated the damage to the colonic mucosa but caused severe gastric mucosal damage. Reduced inflammatory mediator levels were measured from the colon, while elevated levels were measured from the stomach. ASA treatment reduced the plasma TNF-α level. ASA-Tris treatment did not cause damage to either the colon or gastric mucosa. The examined tissue inflammatory parameter levels did not show an increase in the gastric and colon tissues. The plasma TNF-α level did not differ from the control group value. Mesalamine treatment showed similar effectiveness to ASA-Tris treatment.

Effects of ASA-Tris on mitochondrial function: The study showed that the respiratory activity linked to complex IV was significantly reduced in the ASA group compared to the control and ASA-Tris groups, indicating that ASA treatment inhibits mitochondrial respiration, which may lead to a decrease in cellular energy supply. In contrast, in the ASA-Tris group, the respiratory flux was restored to the control group level in response to sodium azide, suggesting that ASA-Tris treatment can compensate for the inhibition caused by ASA and normalize mitochondrial respiration. Mitochondrial function changes, mitochondrial ROS production: Our results

showed that neither ASA nor ASA-Tris administration significantly affected mitochondrial H2O2 production.

Study 3

Evaluation of the GI side effects of Ket-Tris: We demonstrated that 24 hours after Ket treatment, significant tissue damage occurred in the gastric mucosa, accompanied by a significant increase in pro-inflammatory markers (MPO, XOR, NOx, MDA, Cyt c, and TNF-α) compared to the control group. Our OPS examinations measured increased RBCV levels in the stomach, duodenum, jejunum, and ileum. In contrast, Ket-Tris conjugate treatment did not cause tissue damage or elevated inflammatory marker levels; the RBCV remained at normal levels in all examined intestinal segments.

Evaluation of the anti-inflammatory effect of Ket-Tris: In experimental colitis, we demonstrated that TNBS caused colon tissue damage and a significant decrease in the colonic serosal microcirculation, accompanied by elevated inflammatory parameters. Ket and Ket-Tris treatments were effective in preventing the development of tissue damage and microcirculatory impairment. Ket treatment significantly reduced XOR, MDA, and Cyt c levels, while the Ket-Tris conjugate also reduced MPO enzyme activity, along with decreased XOR, MDA, and Cyt c levels.

5. DISCUSSION

The physiological barrier function of the mucosa plays a fundamental role in maintaining the body's homeostasis. The integrity of this barrier function is often impaired with the use of NSAIDs, which can lead to severe complications, such as the development of GI ulcers and bleeding. The pathogenesis of NSAID-induced GI damage is characterized by increased vascular permeability and leukocyte accumulation in the mucosa (Vallance et al., 1990). NSAIDs can influence the functions of gastric mucosal cells (Alino et al., 2008; Pizzuto et al., 1997) and paradoxically stimulate the Ca2+-dependent release of TNF-α from activated macrophages, which can have a direct cytotoxic effect (Fiorucci et al., 1998).

To mitigate the adverse effects associated with NSAID treatment, several different strategies have been investigated, including enhancing the bioavailability of the drugs (Kumar et al., 2022) and combining NSAIDs with other analgesics or adjuvant agents (Pergolizzi et al., 2023). Current treatment strategies for the prevention of ulcers generally involve "modified-release", enteric-coated formulations, acid-suppressing agents, and antacids; however, achieving

effective multimodal therapy is challenging, especially in non-compliant patients (Evans et al., 1983, Scheen et al., 1983). Molecular modification of NSAIDs may be promising for reducing ulcerogenic potential, as experimental studies have demonstrated significantly reduced ulcerogenic effects with diclofenac derivatives (Bhandari et al., 2008, Ilango et al., 2015).

The aim of our present research was to synthesize compounds that retain the therapeutic efficacy of the original NSAIDs while having fewer GI side effects. The possibility of conjugation between NSAIDs and amino-alcohols, as well as their mechanisms of action, had not been previously investigated. Therefore, we developed new molecules by conjugating ASA and/or Ket with the Tris precursor. Our results showed that these conjugates are effective anti-inflammatory agents while causing less or no damage to the gastric mucosa.

In our experiments, we demonstrated the hemorrhagic mucosal damage induced by ASA in our in vivo rodent models and in vitro studies. Intravital histological examinations revealed that ASA caused significant loss of mucosal barrier function, leading to impairment of both serosal and mucosal microcirculation and severe endothelial injury. Polymorphonuclear (PMN) leukocytes play a significant role in this process, as indicated by the increased tissue MPO levels reflecting PMN accumulation. These changes were accompanied by an increase in the activity of xanthine oxidoreductase (XOR), which plays a key role in ROS production. We also observed increased tissue MDA, NOx, and Cyt c release, as well as elevated plasma TNF-α concentration, and significant Cyt c release from liver tissue. This demonstrates the spread of the inflammatory signaling - the localized damage resulted in systemic impairment.

In contrast, the ASA-Tris conjugate did not cause PMN accumulation, inflammatory enzyme activation, or an increase in the levels of inflammatory markers, either in the stomach or in the liver. Our in vivo histological examinations showed that the application of ASA-Tris did not result in mucosal damage, suggesting that this molecule could be a potentially safe alternative to traditional NSAIDs. The different responses observed among the various ASA conjugates (ASA-Mono, ASA-Bis) indicate that structural modifications can significantly influence the extent of GI damage, with ASA-Tris showing the most favorable therapeutic potential. Through our standardized platelet function tests (Boeer et al., 2010), we also found that ASA treatment significantly reduces the degree of COX-dependent aggregation and platelet activation. ASA-Tris had a moderate effect on COX-dependent aggregation, suggesting an alternative anti-inflammatory mechanism for this molecule, in which COX inhibition may not be the sole contributing factor.

ROS plays a crucial role in the ASA-induced acute mucosal ulceration (Fiorucci et al., 1998), as it can lead to lipid peroxidation, which compromises the integrity of biomembranes and disrupts cellular homeostasis (DeCuyper and Joniau, 1980; Slater et al., 1984). Lipid peroxidation, as indicated by increased MDA levels, damages membrane phospholipids and can lead to the release of Cyt c from the mitochondria. This release can initiate apoptosis through the activation of the caspase pathway (Linkermann et al., 2014). After ASA treatment, we observed increased MDA and Cyt c levels, as well as mitochondrial damage, while ASA-Tris did not elicit such effects. The administration of ASA-Tris also did not increase NOx levels, suggesting the absence of oxidative and nitrosative stress. These results indicate that ASA-Tris protects the cellular energy state, preserves cell function, and reduces mucosal injury. However, the precise mechanism underlying the effects of ASA-Tris remains unclear. The masked carboxyl group in ASA-Tris may minimize gastric irritation, and the efficacy appears to be proportional to the number of hydroxyl groups in the different ASA conjugates (Mono, Bis, Tris). The structure of ASA-Tris likely facilitates a redox regulatory mechanism; for example, through the hydroxyl groups, similar to flavonoids, which are known to have protective effects against oxidative damage. ASA-Tris may also function through a redox-buffering mechanism, akin to ethanol, which can increase NADH levels, elevate reduced glutathione levels, and shift the cellular redox balance towards a physiological state (Watson et al., 2011).

The unclear etiology and pathomechanism of IBD underscores the importance of developing new therapeutic options that can positively influence the course of the disease. Currently, the role of NSAIDs in the treatment of IBD is controversial, as COX inhibitors can both exacerbate and alleviate disease activity (Takeuchi et al., 2006; Ding et al., 2014). The hemorrhagic GI side effects further complicate and hinder their use. Nevertheless, NSAIDs may be valuable for IBD patients, especially those with concurrent musculoskeletal disorders (Jose and Heyman, 2008). Therefore, we also investigated the bioactivity of the new conjugates in the TNBS rat colitis model, comparing the results to the effects of mesalamine and the original molecules. Our data showed that ASA-Tris and Ket-Tris were effective against the colitis-induced weight loss and the increase in inflammatory markers, while preserving the integrity of the GI mucosa, in contrast to the original (ASA, Ket) molecules. Our results also suggest that the favorable effects of the new conjugates are likely related to their protective potential against oxidative biomembrane damage. In summary, our studies indicate that the ASA-Tris and Ket-Tris

conjugates represent a promising alternative to traditional NSAID therapies, minimizing GI side effects while maintaining therapeutic efficacy.

6. SUMMARY OF NEW FINDINGS

- **Successful synthesis of NSAID derivatives**: New NSAID derivatives have been synthesized by conjugating ASA and Ket with amino-alcohol precursors, demonstrating that biologically active products with promising pharmacological properties can be formed through ASA or Ket and Tris combinations.

- **Effective GI protection through Tris conjugation**: Tris conjugation emerges as an effective approach to minimize NSAID-induced GI side effects. The new Tris compounds offer significant protection against mucosal injury, preserving epithelial structure by reducing cytokine-driven pro-inflammatory processes.

- **Reduced GI damage with ASA-Tris conjugates**: Unlike the less efficient ASA-Mono and ASA-Bis derivatives, ASA-Tris conjugates did not induce GI damage or cause gastric bleeding, underscoring the added safety profile of the Tris conjugate.

- **Analgesic effects and platelet safety of ASA-Tris**: ASA-Tris demonstrated strong analgesic effects in carrageenan-induced paw inflammation without significantly impacting platelet function, contrasting with ASA's influence on platelet aggregation.

- **Anti-inflammatory efficacy in colitis model**: ASA-Tris and Ket-Tris conjugates showed significant anti-inflammatory effects in a TNBS-induced colitis model, achieving this without gastric damage. Mitochondrial assessments suggest that ASA-Tris's benefits do not stem from direct radical scavenging but are likely due to its protective effects against oxidative biomembrane damage, leading to improved cell viability and preserved mucosal morphology compared to traditional NSAIDs.

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