

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

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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**

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ABBREVIATIONS

ADP	adenosine-diphosphate
ASA	acetylsalicylic acid
CLSEM	confocal laser scanning endomicroscope
COX	cyclooxygenase enzyme
Cyt c	cytochrome c oxidase
FITC-dextran	fluorescein isothiocyanate-dextran
GI	gastrointestinal
Ket	ketoprofen
MDA	malondialdehyde
MPO	myeloperoxidase enzyme
NO-NSAID	nitric oxide-releasing non-steroid anti-inflammatory drug
NSAID	non-steroid anti-inflammatory drug
OPS	orthogonal polarization spectral imaging
PG	prostaglandins
RBCV	red blood cell velocity
RET	reverse electron flux
ROS	reactive oxygen species
SUIT	substrate-uncoupler-inhibitor titration
SPRD	Sprague-Dawley
TMPD	N,N,N,N-tetramethyl-p-phenylenediamine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF- α	tumor necrosis factor-alpha
Tris	tris-hydroxymethyl-aminomethane
TXA ₂	thromboxane A ₂
XOR	xanthine oxidoreductase enzyme

LIST OF CHEMICAL COMPOUNDS

H ₂ O ₂	hydrogen peroxide
H ₂ S	hydrogen sulphide
H ₂ SO ₄	sulfuric acid
KOH	potassium hydroxide
K ₃ PO ₄	tripotassium phosphate
NO	nitric oxide

SUMMARY

The integrity of the mucosa is vital for maintaining gastrointestinal (GI) homeostasis. Non-steroidal anti-inflammatory drugs (NSAIDs) are well-documented for causing significant damage to this barrier, resulting in ulcerations and bleeding. NSAIDs disrupt mucosal cell function and paradoxically stimulate the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), which exert cytotoxic effects. These results can lead to microvascular leakage, leukocyte accumulation, and mitochondrial dysfunction within the mucosa. To mitigate these adverse outcomes, several strategies have been explored, including enhancing NSAID bioavailability, utilizing modified-release formulations, co-administering NSAIDs with other analgesics or adjuvant agents, and using acid secretion inhibitors or antacids. However, implementing effective multimodal therapy can be challenging, particularly in non-compliant patients. Consequently, molecular modification of NSAIDs to reduce their ulcerogenic potential has emerged as a promising area of research.

In this series of studies, we hypothesized that the chemical conjugation of NSAIDs with tris(hydroxymethyl)aminomethane (Tris), an amino alcohol, could achieve this goal. Specifically, we investigated the effects of newly synthesized NSAID compounds - acetylsalicylic acid (ASA)-Tris and ketoprofen (2-(3-benzoylphenyl) propanoic acid; Ket)-Tris – which were designed to retain the anti-inflammatory and analgesic properties of their parent drugs while minimizing their ulcerogenic potential.

Extensive experimental evidence demonstrated that ASA-Tris and Ket-Tris conjugates caused significantly less mucosal damage compared to their parent NSAIDs. In vivo studies confirmed that ASA-Tris neither induced leukocyte accumulation nor activated inflammatory enzymes. Moreover, it preserved microvascular integrity and prevented increases in inflammatory markers, supporting its potential as a safer alternative to conventional NSAIDs. Further investigation into ASA-induced mucosal injury revealed that ASA administration led to endothelial damage, loss of epithelial integrity, and elevated oxidative stress markers. In contrast, ASA-Tris administration did not increase these markers, suggesting a protective mechanism against oxidative and nitrosative stress. While the exact mechanism of ASA-Tris action remains unclear, additional studies involving ASA and mono- and bis-hydroxymethylaminomethane conjugates suggested that the protective effects may be attributed to redox control or antioxidant properties conferred by the hydroxyl groups of these novel compounds.

In the context of inflammatory bowel disease (IBD), where NSAIDs can have dual effects - either exacerbating or alleviating symptoms - the therapeutic potential of ASA-Tris was evaluated in an experimental rodent colitis model. ASA-Tris effectively mitigated weight loss and mucosal damage, demonstrating efficacy comparable to mesalamine, a standard treatment for IBD. Importantly, ASA-Tris did not induce gastric damage or alter inflammatory markers in the stomach, highlighting its safety profile.

Overall, these findings suggest that chemical modifications by conjugating NSAIDs with Tris, can significantly reduce the ulcerogenic potential of these drugs. This approach holds considerable promise for developing safer NSAID formulations that minimize GI side effects, thereby enhancing therapeutic outcomes.

1. INTRODUCTION

1.1. The progression of inflammation

Injuries such as physical trauma, thermal exposure, ionizing radiation, chemical agents, infections, and disruption in local blood flow can all initiate an inflammatory reaction. The primary objective of the response is to eliminate harmful agents, remove damaged tissues, and provide ways to restore tissue integrity and function. This cascade of events begins almost immediately after the harmful stimulus, leading to the production and dissemination of various signalling molecules and intermediates that facilitate the subsequent steps (Kumar et al, 2004). A central component of this process is the release of arachidonic acid (AA) from membrane phospholipids, mediated by the enzyme phospholipase A₂. AA serves as a crucial precursor for two principal pathways in the inflammatory cascade: the cyclooxygenase (COX) pathway and the lipoxygenase (LOX) pathway. Briefly, the LOX pathway leads to the synthesis of leukotrienes, while the COX-1 enzyme produces prostaglandins essential for normal cellular functions, including renal and gastrointestinal (GI) homeostasis. The upregulation of cyclooxygenase-2 (COX-2, also known as prostaglandin-endoperoxide synthase 2) results in an increased production of prostanoids during inflammatory states. Beyond its role in inflammation, COX-2 is implicated in various pathophysiological conditions, including lung and colorectal cancers and neurodegenerative disorders such as Alzheimer's disease (Murakami et al, 2017, Vane et al., 1998),

1.1.1. Potentially protective and detrimental tissue changes

The most notable circulatory changes during acute inflammation primarily occur within the microvasculature. The microcirculatory response is marked by hyperaemia in the affected area, with increased vascular permeability which facilitates the extravasation of plasma proteins and fluid into the surrounding tissues. Simultaneously, leukocyte migration from the bloodstream into the interstitial space is initiated. If the pro-inflammatory condition persists, angiogenesis is stimulated, supplying blood to newly formed tissue. The progression to a subacute or chronic inflammatory state leads to prolonged cellular activation, macrophage infiltration, persistent oedema, and tissue damage (Treppeles et al, 2006, Feletou et al, 2006). Indeed, inflammation can have both beneficial and detrimental effects on tissue and organ function, depending on its cause, type, and severity. Acute, self-limiting inflammation, such as that seen in wound healing, typically progresses to scar formation and tissue remodelling (Maher et al, 2011, Murata et al, 2018). In this process, activated macrophages play pivotal roles in regulating tissue regeneration and repair. They achieve this by activating progenitor cells, promoting extracellular matrix

formation to provide scaffolding for regeneration, and facilitating angiogenesis (Wynn et al, 2016, Vanella et al, 2017, Smigiel et al, 2018). Nonetheless, under prolonged inflammatory conditions these activities may become harmful often leading to irreversible tissue damage and pathological fibrosis (Oishi et al, 2018). Histotoxicity is driven by various further mechanisms, including polymorphonuclear (PMN) leukocyte-endothelium interactions and oxidative-proteolytic pathways (Dallegrì et al, 1997).

1.2. Intraarticular and bone inflammation

Physiologically, bones are not only critical components of the musculoskeletal system but also serve as essential mineral storage sites. They are closely linked to the haematopoietic system and, therefore, play a role in immune responses (Maneerat et al, 2021, Guder et al, 2020). In chronic inflammatory diseases of the bones and joints, such as rheumatoid arthritis or osteoarthritis, significant bone mass loss can occur due to disrupted homeostasis (Pietschmann et al, 2022, van Bodegraven et al, 2020). Osteoarthritis is one of the most common conditions, particularly in ageing individuals, leading to physical limitations and reduced activity (Vincent et al, 2022). Chronic, low-grade inflammation contributes to the disease by triggering catabolic responses in chondrocytes. Moreover, the inflammation affects not only the articular cartilage but also other tissues, including subchondral bone, synovial tissue, ligaments, and the meniscus (Zhang et al, 2021, Griffin et al, 2019, Schulze-Tanzil et al, 2019, Radi et al, 2005).

1.3. Inflammation in the gastrointestinal tract

Inflammation of the gastrointestinal (GI) system presents challenges in selecting appropriate therapies, as the absorption of oral medications is often hindered. Furthermore, the chemical properties of drugs become crucial when treating damaged GI tissues (Barros et al, 2019, Parikh et al, 2019).

Gastritis, particularly chronic gastritis, is a common condition that can persist throughout life and plays a significant role in the development of ulcerative disorders and stomach cancers (Sipponen et al, 2015). It typically begins with superficial mononuclear inflammation, often accompanied by acute PMN cell infiltration. As the condition progresses, it may lead to atrophic gastritis, characterized by the loss of normal mucosal glands. The depletion of mature, functional glandular and epithelial cells further accelerates the progression of atrophic gastritis (Sipponen et al, 2015). *Helicobacter pylori* infection is a well-recognized and prevalent risk factor for chronic gastritis, significantly influencing treatment decisions for the disease (Lahner et al, 2014, Lahner et al, 2009, Kaptan et al, 2000).

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic disorders characterized by inflammation of the GI tract. These conditions arise from a complex interplay of genetic, environmental, and immune factors, leading to immune dysregulation. Ulcerative colitis primarily affects the colon and rectum, whereas Crohn's disease can impact any part of the GI tract. Symptoms commonly include abdominal pain, diarrhoea, and weight loss, with alternating periods of flare-ups and remission. In IBD, various local and systemic immune processes contribute to tissue damage and disruption of the mucosal barrier (Parikh et al, 2019, Xavier et al, 2007).

Increased intestinal permeability is an early event in IBD pathogenesis, disrupting the delicate balance between the mucosal barrier and luminal components. When the symbiotic relationship between the microbiota and mucosal barrier integrity is compromised, dysbiosis may occur, further exacerbating permeability dysfunction (Stange et al, 2019, Liu et al, 2021). IBD is characterized by both local and systemic immune responses, which involve the recruitment of effector cells and the release of humoral mediators, collectively exacerbating the breakdown of the mucosal barrier (Appleyard et al. 2002; Hatoum et al. 2003; Hatoum and Binion 2005). The exact pathway through which intestinal tissue damage develops in IBD remains unclear. However, evidence suggests that impaired mitochondrial oxidative phosphorylation (OxPhos) plays a critical role in the disease's pathomechanism. In IBD patients, the colonic mucosa often exhibits lower adenosine triphosphate (ATP) levels, and significantly decreased mitochondrial Complex II activity has also been observed (Kameyama et al. 1984; Santhanam et al. 2012).

1.3.1. Therapeutic possibilities in GI inflammatory conditions

In cases of gastritis, current treatments focus on reducing inflammation, managing symptoms, and maintaining remission. Proton pump inhibitors (PPIs) and H₂ receptor antagonists are commonly prescribed to decrease gastric acid production, thereby promoting the healing of the gastric mucosa. Antacids offer quick relief by neutralizing stomach acid. When *Helicobacter pylori* infection is detected, a combination of antibiotics and PPIs is used to eradicate the bacteria. Additionally, lifestyle modifications - such as dietary adjustments and avoiding substances that may damage the mucosa, like certain medications or alcohol - can help alleviate symptoms and prevent recurrence.

For IBD, current treatment strategies include immune modulators, anti-inflammatory agents, and anti-cytokine therapies such as TNF- α blockers (Rutgeerts et al. 2004). Non-steroidal anti-inflammatory drugs (NSAIDs) may also be beneficial for some IBD patients, particularly those with extraintestinal musculoskeletal disorders (Jose and Heyman 2008). However, these

therapies carry a risk of potentially severe side effects, raising concerns about their long-term tolerability (Blackburn et al. 2002; Donihi et al. 2006; Hasselgren et al. 2010). NSAIDs, in particular, are associated with an increased risk of GI mucosal injury and ulcerative damage, especially with regular, long-term use.

Mesalamine, a 5-aminosalicylic acid compound (5-ASA) (Managlia et al. 2013), is frequently used as a first-line therapy for IBD (Braus and Elliott 2009). It inhibits the production of pro-inflammatory compounds, including leukotrienes and prostaglandins, thereby helping to manage symptoms and maintain remission. Because mesalamine acts locally in the bowel, it tends to have relatively few systemic side effects (Bickston and Cominelli 2003).

1.3.2. Experimental models of human IBD

Dextran sodium sulfate (DSS) or 2,4,6-trinitrobenzene-sulfonic acid (TNBS) administration in rodents are widely used experimental methods for modelling human IBD. TNBS-induced colon damage is a common murine model for IBD in preclinical efficacy studies, a single intracolonic enema of TNBS induces transmural colitis that lasts over eight weeks and replicates several symptoms of human IBD (Morris 1989). This model is characterised by weight loss (Morris 1989), visceral hyperalgesia (Zhou 2008), significant elevations in pro-inflammatory cytokines, including TNF- α (Neurath 1997) and interleukin-6 (IL-6) (Hove 2001), extensive ulcerations, morphological changes (Tatsumi 1996), and tissue leukocyte accumulation (Kiss 1997). Additionally, TNBS administration has been shown to cause significant structural damage to epithelial mitochondria, leading to swelling and disruption of the inner membrane (Bou-Fersen et al. 2008). Reduced Complex II activity has also been demonstrated in TNBS- and DSS-induced experimental colitis as well (Santhanam et al. 2012).

1.4. Steroids and NSAIDs

Steroid compounds such as prednisone and hydrocortisone, are commonly used in human pharmacology and pharmacotherapy (Dinarello et al, 2010). The anti-inflammatory effects are mediated through inhibition of the production of pro-inflammatory mediators, thereby reducing immune cell activation. The rapid onset of action and effectiveness could make them a first-line treatment option in controlling inflammation but the potential for significant side effects, such as increased susceptibility to infections, osteoporosis, and metabolic disturbances, necessitates careful consideration of their use, especially for long-term treatment. While NSAIDs are effective for mild to moderate inflammation, especially in musculoskeletal pain, they are generally less potent than corticosteroids for severe conditions and carry also risks of

side effects such as GI bleeding, renal impairment, and cardiovascular issues, especially with prolonged use.

1.4.1. NSAID classifications

NSAIDs embrace a large class of drugs with structural and functional diversity with different selectivity to inhibit the COX/prostaglandin-endoperoxide synthase (PGHS) enzymes (Kumar et al, 2018). Salicylates have dose-dependent antipyretic, anti-inflammatory, antithrombotic, and analgesic properties, achieved through the inhibition of COX enzymes (Rao et al, 2008, Melmon et al, 1969, Higgs et al, 1987, Huang et al, 2011, Rodriguez et al, 2019). Propionic acid derivatives, which inhibit COX enzymes non-selectively, are commonly used in the treatment of rheumatic and other joint diseases (Cathcart et al, 1973). Indomethacin, the most commonly used acetic acid derivative, is administered in joint diseases and obstetrics for tocolysis (Baber et al, 1979, Amin et al, 2007, Rath et al, 2018). In addition to its positive properties, it has serious side effects, including sodium retention, leading to oedema and hypertension, tachycardia, and bronchospasm (Donker et al, 1976, Michell et al, 1983). Pyrazolone derivatives, unlike other NSAIDs, have rapidly reversible effects on inflammatory processes and bind minimally to plasma proteins (Volz et al, 1980). Oxycam derivatives can act as non-selective COX inhibitors (e.g. piroxicam) and as selective COX-2 inhibitors (e.g. meloxicam), depending on the structure of the molecule. Selective COX-2 inhibitors may cause fewer GI side effects, although long-term use can increase blood clotting and lead to cardiovascular problems (Xu et al, 2014, Lipscomb et al, 1998, Mukherjee et al, 2001).

Classification	Examples	Characteristics	Common Uses
Salicylates	Acetylsalicylic Acid (ASA)	Irreversibly inhibits COX enzymes, reducing pain and inflammation.	Pain relief, anti-inflammatory, cardiovascular protection
Propionic Acid Derivatives	Ibuprofen, Naproxen, Ketoprofen	Reversibly inhibits COX enzymes, lower GI irritation compared to ASA.	Pain, fever, arthritis, menstrual pain
Acetic Acid Derivatives	Indomethacin, Diclofenac, Ketorolac	Potent anti-inflammatory agents, more selective for COX-2.	Acute pain, rheumatoid arthritis, osteoarthritis
Oxycam Derivatives	Piroxicam, Meloxicam	Long half-life, better selectivity for COX-2.	Chronic pain conditions like arthritis
COX-2 Inhibitors	Celecoxib, Etoricoxib	Selectively inhibit COX-2, reduced GI side effects.	Osteoarthritis, rheumatoid arthritis, acute pain

Classification	Examples	Characteristics	Common Uses
Fenamate Derivatives	Mefenamic Acid, Meclofenamate	Less commonly used, moderate inhibition of COX enzymes.	Short-term management of mild to moderate pain
Naphthylacetic Acid Derivatives	Nabumetone	Prodrug with lower GI toxicity, converted to active metabolite.	Long-term management of arthritis
Pyrazolone Derivatives	Phenylbutazone, Metamizole	Anti-inflammatory and analgesic, less used due to adverse effects.	Severe pain and inflammation

Table 1. Classification of NSAIDs

1.4.2. ASA and ketoprofen

ASA is a prototype NSAID and one of the most widely used salicylate drugs in general medical practice. At higher doses, it has analgesic, antipyretic, and anti-inflammatory effects, while lower doses are commonly used to prevent cardiovascular thrombotic events (Baigent et al., 2009). ASA is a potent inhibitor of the constitutive COX-1 isoform in platelets, but it also has a range of other pharmacological effects, such as reducing the synthesis of coagulation factors (Meade et al., 1992; Schrör et al., 1997). Regular use of ASA can lead to upper GI tract toxicity (Sørensen et al., 2000; Delaney et al., 2007).

Ket is also frequently used NSAID with more potent anti-inflammatory activity than ASA [6]. It inhibits both COX-1 and COX-2 isoforms, leading to decreased synthesis of prostaglandin and thromboxane precursors and thereby also reducing platelet aggregation. Ket is primarily indicated for the symptomatic treatment of acute and chronic rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, primary dysmenorrhea, and mild to moderate pain associated with musculoskeletal injuries. However, it is also among the most GI-toxic NSAIDs, posing a significant risk of GI adverse effects (Kantor et al, 1986, Lastra et al, 2002, Jerussi et al, 1998).

1.4.3. Mechanisms of NSAID side effects

The general mechanism of action of NSAIDs involves the inhibition of COX/prostaglandin-endoperoxide synthase (PGHS-1 and PGHS-2), regulatory enzymes involved in the biosynthesis of prostaglandins (PG), which play a significant role in inflammation. PGHS-1 is considered a housekeeping gene, participating in various biological functions, including the protection of the gastric mucosa, whereas PGHS-2 is primarily responsible for inflammation. Some NSAIDs are non-selective inhibitors of both enzymes, while others are specific, particularly the "coxibs", which selectively inhibit PGHS-2. Previously, it was thought that

inhibitors of PGHS-1 might lead to GI complications by inhibiting PG synthesis within the gastric mucosa. However, substantial evidence contrary to this view has emerged in recent years (Bindu et al, 2020). At one point, a switch to coxibs was regarded as effective. Since PGHS-2 is inducible and not directly involved in gastric mucosal protection but rather in inflammation, inhibition of PGHS-2 by coxibs was thought to be relatively safe. Nevertheless, emerging evidence has challenged this theory as well and placebo-controlled trials also demonstrated that these inhibitors were linked with an increased risk of atherothrombotic vascular events (Masclée et al, 2013).

Alongside the development and establishment of the PGHS/PG-dependent pathway of NSAID toxicity, other studies demonstrated direct impact of NSAIDs on mitochondria, followed by the onset of cellular oxidative stress and apoptosis, as an independent pathway of NSAID-induced cytopathology. Beyond GI and cardiovascular complications, regular NSAID use is also associated with nephrotoxicity and occurrences of hepatotoxicity. Nevertheless, the exact mechanisms of these adverse effects induced by NSAIDs remain unknown.

1.4.4. Mechanisms of NSAID side effects in the GI tract

It is rather unfortunate that the mucosal side effects of such effective compounds are also significant. Adverse effects range from mild to severe structural and functional damage to the mucosal layers (Appleyard et al., 2002), which can result in hemorrhage, ulcer formation, or even perforation. The causes of bleeding are often multifactorial, but two primary mechanisms have been identified, the irreversible inhibition of COX-1 and direct, non-prostaglandin-mediated topical irritation that alters endothelial function and mucosal permeability (Hochain et al., 2000). Additionally, NSAIDs, like ASA can impact mitochondrial respiratory activity and thiol redox functions, leading to apoptosis-inducing signals and changes in mitochondrial membrane potential (Nulton-Persson et al., 2004; Raza et al., 2012; Redlak et al., 2005).

1. NSAIDs achieve their effects through inhibition of the COX pathway, leading to a reduction in prostaglandin-E2 (PGE2) levels. While this decrease alleviates inflammation-related edema, fever, and pain, it also reduces TXA2 levels, diminishing platelet aggregation, prolonging bleeding time, and increasing the risk of bleeding. NSAIDs also reduce prostacyclin (PGI2) production, which worsens the loss of cytoprotective effects on the GI mucosa (Bacchi et al., 2012, Henry et al., 1988, Vane et al., 1994). The reduction in prostaglandin levels also decreases vasodilation, which in turn reduces edema at inflammation sites. Additionally, this reduction lowers the sensitivity of nociceptive nerve endings to pain-inducing substances, providing

analgesic relief (Jang et al., 2020). NSAIDs' antipyretic effects also arise from this mechanism, as pyrogens typically increase hypothalamic prostaglandin levels to induce fever; without these prostaglandins, fever does not occur (Yang et al., 2020).

2. NSAIDs can also cause indirect damage by weakening the GI tract's defence mechanisms (Vallace et al., 2008). In this line the acidic carboxyl group of NSAID molecules can contribute to tissue lesions and can directly harm the GI mucosa due to their acidic nature (Bjarnason et al., 1993, Price et al., 1990).
3. Interference with mitochondrial energy production by inhibiting oxidative phosphorylation (OxPhos) is another possibility. The reduction in OxPhos function can impair intercellular junctions, increasing mucosal permeability and facilitating the translocation of bacteria or bacterial products from the lumen (Chan et al., 2005, Bjarnason et al., 2018).
4. Nephrotoxicity is another significant adverse effect, as certain NSAIDs can stimulate angiotensin-II-mediated vasoconstriction, thereby reducing renal blood flow and glomerular filtration rate (Dunn et al., 1984).

To sum up, NSAIDs exhibit varying effects and COX-2 selectivity based on their chemical characteristics. By lowering prostaglandin levels, NSAIDs compromise the GI mucosa's protective mechanisms, potentially leading to tissue lesions (Price et al., 1990, Bjarnason et al., 2018, Huang et al., 2011, Rodriguez et al., 2019). Such mucosal damage can cause bleeding, ulcers, and, in severe cases, mucosal necrosis.

1.4.5. The possibilities of prevention of NSAID-induced cell or organ damage

To avoid superficial direct harms and ulcers, the reduction of acidic character of a drug molecule can be possible solution (Narsinghani et al., 2014). Further drug categories that can protect the gastric mucosa can be listed as follows:

- Acid secretion inhibitors: H₂-receptor blockers, muscarinic receptor antagonists, proton pump inhibitors, and others, such as gastrin antagonists.
- Neutralizers of secreted acid: sucralfate, prostaglandins, colloidal bismuth.
- H. pylori-eradicating antibiotics: metronidazole, tetracycline, amoxicillin, clarithromycin, among others (Deakin et al., 1992, Londong et al., 1986, Minalyan et al., 2017, Zhou et al., 2022, Calam et al., 1993, Shea-Donohue et al., 1986, Suzuki et al., 2010, Marcus et al., 2016).

In case of NSAID-induced ulcers, H₂ receptor antagonists can provide rapid and significant relief if NSAID therapy is discontinued concurrently (Deakin et al., 1992, Tuskey et al., 2013). When NSAIDs are used long-term, proton pump inhibitors are generally necessary to prevent gastric and duodenal ulcers (Scheiman et al., 2013, Gwee et al., 2018). While gastric protective agents are often effective, the severity and nature of harmful factors can impact therapy effectiveness, making it challenging to adjust dosages for optimal patient safety (Gwee et al., 2018, Lazzaroni et al., 2001, Patel et al., 2020). Additionally, patient non-compliance and multi-drug regimens complicate effective treatment (Thummak et al., 2023, Scheen et al., 2010, Evans et al., 1983). To mitigate NSAID-induced side effects and improve patient safety, ongoing evaluation and timely modification of NSAID therapies are essential.

To-date several other methods were developed to widen NSAID usability with increased patient safety. In general terms, these strategies focus on restoring the balance between harmful factors and protective mechanisms. Most approaches aim to reduce damaging influences, while some others target the enhancement of mucosal protective processes (Gautam et al., 2013, Shin et al., 2008). A prerequisite of NSAID modifications is to keep up or even improve the anti-inflammatory, analgesic, and cardioprotective properties while minimizing GI-related complications. In this line several modifications have been developed, such as the maintenance of ASA in the ionic form or preventing it from dissolving until it reaches the small intestine, but the efficacy of these approaches is still suboptimal. Further approaches involved the linking of NSAIDs to hydrogen sulphide (H₂S) or nitric oxide (NO) release to protect against the ulcerogenic effects of NSAID therapy (Fiorucci et al., 2001, Gemici et al., 2015). H₂S, which is naturally produced by the gastric mucosa, plays a significant protective role by inhibiting leukocyte adhesion to vascular endothelium during inflammation (Gemici et al., 2015). In the presence of direct harmful effects, such as those from acidic NSAID molecules, excess H₂S in theory, can counteract destructive processes through its anti-inflammatory properties (Vallace et al., 2007). Additionally, H₂S participates in reparative mechanisms, with elevated levels accelerating healing, as the physiological response to GI damage includes an increase in H₂S production (Vallace et al., 2007).

NO-releasing NSAIDs (NO-NSAIDs) are typically created by attaching a nitroxybutyl or nitrosothiol group to the NSAID molecule via a short-chain linker (Fiorucci et al., 2001). Experimental studies have demonstrated that NO-NSAIDs retain the anti-inflammatory and antipyretic efficacy of their parent NSAIDs, but with reduced GI toxicity (Keeble et al., 2002, Konturek et al., 2002). NO-aspirin (NO-ASA), for instance, has been shown to have enhanced

antithrombotic effects compared to standard aspirin, likely due to the combination of COX-1 inhibition and NO's anti-adhesive action (Gresele et al., 2006). Additionally, NO derivatives of NSAIDs are better suited for topical delivery, targeting the brain more effectively and potentially enhancing both analgesic and anti-inflammatory effects (Fonseca et al., 2015).

1.4.6. Approaches to eliminate the acidic characteristic of NSAID molecules

Another approach involves modifying the NSAID molecule through its carboxyl group, which changes its acidic properties, with the final compound's effectiveness determined by the attached side group (Narsinghani et al., 2014). Diclofenac ((2-(2-((2,6-dichlorophenyl)amino)phenyl)acetic acid), widely used for managing musculoskeletal pain and inflammation, exemplifies both direct and indirect harmful effects typical of NSAIDs. Due to its widespread use, diclofenac is frequently studied to mitigate its GI toxicity. Modifying its carboxyl group, with azoles like oxadiazole or thiazole, has shown promise (Bhandari et al., 2008). Studies on animal models indicate that certain 1,3,4-oxadiazole derivatives of diclofenac exhibit stronger anti-inflammatory effects than the standard molecule while causing significantly fewer GI ulcerations (Ilango et al., 2015, Somani et al., 2011).

Mefenamic acid (2-[(2,3-dimethylphenyl)amino] benzoic acid), another commonly used NSAID, has also been conjugated with oxadiazole. Results are similar to those seen with diclofenac-oxadiazole: the conjugate retains substantial anti-inflammatory properties while causing only mild GI side effects (Somani et al., 2011).

Among newly developed NSAIDs, dexketoprofen trometamol (S(+)-ketoprofen; (S)-2-(3-benzoylphenyl)propanoic acid) has proven particularly effective and is now used clinically. Racemic ketoprofen is one of the most potent inhibitors of prostaglandin synthesis, but it reaches maximum plasma concentration more slowly than dexketoprofen, resulting in about half the analgesic efficacy of the modified compound (Mauleon et al., 1996, McGurk et al., 1998). Along with its faster and more potent effects, dexketoprofen (and dexibuprofen) has demonstrated higher tolerability in humans, making it well-suited for acute pain and short-term symptomatic relief in osteoarthritis (Beltran et al., 1998, Zamani et al., 2014).

1.4.7. Chemical modifications of the NSAID structure to develop novel NSAID-derivatives

In view of this background, it seems plausible that a new drug synthesized from NSAID and suitable precursors might favourably affect the degree of mucosal damage. In theory, a harmful effect can be reduced by the modification of the chemical structure of a drug molecule, but preserving the original biochemical function can be a great challenge (Uzzaman et al., 2020,

Narsinghani et al., 2014, Rahman et al., 2024). Tris(hydroxymethyl)aminomethane or 2-amino-2-(hydroxymethyl)-1,3-propanediol (commonly known as Tris) is widely recognized for its applications in biochemistry (Gomori et al., 1955). Tris is frequently utilized as a component in buffer solutions that are effective within a pH range of 7.07 to 9.07. The effects of Tris buffers significantly impact arterial pH and base deficit, as well as reduce the partial pressure of carbon dioxide in arterial blood, making it a preferred alternative for patients with mixed acidosis (Hoste et al., 2005; Kallet et al., 2000). In addition to its influence on acid-base balance, Tris can also inhibit the activity of certain enzymes, including aminopeptidases and alpha-amylases (Desmarais et al., 2002; Ghalanbor et al., 2008). Tris was also tested as a cationic salt forming agent in a 1:1 combination with the NSAID ketorolac-tromethamine to increase the solubility of the formulation (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 $200 \text{ g} \pm 10 \text{ g}$) housed in plastic cages under a 12-h dark-light cycle, in a thermoneutral environment ($21 \pm 2 \text{ }^\circ\text{C}$). 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. Chemical synthesis and characterizations of ASA-Mono-, Bis-, Tris-conjugates

The synthesis of the compounds and the supporting ^1H NMR and HPLC studies were carried out in the Department of Medical Chemistry, University of Szeged. Briefly, ASA was dissolved in absolute tetrahydrofuran and cooled to $-15 \text{ }^\circ\text{C}$, then isobutyl chloroformate and triethylamine were added under stirring. After stirring at $-15 \text{ }^\circ\text{C}$ for 25 min, an equimolar amount of ethanolamine (Mono), or 2-amino-1,3-propanediol (Bis) or tris-hydroxymethyl-aminomethane (Tris) was added and the mixture was stirred for an additional 1 h at $0 \text{ }^\circ\text{C}$, and then at room temperature overnight. The reaction mixtures were filtered, evaporated and the resulting crystalline materials were washed with diethyl ether-hexane, resulting in the pure products (Figure 1). Chemical properties of ASA-Mono, -Bis and -Tris are presented in Supplement I.

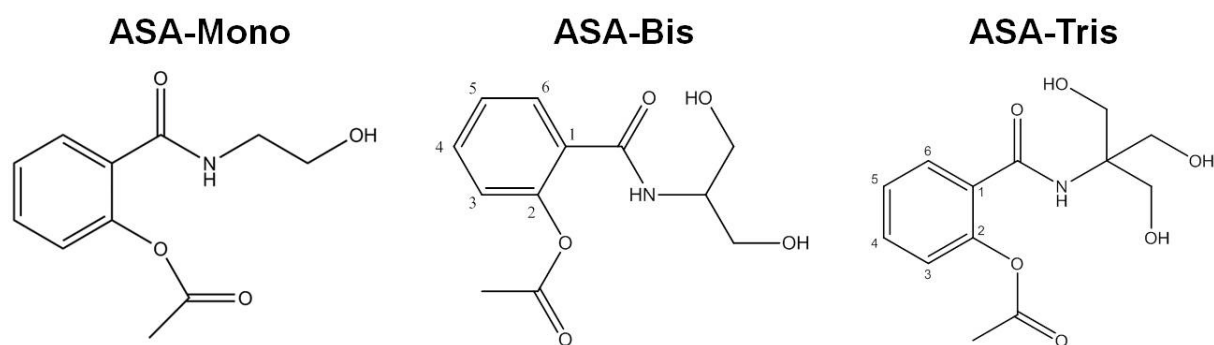


Figure 1. Structural formulae for the ASA-Mono, ASA-Bis and ASA-Tris conjugates (Miklós Glyczy was until 09.2016 applicant and proprietor of European patent application EP 2889286A1 and International patent application WO 2015/101501 (PCT/EP2014/078296) entitled “Pharmaceutically active compound for use as anti-inflammatory agent”).

3.2.2. Chemical synthesis and characterization of Ket-Tris conjugate

The synthesis and the supporting ^1H NMR and HPLC studies were carried out at the Department of Medical Chemistry, University of Szeged. Briefly, Ket (5.09 g) was dissolved in absolute tetrahydrofuran (200 ml) and cooled to -15°C . Isobutyl chloroformate (2.62 ml) and triethylamine (2.79 ml) were added while stirring. After stirring at -15°C for 25 min, Tris (2.42 g) was added and the mixture was stirred for an additional 1 h at 0°C and then at room temperature over-night. The reaction mixtures were filtered and evaporated, and the resulting crystalline materials were washed with diethyl ether-hexane, resulting in the pure products (2.9 g, 40.56 %, Ms 358.06) (Figure 2). Chemical properties of Ket-Tris are demonstrated in Supplement II.

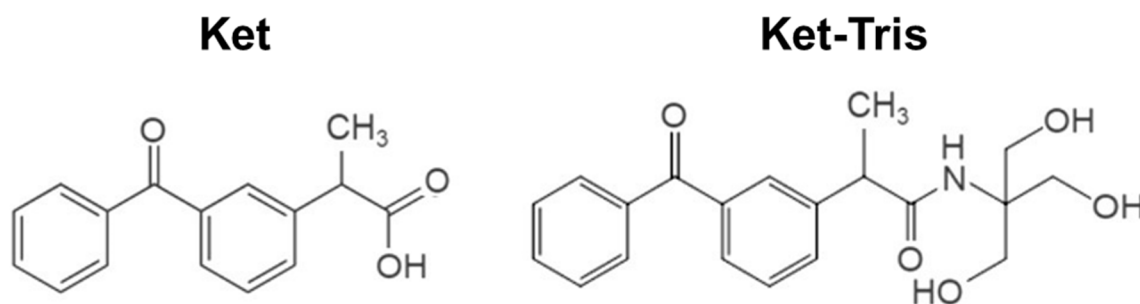


Figure 2. Structural formulae for the Ket and developed Ket-Tris conjugate (Miklós Ghyczy was until 09.2016 applicant and proprietor of European patent application EP 2889286A1 and International patent application WO 2015/101501 (PCT/EP2014/078296) entitled “Pharmaceutically active compound for use as anti-inflammatory agent”).

3.3. *In vivo* experimental methods

3.3.1. Direct measurements of the intestinal microcirculation

For the non-invasive visualization of the gastric or colon serosal microcirculation we applied orthogonal polarization spectral (OPS) imaging (Cytoscan A/R, Cytometrics, Philadelphia, PA, USA). This technique utilizes reflected polarized light at the wavelength of the isobestic point of oxy- and deoxyhaemoglobin (548 nm). Only photons scattered from a depth of 2-300 μm contribute to image formation, as polarization is preserved in reflection. A 10x objective was placed onto the serosal surface of the stomach, and microscopic images were recorded with an S-VHS video recorder 1 (Panasonic AG-TL 700; 7 Matsushita Electric Ind. Co. Ltd, Osaka, Japan). Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Changes of red blood cell velocity (RBCV;

$\mu\text{m/s}$) in the postcapillary venules were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). All the microcirculatory evaluations were performed by the same investigators (Melinda Ugocsai, Norbert Lajkó and Gabriella Varga).

3.3.2. Detection of mucosal damage with *in vivo* histology

The severity of damages of the gastric or colon mucosa were characterized by means of fluorescence confocal laser scanning endomicroscopy (CLSEM) (Five1, Optiscan Pty. Ltd., Melbourne, Victoria, Australia) developed especially for *in vivo* histology. Two different investigators conducted the analysis, all measurements were taken twice. The mucosal surface of the stomach and the colon was surgically exposed and laid flat for examination. After the *iv.* administration of 0.3 ml of fluorescein isothiocyanate-dextran (FITC-dextran, 150 KDa, 20 mg/ml solution dissolved in saline, Sigma Chem.) the microvascular structure was recorded. The objective of the device was placed onto the mucosal surface of the stomach and the colon, and confocal imaging was performed 5 min after dye administration (1 scan/image, 1024 x 512 pixels and 475 x 475 μm per image). The changes in the mucosal structure were examined following topical application of the fluorescent dye acridine orange (Sigma-Aldrich Inc, St. Louis, MO, USA). 2 min before imaging the excess dye was washed off the mucosal surface with saline. We employed three criteria to establish a scoring system for semiquantitative assessment of the changes: I. the structure of the microvessels (0 = normal, 1 = dye extravasation, but the vessel structure recognizable, 2 = destruction, and the vessel structure unrecognizable); II. oedema (0 = no oedema, 1 = moderate epithelial swelling, 2 = severe oedema); and III. epithelial cell outlines (0 = normal, clearly, well-defined outlines, 1 = blurred outlines, 2 = lack of normal cellular contours).

3.3.2. Direct measurements of analgesic effect of ASA-Tris conjugate

The carrageenan-induced paw inflammation model was used to test analgesic effects. The induction of paw inflammation was established by injection of carrageenan (300 $\mu\text{g}/30 \mu\text{l}$) into the tibiotarsal joint of the right hind limb, then the analgesic effect was determined by using a dynamic plantar aesthesiometer (mod-37450; Ugo Basile, Comerio, Italy). Before the baseline measurements the animals were habituated to a testing box with wire mesh grid floor for at least 20 min. After that the measurements were performed with a straight metal 0,5 mm diameter needle, which exerts an increasing upward force at a constant rate of 4.25 g/s. The maximum cut-off force of the needle was 50 g. The filament was placed under the plantar surface of the

right hind paw, and the measurements were stopped when the paw was withdrawn, and results were expressed as paw withdrawal thresholds in grams.

3.4. *In vitro* experimental methods

3.4.1. Preparation of tissue biopsies for the measurement of inflammatory markers

Gastric and colon biopsies were kept on ice, then were homogenized in phosphate buffer (pH 7.4) which contained 50 mM Tris-HCl (Reanal, Budapest, Hungary), 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml soybean trypsin inhibitor and 10 µg/ml leupeptin (Sigma-Aldrich GmbH, Germany). The homogenate was centrifuged at 4 °C for 20 min at 24,000 g and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100,000 MW cut-off ultrafilter). The activity of xanthine oxidoreductase (XOR) was determined in the ultrafiltered supernatant (Beckman et al., 1989), while that of myeloperoxidase (MPO) was measured on the pellet of the homogenate (Kuebler et al., 1996).

3.4.2. Measurement of tissue MPO activity

As a marker of tissue leukocyte infiltration, the activity of MPO was measured on the pellet of the homogenate. Briefly, the pellet was resuspended in K₃PO₄ buffer (0.05 M; pH 6.0) containing 0.5% hexa-1,6-bis-decyltriethylammonium bromide. After three repeated freeze-thaw procedures, the material was centrifuged at 4 °C for 20 min at 24,000 g and the supernatant was used for MPO determination. Subsequently, 0.15 ml of 3,3',5,5'-tetramethylbenzidine (dissolved in DMSO; 1.6 mM) and 0.75 ml of hydrogen peroxide (dissolved in K₃PO₄ buffer; 0.6 mM) were added to 0.1 ml of the sample. The reaction led to the hydrogen peroxide-dependent oxidation of tetramethylbenzidine, which could be detected spectrophotometrically at 450 nm (UV-1601 spectrophotometer; Shimadzu, Kyoto, Japan). MPO activities were measured at 37 °C; the reaction was stopped after 5 min by the addition of 0.2 ml of H₂SO₄ (2 M) and the resulting data were referred to the protein content (Kuebler et al., 1996).

3.4.3. Tissue xanthine oxidoreductase (XOR) activity

XOR activity was determined in the ultrafiltered, concentrated supernatant by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterin in the presence (total XOR) or absence (xanthine oxidase activity) of the electron acceptor methylene blue (Beckman et al., 1989).

3.4.4. Cyt c oxidase level

The release of Cyt c was calculated via the time-dependent oxidation of Cyt c at 550 nm. Liver and gastric tissue samples were homogenized in 10x ice-cold MitOx2 medium with a Potter grinder, and then centrifuged at 800 g for 5 min at 4 °C. Then 50 µl supernatant was added to 2.5 ml Cyt c stock solution (10.6 mg Cyt c dissolved in 20 ml distilled water) (Sigma-Aldrich, Budapest, Hungary) and the measurement of decrease was made in optical density at 550 nm spectrophotometrically during 1-min intervals at 0, 30 and 60 min.

3.4.5. Plasma TNF- α level

0.5 ml of blood samples were taken from the inferior caval vein into precooled, heparinised (100 U/ml) polypropylene tubes, then were centrifuged at 1000 g at 4 °C for 30 min and stored at -70 °C until assay. Plasma TNF- α concentrations were determined in duplicate by means of a commercially available enzyme-linked immunosorbent assay (Quantikine ultrasensitive ELISA kit for rat TNF-alpha; Biomedica Hungaria Kft, Budapest, Hungary). The minimum detectable level was less than 5 pg/ml, and the interassay and intraassay coefficients of variation were less than 10%.

3.4.6 Tissue NO products

Nitrite and nitrate (NO_x), stable end-products of NO were determined in gastric homogenate by the Griess reaction. This assay depends on the enzymatic reduction of nitrate to nitrite, which is then converted into a coloured azo compound that is detected spectrophotometrically at 540 nm. Total NO_x was calculated and expressed as µmol/mg protein.

3.4.7. Malondialdehyde (MDA) level

The level of MDA shows the oxidative damage of lipid membranes, thereby the degree of lipid peroxidation was estimated via the amount of MDA. MDA level was measured through the reaction with thiobarbituric acid, and the values were corrected for the tissue protein content. The MDA concentration was determined on a standard curve (nmol/ml).

3.4.8. Mitochondrial function of isolated mitochondria

High-resolution respirometry (Oxygraph- 2k respirometer, Oroboros Instruments, Innsbruck, Austria) was used to determine changes in respiratory activity and reactive oxygen species (ROS)-producing capacity of rat-liver mitochondria. Tissue samples taken from the left liver lobes were homogenized, then followed by centrifugation in an isotonic sucrose medium (300 mM sucrose, 0.2 mM EDTA and 10 mM HEPES, adjusted to pH 7.4 with KOH at 4 °C) using

the Gnaiger method. After that mitochondrial pellets were resuspended in 3ml MitOx2 (mitochondrial respiration) medium and utilized for high-resolution respirometry.

For the respirometric analysis of the mitochondrial OxPhos, the Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol was applied. We determined the rate of Complex I-linked respiration after the addition of 2 mM glutamate, and 10 mM malate (state II respiration), and 2.5mM ADP (state III respiration). To determine the Complex I and Complex II state III respiration 10mM of succinate was added along with 0.5 μ M of rotenone for Complex II-linked respiration. To determine Complex IV-linked respiration 2.5 μ M of antimycin A (Complex III inhibitor), 2 mM ascorbate and 20 μ M N,N,N,N-tetramethyl-p-phenylenediamine was added to the mixture. In last step 5 μ M of Complex IV inhibitor sodium-azid was added. For the data analysis DatLab software (Oroboros Instruments) was used, and the respirometry data were normalized to the mitochondrial biomass.

Measurements of mitochondrial ROS production

Mitochondrial ROS production (i.e. superoxide anion radical production) was estimated from hydrogen peroxide (H₂O₂) release and monitored fluorometrically via the Amplex Red/horseradish peroxidase system, whereby Amplex Red (non-fluorescent) becomes oxidized to Resorufin. The calibration of H₂O₂ production was performed by the addition of known amounts of H₂O₂. Rat-liver mitochondria were incubated with oxidizing Complex II-substrate succinate in the presence of respiratory chain inhibitors (rotenone and antimycin-A). The H₂O₂ production at Complex I was estimated based on the reverse electron flux (RET) from Complex II also using this protocol.

3.4.9. Measurement of thrombocyte function

To measure the platelet aggregation, multiplate electrode aggregometry (Multiplate analyser, Roche, Basel, Switzerland) was used. After platelet activation, the analyser recorded the impedance between two electrodes reflecting thrombocyte aggregation on the surface of the electrodes. 300 μ l blood samples were placed in hirudinized tubes to perform multiplate aggregation tests using AA, the substrate of COX, which subsequently forms the platelet activator TXA₂ (ASPI-test), collagen, which leads to a release of endogenous AA and TXA₂ via the collagen receptors (Col-test), and the adenosine-diphosphate (ADP)-induced platelet activation test (ADP-test).

3.5. Experimental protocol of *Study I*

3.5.1. Experimental protocol to test the gastric side effects of ASA-Mono, ASA-Bis and ASA-Tris

35 animals were randomly allocated into 5 groups (n=7, each). Group 1 served as control group and was treated with vehicle liquid (10 ml/kg buffered 0.11 M potassium hydroxide (KOH) was given orally, three times daily, on three consecutive days). In group 2, high doses of ASA solution (0.55 mmol/kg, in a volume of 10 ml/kg; three times daily for 3 days) were gavaged via a flexible oesophageal tube to the animals. After the treatments, the animals were always returned to their cages and were fed ad libitum with a carbohydrate-rich diet. Groups 3-5 were treated with the ASA-conjugates in equimolar doses to ASA (0.55 mmol/kg, in a volume of 10 ml/kg; three times daily for 3 days). Group 3 was treated with ASA-Mono; group 4 with ASA-Bis and group 5 with ASA-Tris. On day 3, 2 h after the last treatments, the animals were anaesthetized with sodium pentobarbital (50 mg/kg *i.p.* and were placed in a supine position on heating pads, and the trachea and right jugular vein were cannulated to secure spontaneous breathing and i.v. administration of fluids and fluorescence dye, respectively. After a midline abdominal incision, intravital videomicroscopy was performed to examine the microcirculatory changes in the gastric serosa. The stomach was then incised along a greater curvature and rinsed with saline to remove the gastric contents. In each group, *in vivo* histology of the gastric mucosa was performed by CLSEM. At the end of the protocol, tissue biopsies were obtained from the stomach and the liver, to measure mucosal biochemical changes (XOR and MPO enzyme activity, NO_x, MDA level, Cyt c oxidase release) and venous blood samples were taken from the inferior caval vein to determine the plasma TNF- α level.

3.5.2. Experimental protocol to test the antithrombotic effect of ASA-Tris conjugate

The efficacy of ASA-Tris was tested on platelet functions in a separate series; the protocol was identical to that described above. Briefly, 15 animals were randomly allocated into 3 groups (n=5, each), group 1 served as the vehicle-treated control, and repeated doses of ASA, or ASA-Tris solution (0.55 mmol kg⁻¹, in a volume of 10 ml kg⁻¹; three times daily for 3 days) were gavaged via a flexible oesophageal tube to the animals in groups 2 and 3, respectively. Two h after the last treatment, the animals were anaesthetized with sodium pentobarbital and blood samples were taken from the inferior caval vein. The measurement of platelet aggregation was carried out with multiplate electrode aggregometry (Multiplate analyzer, Roche, Basel, Switzerland).

3.5.3. Experimental protocol to test the analgesic effect of ASA-Tris conjugate

Carrageenan-induced paw inflammation was used to test the analgesic effect of ASA-Tris, using 18 male SPRD rats randomly allocated into 3 groups (n=6, each). Group 1 served as positive control, while group 2 was treated with ASA, and group 3 with ASA-Tris conjugate in identical doses. 3 hours after the induction of paw inflammation by the injection of carrageenan (300 µg/30 µl) into the tibiotarsal joint of the right hind limb, the treatment of groups 2 and 3 was administered. All treatments were given to gently restrained conscious animals via a 27-gauge needle without anaesthesia. The analgesic effects of ASA and the ASA-Tris derivative treatments were determined by using a dynamic plantar aesthesiometer (mod-37450; Ugo Basile, Comerio, Italy), after habituating the rats. The measurements were performed with a straight metal filament which was placed under the plantar surface of the right hind paw, and measurements were stopped when the paw was withdrawn. Results were expressed as paw withdrawal thresholds in grams. The baseline measurements were performed 15 min before the induction of inflammation, while the development of inflammation was investigated 3 h after the carrageenan induction. The ASA or ASA-Tris treatments were administered 10 min after the second measurements; subsequent measurements were performed at 60, 120 and 180 min.

3.6. Experimental protocol of *Study II*.

3.6.1. Examination of efficacy of ASA-Tris in TNBS-induced colitis

In Study II. 30 animals were randomly allocated into 5 group (n=6 each). The animals were deprived of food, but not water, for 12 h prior to the enemas. Intracolonic (*ic*) administration of TNBS (40 mg/kg in 0.25 ml of 25% ethanol) through an 8 cm-long soft plastic catheter under transient light inhalation anaesthesia induced the colonic inflammation. In control groups, only the vehicle for TNBS was administered. The animals were then returned to their cages and were fed ad libitum with standard laboratory chow. In control group 1 (n = 6), the animals received enemas with a total volume of 0.25 ml containing 25% of ethanol (the solvent for TNBS). In groups 2, 3, 4 and 5 (n=6, each) colitis was induced with a TNBS enema (40 mg/kg). In groups 3, 4 and 5 repeated doses of ASA (Col + ASA; 0.55 mmol/kg (100 mg/kg), in a volume of 10 ml/kg; three times per day for 3 days), ASA-Tris conjugate (Col + ASA-Tris; 0.55 mmol/kg (161 mg/kg), in a volume of 10 ml/kg; three times per day for 3 days), or mesalamine (Col + Mes; 0.77 mmol/kg (118 mg/kg), in a volume of 10 ml/kg; three times per day for 3 days) were gavaged to the animals 12 h after colitis induction. The animals in the control and the non-treated colitis groups were gavaged with the solvent for ASA (10 ml/kg buffered 0.11 M of potassium hydroxide). On day 3, 2 h after the last treatment, the animals were anesthetized and

surgery was performed. For instrumentation purposes, the animals were placed in a supine position on heating pads. The trachea and the right jugular vein were cannulated and after a midline abdominal incision, the lumen of the stomach and the distal colon was exposed; then the mucosa was rinsed with saline to remove bowel or gastric contents, and then different measurements were carried out. In each group, an *in vivo* histology of the colonic and gastric mucosa was performed by CLSEM to examine the changes in microvasculature and superficial morphology. At the end of the experiments, full-thickness tissue samples were taken to measure mucosal biochemical changes, and venous blood samples were taken to determine the plasma TNF- α level.

3.6.2. Examination of changes in mitochondrial function

Fifteen animals were randomly allocated to 3 groups ($n = 5$, each). Group 1 served as the vehicle-treated control (10 ml/kg buffered 0.11 M of potassium hydroxide was given orally three times per day for three consecutive days), while in group 2, ASA solution [0.55 mmol/kg (100 mg/kg), in a volume of 10 ml/kg; three times per day for 3 days] was gavaged via a flexible oesophageal tube to the animals. Group 3 was treated with the ASA-Tris conjugate in equimolar doses to ASA [0.55 mmol/kg (161 mg/kg), in a volume of 10 ml/kg; three times per day for 3 days]. After the treatments, the animals were then returned to their cages and were fed *ad libitum* with a carbohydrate-rich diet. On day 3, 2 h after the last treatments, the animals were anaesthetized with sodium pentobarbital (50 mg/kg *ip*) and in each group, liver samples were taken for an analysis of the mitochondrial respiration.

3.7. Experimental protocol of *Study III*

3.7.1. Examination the gastric side-effects of Ket-Tris in healthy condition

In this study 18 animals were allocated into 3 groups randomly. Group 1 served as a vehicle-treated control ($n=6$), where 10 ml/kg of buffered 0.11 M potassium hydroxide (KOH) was administered orally. In group 2 ($n=6$), high doses of Ket solution (0.56 mmol/kg, in a volume of 10 ml/kg) were gavaged via a flexible oesophageal tube to the animals. After the treatment, the animals were returned to their cages and fed a carbohydrate-rich diet *ad libitum*. Rodents of group 3 ($n=6$) were treated with the Ket-Tris conjugate in equimolar doses to Ket (0.56 mmol/kg, in a volume of 10 ml/kg). On day 2 the animals were anaesthetized with sodium pentobarbital (50 mg/kg *i.p.*). For instrumentation, the animals were placed in a supine position on heating pads, and the trachea and right jugular vein were cannulated to secure spontaneous breathing and *iv* administration of fluids and fluorescence dye, respectively. After a midline

abdominal incision, intravital videomicroscopy was performed to examine the microcirculatory changes on the serosal surfaces of the stomach, duodenum, jejunum, ileum and colon. The stomach was opened along the great curvature and rinsed with saline to remove the gastric contents, and *in vivo* histology of the gastric mucosa was performed in each group by fluorescent confocal laser scanning endomicroscopy (CLSEM). At the end of the protocol, tissue biopsies were obtained from the stomach, and blood samples (0.5 ml) were taken from the inferior vena cava for further investigation.

3.7.2. Examination of efficacy of Ket-Tris in TNBS-induced colitis

In this study (n=24), the control group (n=6) received enemas with a total volume of 0.25 ml containing 25% ethanol (the solvent for TNBS). In groups 2, 3 and 4 (n=6 each), colitis was induced with a TNBS enema (40 mg/kg). In groups 3 and 4, the animals were gavaged with Ket (Col+Ket; 0.08 mmol/kg, 20 mg/kg, in a volume of 10 ml/kg) or Ket-Tris conjugate (Col+Ket-Tris; 0.09 mmol/kg, 30 mg/kg, in a volume of 10 ml/kg) 12 h after colitis induction. The animals in the control and non-treated colitis groups were gavaged with the solvent for Ket (10 ml/kg buffered 0.11 M of potassium hydroxide). On day 2 (24 h after Ket or Ket-Tris treatments), the animals were anaesthetized (sodium pentobarbital; 50 mg/kg i.p.), and surgery was performed. They were placed in a supine position on heating pads, the trachea and the right jugular vein were cannulated, and the lumen of the distal colon was exposed after a midline abdominal incision. Then the mucosa was rinsed with saline to remove bowel content. In each group, *in vivo* histology of the colonic mucosa was performed (see later) to examine the changes in microvasculature and superficial morphology. At the end of the experiments, full-thickness tissue samples were taken to measure mucosal biochemical changes, and venous blood samples were taken to determine the plasma TNF- α level.

3.8. 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

4.1. Estimation of the severity of gastritis in ASA, ASA-Mono, ASA-Bis and ASA-Tris-treated animals

ASA treatment resulted in manifest, visible bleeding and significant loss of bodyweight relative to the control group. By using ASA-Mono, ASA-Bis and ASA-Tris significant changes in bodyweight did not occur.

4.1.2. Changes in microcirculation

The RBCV of the serosa was measured as a quantitative marker of the gastric microcirculatory condition. In the ASA-treated group the RBCV was significantly decreased as compared with the control group. ASA-Mono and ASA-Bis treatments caused significant, but moderate reductions in the microcirculation in contrast with the control group, and these changes were significantly lower relative to that in the ASA-treated group. The ASA-Tris treatment prevented the reduction in RBCV (Table 2).

4.1.3. In vivo histological detection of gastric mucosal injury

FITC-dextran administration was used to visualize gastric microvessels, while the morphology of the gastric mucosa was examined by topical application of acriflavine dye (Figure 3). Capillary network and gastric mucosal epithelium exhibited a normal pattern (M=0; p25=0; p75=0.15) in control group, while the evaluation of the confocal microscopic records demonstrated significant tissue damage in the ASA-treated group in contrast with the control group (Figures 3, 4). By severe injuries of the capillary network, fluorescent dye leakage pointed to an elevated endothelial permeability and oedema formation (M=5; p25=3.75; p75=5.25). In the ASA-Mono (M=2.5; p25=1.8; p75=3) and ASA-Bis (M=2.5; p25=2; p75=3) treated groups, increased vascular permeability was observed, but changes were accompanied by a normal mucosal pattern. The ASA-Tris treatment did not lead to structural damage or morphological changes in the gastric mucosa. The loss of epithelium accompanying ASA administration was not present, the changes were similar to those observed in the untreated control group (M=0; p25=0; p75=0.15).

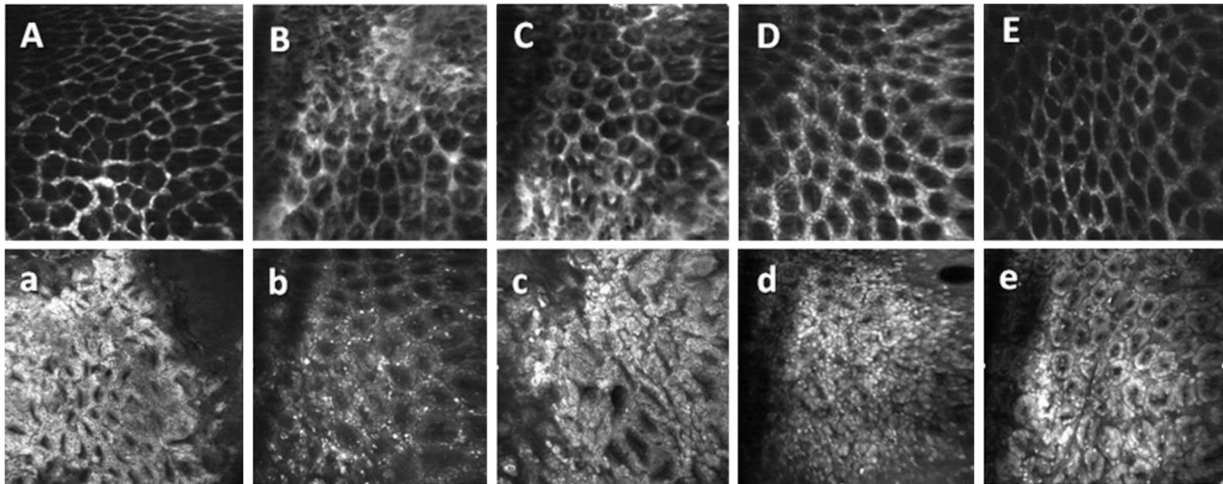


Figure 3. Upper panel: *In vivo* histology images of the mucosal surface of the stomach recorded by confocal laser scanning endomicroscopy (CLSEM) after iv administration of FITC-dextran: A: The mucosal vasculature in the control group. B: Dye leakage from the vessel lumina after 3 days of ASA-treatment. C: 3 days of ASA-Mono treatment. D: 3 days of ASA-Bis treatment. E: Normal mucosal vasculature after 3 days of ASA-Tris-treatment.

Lower panel: CLSEM after topical administration of acriflavine: a: Normal structure of the mucosa in the control group. b: Total loss of epithelium on the surface after 3 days of ASA treatment. c: Normal structure of the mucosa after 3 days of ASA-Mono treatment. d: ASA-Bis treatment. e: Normal structure of the mucosa after 3 days of ASA-Tris treatment.

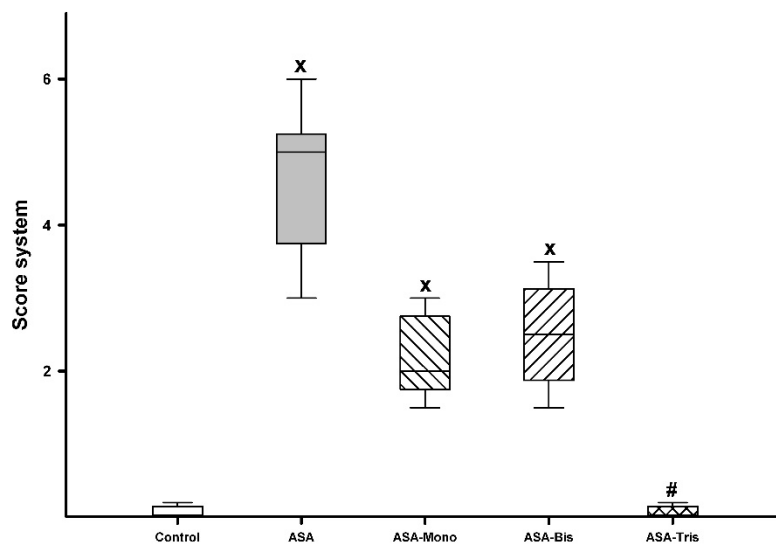


Figure 4. Grading of *in vivo* histology. Control (white empty box), ASA-treated (grey empty box), ASA-Mono-treated (striped white box on the right side), ASA-Bis-treated (striped white box on the left side) and ASA-Tris-treated (checked white box) groups. The plots demonstrate the median (horizontal line in the box) and

the 25th (lower whisker) and 75th (upper whisker) percentiles. ^x $P < 0.05$ between groups vs control group, [#] $P < 0.05$ between ASA-treated vs ASA-Tris-treated groups.

4.1.4. Changes in TNF- α levels

Significant increase was detected in the plasma level of TNF- α after ASA and ASA-Mono administration as compared with the control group. The plasma level of TNF- α in the ASA-Bis, or ASA-Tris-treated groups was kept at a significantly lower level relative to the ASA-treated group (Table 2).

4.1.5. Changes in MPO activity

The ASA treatment caused tissue leukocyte accumulation as revealed via measurement of the MPO activity. The median MPO activity in the control animals at the end of the observation period was 1035 (p25=1020; p75=1508) mU/(mg protein). 2 h after the last ASA treatment, the gastric MPO activity was increased significantly (M=2069; p25=1951; p75=2247 mU/(mg protein)) relative to the control group. The ASA-Mono treatment resulted in a significant elevation in the MPO activity as compared with the control group, while the ASA-Bis and ASA-Tris (M=970; p25=927; p75=1176 mU/(mg protein)) treatments prevented the increase of MPO activity in the gastric tissue (Figure 5).

4.1.6. Changes in xanthine oxidoreductase enzyme (XOR) activity

The XOR enzyme activity 2 h after the last ASA treatment was significantly elevated in contrast with the control group. The ASA-Mono treatment did not influence while the ASA-Bis and ASA-Tris treatments significantly decreased the activity of XOR (Figure 5).

4.1.7. Changes in nitric oxide (NO_x) products level

The ASA treatment significantly elevated the NO_x level in the gastric tissue relative to the control group. The elevation of NO_x was significantly higher in comparison with the control group in the ASA-Mono and ASA-Bis-treated groups. ASA-Tris treatment decreased the NO_x elevation in contrast with the non-treated or other ASA conjugate-treated groups (Figure 5).

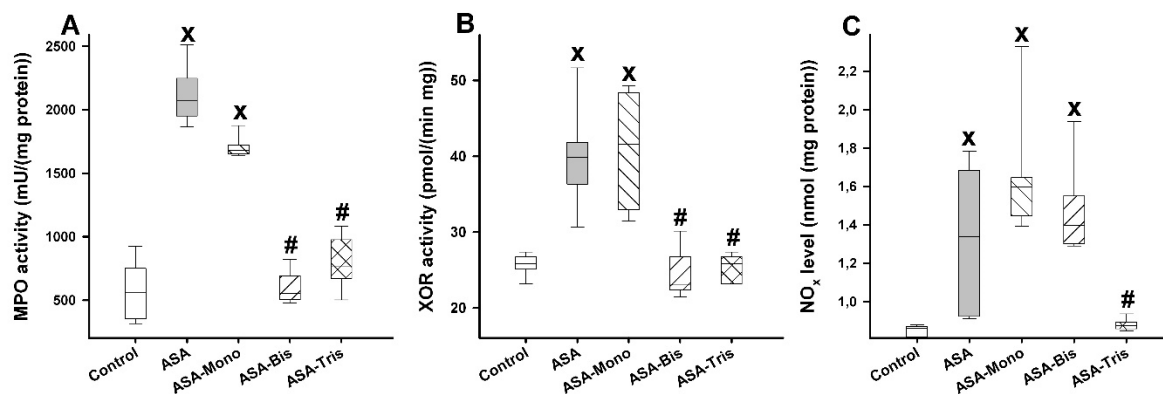


Figure 5. Changes in gastric MPO activity (A), XOR activity (B) and the level of the NO_x (C) in the control (white empty box), ASA-treated (grey empty box), ASA-Mono-treated (striped white box on the right side), ASA-Bis-treated (striped white box on the left side) and ASA-Tris-

treated (checked white box) groups. The plots demonstrate the median (horizontal line in 26 the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^x $P < 0.05$ between groups vs control group, [#] $P < 0.05$ between ASA-treated vs ASA-Tris-treated groups.

4.1.8. Changes in MDA levels

The MDA level was significantly increased in the gastric tissue in the ASA-treated group as compared with the control group. The ASA-Mono, ASA-Bis, or ASA-Tris treatment significantly prevented the elevation in MDA level (Figure 6).

4.1.9. Changes in Cyt c oxidase levels

Cyt c release from the mitochondria as an indicator of mitochondrial membrane damage was determined in the liver and gastric tissues. The hepatic Cyt c level was significantly increased in the ASA- or ASA-Mono-treated groups as compared with the control group. The ASA-Bis or ASA-Tris treatment significantly prevented the elevation in Cyt c release (Fig. IV). The Cyt c release was also measured in the gastric tissue. In these samples, the Cyt c level was significantly elevated as a result of ASA treatment, while the release of Cyt c could not be demonstrated in the ASA-Tris group (Figure 6).

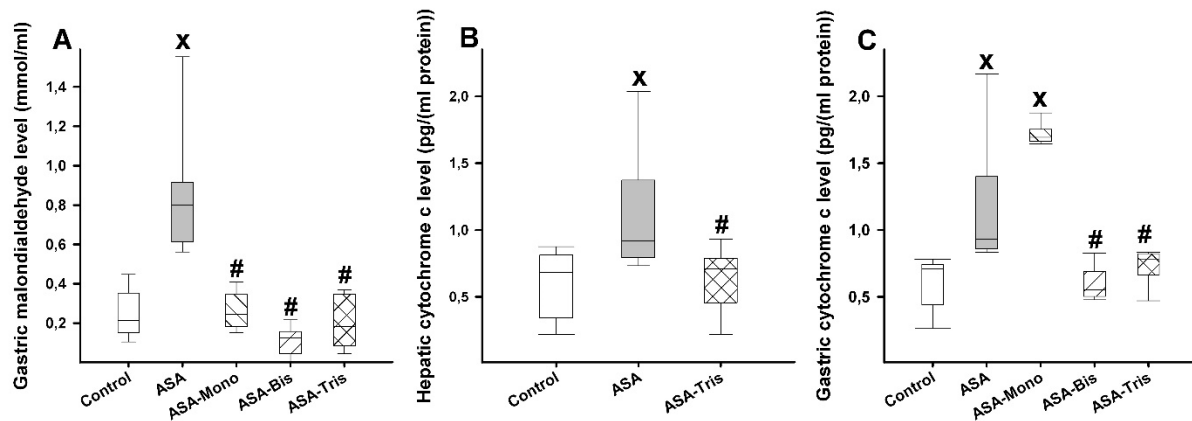


Figure 6. Malondialdehyde (MDA) level in the gastric tissues (A); cytochrome c release in the hepatic- (B) and in the gastric (C) tissues in the control (white empty box), ASA-treated (grey empty box), ASA-Mono-treated (striped white box on the right side), ASA-Bis-treated (striped white box on the left side) and ASA-Tris-treated (checked white box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^x $P < 0.05$ between groups vs control group, [#] $P < 0.05$ between ASA-treated vs ASA-Tris-treated groups.

	ASA	ASA-Mono	ASA-Bis	ASA-Tris
Changes in body weight	xxx	-	-	-
Mucosal damage	xxx	xx	xx	-

Microvascular damage	xxx	xx	xx	-
Haemodynamic damage	xxx	x	x	-
MPO activity	xxx	xx	-	-
XOR activity	xxx	xxx	-	-
NO_x	xxx	xxx	xxx	-
MDA	xxx	-	-	-
Plasma TNF-α	xxx	xxx	-	-
Gastric Cyt c release	xxx	not tested	not tested	-
Liver Cyt c release	xxx	xxx	-	-
Sum of scores of tested effects	33	19	8	0

Table 2. Summary of key findings on gastric side effects of ASA-Mono, ASA-Bis and ASA-Tris

4.1.10. Changes of thrombocyte aggregation

In our study ASPI-test, ADP-test and Col-test were used to test the different pathways of platelet aggregation, which demonstrated an approximately 80% decrease in platelet function after ASA treatment as compared with the control group. After ASA-Tris treatment, the ASPI-test showed that the rate of thrombocyte aggregation was 20% lower than in the control group, but the Col-test and ADP-test results were not affected (Figure 7).

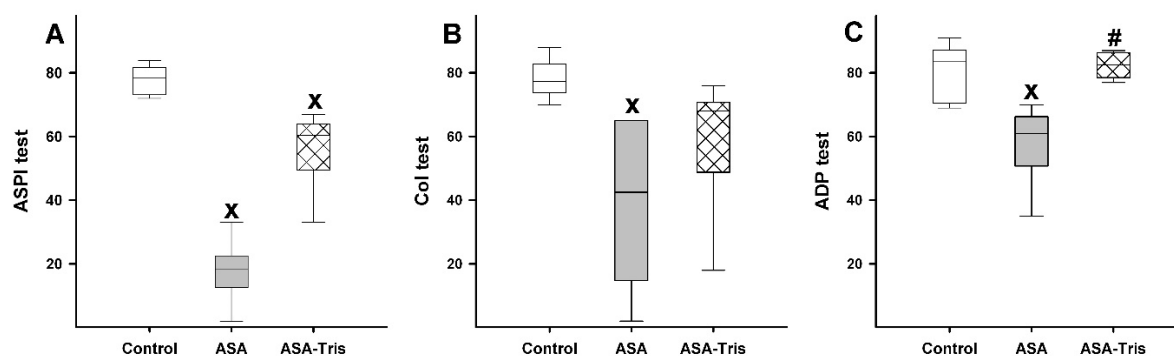


Figure 7. ASPI-test (A), ADP-test (B), Col-test results (C) in the control (white empty box), ASA-treated (grey empty box), and ASA-Tris-treated (checked white box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^x $P < 0.05$ between groups vs control group, [#] $P < 0.05$ between ASA-treated vs ASA-Tris-treated groups.

4.1.11. Changes in nociception

In all groups of rodents, a significant decrease in the paw pressing force was detected 3 h after carrageenan administration on the treated side, referring to the development of inflammation

induced pain. Acute, single-dose ASA or ASA-Tris administrations significantly increased these values. This effect was transitional after ASA treatment, while equimolar ASA-Tris resulted in a sustained and prolonged effect lasting until the end of the 180 min observation period (Figure 8).

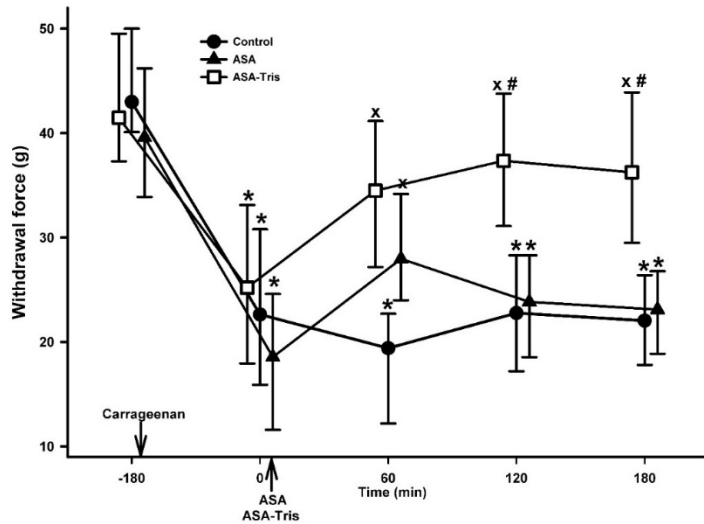


Figure 8. Changes in nociception in the control (white square with a thin continuous line), ASA-treated (black triangle with a thin continuous line) and ASA-Tris treated (black circle with a thin continuous line) groups. The plots demonstrate the median and the 25th (lower whisker) and 75th (upper whisker) percentiles. * $P < 0.05$ within groups vs baseline

values (0 h), ^x $P < 0.05$ between groups vs control group; [#] $P < 0.05$ between ASA and ASA-Tris-treated groups.

4.2. Results of Study II

4.2.1. Changes in bodyweight

The severity of colitis was estimated with macroscopic changes in the stool and the measurement of body weight. TNBS induction was accompanied by diarrhoea and the appearance of blood in the faeces in contrast with that in the control group. On day 3 of TNBS colitis the animals exhibited a significant weight loss relative to the baseline body weight (day 0) and the average weight in control group. ASA-Tris and mesalamine treatments prevented the weight loss relative to that in the control groups (Figure 9).

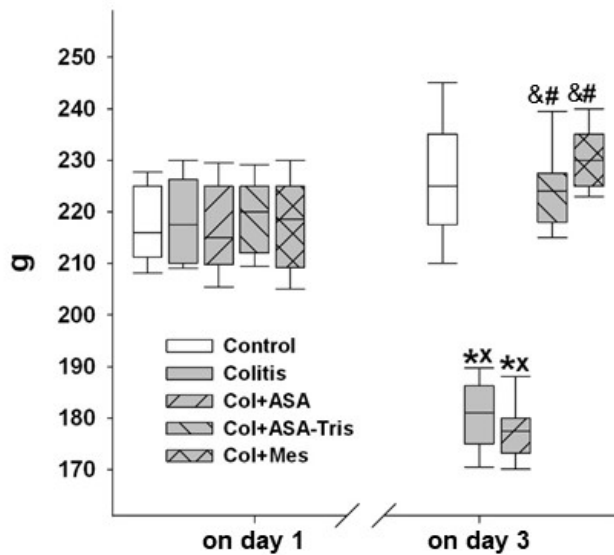


Figure 9. Changes in body weight between days 1 and 3 of colitis in control (white empty box), colitis (grey empty box), ASA-treated colitis (striped grey box on the left), ASA-Tris-treated colitis (stripped grey box on the right), mesalamine-treated colitis (checked grey box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. * $p < 0.05$ within groups vs baseline values, ^x $p < 0.05$ between groups vs control group, # $p < 0.05$ between colitis group vs ASA-Tris or mesalamine treated groups, & $p < 0.05$ between ASA-treated vs ASA-Tris or mesalamine treated groups.

baseline values, ^x $p < 0.05$ between groups vs control group, # $p < 0.05$ between colitis group vs ASA-Tris or mesalamine treated groups, & $p < 0.05$ between ASA-treated vs ASA-Tris or mesalamine treated groups.

4.2.2. In vivo histological detection of colon mucosal injury

In control group, the network of capillaries exhibited a honeycomb pattern and the luminal openings of the crypts were covered with a continuous layer of epithelial cells, which appeared as black holes that opened onto the surface of the mucosa (Figure 10A, 10F, Table 3.). Evident tissue damage appeared after TNBS enemas. Fluorescent dye leakage with edema formation was present 3 days after TNBS treatments (Figure 10B) and the complete loss of the epithelium was commonly observed (Figure 10G, Table 3.). ASA treatment moderated the severity of injury, but these changes were still apparent (Figure 10C, 10H, Table 3.). ASA-Tris (Figure 10D, 10I, Table 3.) and mesalamine (Figure 10E, 10J, Table 3.) treatments prevented the structural changes in the microvasculature and the loss of epithelium of the inflamed colonic mucosa (Figure 10).

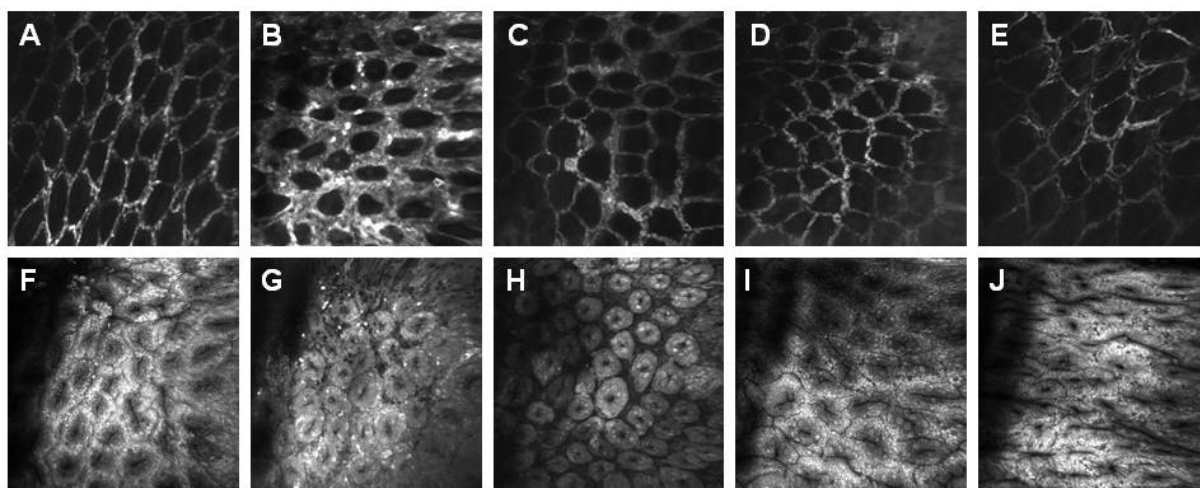


Figure 10. Upper panel: *In vivo* histology images of the mucosal surface of the ascending colon recorded by confocal laser scanning endomicroscopy (CLSEM) after iv administration of FITC-dextran: A, the mucosal vasculature in the control group. B, Dye leakage from the vessel lumina on day 3 of colitis. C, Moderate dye leakage from the vessel lumina on day 3 of colitis and ASA treatment. D, Normal mucosal vasculature on day 3 of colitis and ASA-Tris treatment. E, Normal mucosal vasculature after 3 days of colitis and mesalamine treatment. Lower panel: CLSEM after topical administration of acriflavine: F, the normal structure of the mucosa in the control group. G, The total loss of epithelium on the surface on day 3 of colitis. H, The enlarged spaces between the glands on day 3 of colitis and ASA treatment. I, The normal structure of the mucosa on day 3 of colitis and ASA-Tris treatment. J, The normal structure of the mucosa on day 3 of colitis and mesalamine treatment.

		Tissue damage	TNF-alpha level
Control	Median; 25p; 75p	0.1; 0.025; 0.2	75; 59; 80
Colitis	Median; 25p; 75p	6.0^a; 5.5; 6.6	148^a; 97; 175
Col+ASA	Median; 25p; 75p	2.5^{ab}; 1.875; 3.0	55^b 37; 70
Col+ASA-Tris	Median; 25p; 75p	0.5^{bc}; 0.0; 0.5	51^b; 28.5; 69
Col+Mes	Median; 25p; 75p	0.5^{bc}; 0.0; 1.0	55^b; 41; 87

Table 3. The effects of ASA, ASA-Tris or mesalamine treatments on changes in mucosal structure of the colon and TNF-alpha levels [pmol ml^{-1}]. ^a $p < 0.05$ between groups vs control group; ^b $p < 0.05$ between ASA, ASA-Tris and mesalamine groups vs colitis group; ^c $p < 0.05$ between ASA-Tris and mesalaminde groups vs ASA group.

4.2.3. *In vivo* detection of mucosal injury in the stomach

In control group, the network of capillaries and gastric mucosal epithelium exhibited a normal pattern (M = 0; p25 = 0; p75 = 0.3 Table 3). The TNBS enema did not cause gastritis (M = 0.6; p25 = 0; p75 = 1.7), while significant tissue damage was present in the ASA-treated colitis group in contrast with the control group. Fluorescent dye leakage suggested increased endothelial permeability and injured capillary network, which resulted in oedema formation and structural tissue damage (M = 5.3; p25 = 4.8; p75 = 5.7). In the ASA-Tris-treated group this damage was not present (M = 0; p25 = 0; p75 = 0.5 Table 3) and also in the case of mesalamine-treated colitis groups the normal capillary and mucosal patterns were observed (M = 0; p25 = 0; p75 = 0.4 Table 3).

4.2.4. Changes in TNF alpha levels

The source of TNF- α may be the lymphocytes or activated PMN leukocytes. It can further enhance ROS production and the release of other proinflammatory cytokines. The importance of TNF- α was demonstrated in the pathogenesis of IBDs and it is a significant target of biological therapies. The plasma level of TNF- α significantly increased after colitis induction as compared with that in the control group. The plasma level of TNF- α in the ASA-treated group decreased relative to the colitis group. ASA-Tris and mesalamin treatment significantly decreased the plasma TNF- α level, in contrast to that in the non-treated colitis and ASA-treated colitis (Table 3).

4.2.5. Changes in XOR activity, MPO activity and NO_x level in colon

The activity of XOR is one of the most important sources of ROS in the GI tissues that can greatly increase tissue damage. Another important effect of the ROS produced by the XOR system is the activation and local accumulation of PMN leukocytes. Activated neutrophils can contribute to the NO production along with the endothelium and the increased level of NO and ROS production can further enhance tissue damage with the formation of peroxynitrite, a highly oxidative agent with relatively long biological half-life. The NO_x level refers directly to the intensity of NO production. The MPO and XOR activities and NO_x levels in the distal colon significantly increased 3 days after colitis compared to the controls. Two hours after the last ASA-treatment, the colonic MPO activity was decreased 5 significantly relative to the colitis group. The ASA-Tris and mesalamine treatments resulted in a significant decrease in MPO activity as compared with that in the colitis or ASA treated colitis groups (Figure 11A). ASA treatment did not influence the elevated XOR activity, but both ASA-Tris and mesalamine

treatment effectively reduced the activation of XOR (Figure 11B). In the ASA-treated colitis group, the elevation of NO_x was significantly higher in comparison with the control group. Both ASA-Tris and mesalamine treatments decreased the NO_x elevation, unlike that in the non-treated colitis or ASA-treated colitis groups (Figure 11C).

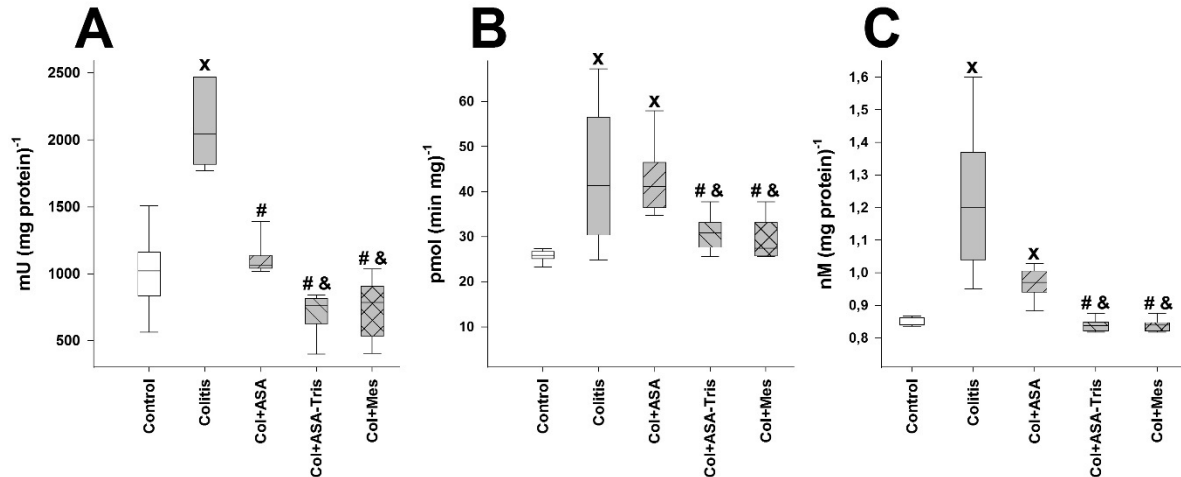


Figure 11. Changes in gastric MPO activity (a), XOR activity (b) and the level of the NO_x (c) in the control (white empty box), colitis (grey empty box), ASA-treated colitis (striped grey box on the left), ASA-Tris-treated colitis (stripped grey box on the right), mesalamine treated colitis (checked grey box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. *x* *p* < 0.05 between groups vs control group, #*p* < 0.05 between colitis group vs ASA-Tris or mesalamine treated groups, &*p* < 0.05 between ASA-treated vs ASA-Tris or mesalamine treated groups.

4.2.6. Changes in XOR activity, MPO activity and NO level in stomach

On day 3 of colitis, the gastric MPO and XOR activities were significantly increased in contrast to those in the control group, while the NO_x level did not change in the gastric tissue. Two hours after the last ASA treatment, the MPO activity remained significantly elevated relative to that in the control group. The ASA-Tris and mesalamine treatments reduced the elevation in the MPO activity in the stomach as compared to that in the colitis or ASA-treated colitis groups. ASA treatment did not influence the increased XOR activity, while ASA-Tris and mesalamine treatment prevented the elevated activation of XOR. In the ASA-treated colitis group, the elevation of NO_x was significantly higher than that in the control group. ASA-Tris and mesalamine treatments did not increase NO_x level, in contrast with that in the ASA-treated colitis group (Table 4).

		Tissue damage	MPO activity	XOR activity	NO_x level
Control	Median 25p; 75p	0 0; 0.3	325 300; 584	25.8 25.1; 26.7	0.86 0.84; 0.88
Colitis	Median 25p; 75p	0.6 0; 1.7	933^a 850; 1030	41.4^a 28.5; 59.6	0.87 0.85; 0.97
Col+ASA	Median 25p; 75p	5.3^a 4.8; 5.7	786^a 611; 1055	42.6^a 39.1; 57.8	1.04^a 0.99; 1.34
Col+ASA-Tris	Median 25p; 75p	0^c 0; 0.5	342^b 228; 385	27.8^{bc} 25.6; 31.4	0.84^c 0.81; 0.84
Col+Mes	Median 25p; 75p	0^c 0; 0.4	357^b 229; 393	25.8^{bc} 25.7; 30.1	0.84^c 0.8; 0.85

Table 4. The effects of colitis and ASA, ASA-Tris or mesalamine treatments on changes in mucosal structure, myeloperoxidase (MPO) [mU/(mg protein)], xanthine oxidoreductase enzyme activity [pmol/(min mg)] and NO_x level [nM/(mg protein)] in gastric tissue. ^a $p < 0.05$ between groups vs control group; ^b $p < 0.05$ between colitis group vs ASA, ASA-Tris or mesalamine treated groups; ^c $p < 0.05$ between ASA-treated group vs ASA-Tris or mesalamine-treated groups.

4.2.7. Changes of mitochondrial functions - respiratory activity of mitochondria

Basal mitochondrial respiration was induced by adding substrates of Complex I. The saturating concentration of ADP resulted in a twofold increase in Complex I-linked respiration. After a stable signal had been attained, Complex II-dependent respiration was stimulated by adding succinate, which caused a further 1.5-fold increase in all the groups. Complex I was then inhibited with rotenone to assess Complex II-linked respiration. There was a tendency in the ASA group to display lower respiratory values in Complex I- and II-dependent oxygen consumption, but these differences were statistically not significant among the groups. After Complex III inhibition with antimycin-A, the residual O₂ consumption was identical in all the groups. However, when ascorbate and TMPD were added to the medium, Complex IV-linked respiration significantly decreased in the ASA group as compared with that in the control and ASA-Tris groups. Next, the inhibition of Complex IV by adding sodium-azide resulted in significantly lower residual respiratory flux in the ASA-treated group, but in the ASA-Tris group this was restored to the level of the control group (Figure 12).

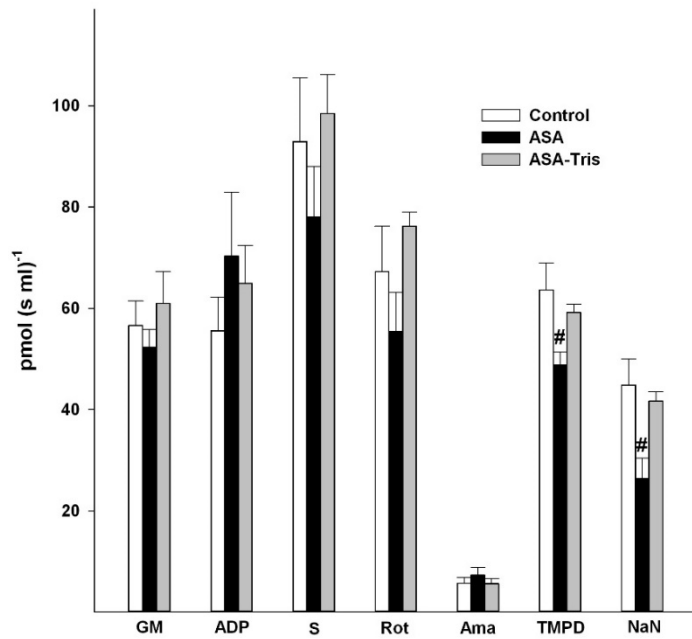


Figure 12. Oxygen consumption of liver mitochondria measured by means of high-resolution respirometry. Animals received ASA treatment (ASA group, black column) or ASA-Tris treatment (ASA-Tris group, grey column), or vehicle (control group, white column). Data are presented as mean \pm SEM. # $p < 0.05$ vs control group (one way ANOVA, Bonferroni test). G glutamate, M malate, D ADP, S succinate, Rot rotenone, Ama antimycin A, TMPD ascorbate and TMPD, NaN₃ sodium-azide.

4.2.8. Changes of mitochondrial functions - ROS production of mitochondria

In the control group, H₂O₂ generation with succinate as the sole substrate was partly inhibited by rotenone; however, the subsequent addition of antimycin-A resulted in a fourfold increase in H₂O₂ formation. From these results, it seems that in this set-up H₂O₂ production was linked to the leakage of electrons at Complex I and Complex III, but Complex III-linked events were predominant. ASA and ASA-Tris administrations did not influence mitochondrial H₂O₂ production significantly (Figure 13).

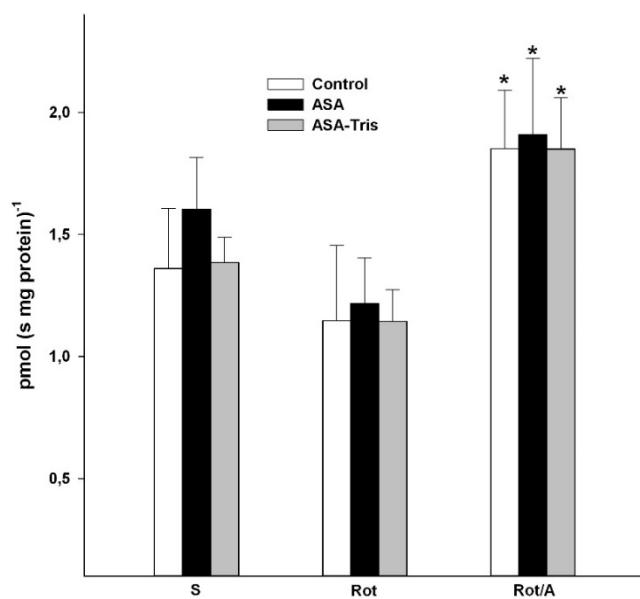


Figure 13. Hydrogen peroxide (H₂O₂) production of liver mitochondria measured by means of high-resolution respirometry. Animals received ASA (ASA group, black column) or ASA-Tris (ASA-Tris group, grey column), or vehicle (control group, white column). Data are presented as mean \pm SEM. * $p < 0.05$ vs S (one-way ANOVA, Bonferroni test). S succinate, Rot rotenone, Ama antimycin A.

4.3. Results of Study III

4.3.1. *In vivo* detection of gastric mucosal injury

The morphological changes in the gastric mucosa were evaluated by means of *in vivo* imaging, using CLSEM. The FITC-dextran and acriflavine staining demonstrated significant tissue damage in the Ket group. A severely injured capillary network, fluorescent dye leakage, elevated endothelial permeability and oedema formation (Figure 14 BE) were observed in contrast to the normal mucosal pattern of the control group (Figure 14 AD). The Ket-Tris treatment effectively attenuated the structural damage or morphological changes in gastric mucosa (Figure 14 CF, Table 5).

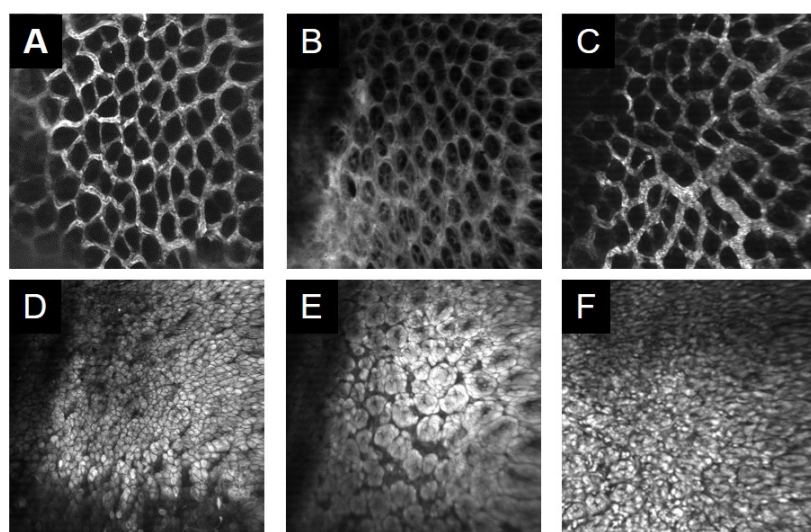


Figure 14. *In vivo* detection of gastric mucosal injury. *In vivo* histology images of the mucosal surface of the stomach recorded by confocal laser scanning endomicroscopy (CLSEM) after *iv* administration of FITC-dextran (A, B, C) or topical administration of

acriflavine (D, E, F). A, D: The normal mucosal vasculature and normal structure of the mucosa in the control group. B, E: Dye leakage from the vessel lumina, loss of epithelium and oedema formation on the surface after Ket treatment. C, F: Normal structure of the mucosal vasculature and normal mucosa surface after Ket-Tris treatment.

4.3.3. Changes in biochemical parameters

Pro-inflammatory biochemical markers, MPO, XOR, NO_x, MDA, Cyt c and TNF- α 2 were significantly increased 24 h after Ket treatment relative to the control group. Ket-Tris administration did not induce elevation of these molecules (Table 5).

	Parameters	Control	Ket	Ket-Tris
Tissue damage	Median	0.15	5 ^a	0.275 ^b
	25 ^p ; 75 ^p	0; 0.3	4.12; 5.87	0; 0.3
MPO	Median	1.7	2.4 ^a	1.6 ^b

	<i>25p; 75p</i>	<i>1.06; 1.85</i>	<i>2.16; 2.16</i>	<i>1.5; 1.83</i>
XOR	Median	18.7	41.9^a	23.5^b
	<i>25p; 75p</i>	<i>14.9; 27.2</i>	<i>33.9; 46.2</i>	<i>18.9; 26.1</i>
NO_x	Median	0.62	1.37^a	0.65^b
	<i>25p; 75p</i>	<i>0.51; 20</i>	<i>1.14; 1.84</i>	<i>0.61; 0.68</i>
MDA	Median	0.21	0.88^a	0.35^b
	<i>25p; 75p</i>	<i>0.15; 0.42</i>	<i>0.81; 1.38</i>	<i>0.29; 0.39</i>
Cyt c	Median	0.68	1.89^a	0.67^b
	<i>25p; 75p</i>	<i>0.34; 0.8</i>	<i>1.35; 1.95</i>	<i>0.53; 0.7</i>
TNF-α	Median	3.17	4.4^a	3.23^b
	<i>25p; 75p</i>	<i>2.7; 3.3</i>	<i>3.7; 7.4</i>	<i>2.97; 3.55</i>

Table 5: Extent of tissue damage and changes in biochemical parameters The effects of Ket and Ket-Tris treatment on mucosal structure in gastric tissue and changes in MPO activity [mU/(mg protein)], XOR activity [pmol/(min mg)], NO_x level [nmol/(mg protein)], MDA level [pmol/ml], Cyt c [pg/(ml protein)] and plasma TNF- α level [pg/ml]. ^a $p < 0.05$ between groups vs the control group; ^b $p < 0.05$ between the Ket-treated group vs the Ket-Tris-treated group.

4.3.4. In vivo detection of the microcirculation

RBCV of the mucosa was measured as a quantitative marker of GI microcirculatory condition, baseline microcirculatory values were measured in the entire GI tract in the Ket-Tris-treated group (Table 6). The RBCV in gastric serosa was significantly higher in the Ket-treated group as compared to the control group (Table 6), and a similar change was detected in the duodenum, jejunum and ileum, but not in the colon.

	Parameters	Control	Ket	Ket-Tris
Stomach	Median	882	1084^a	763^b
	<i>25p; 75p</i>	<i>779; 967</i>	<i>982; 1245</i>	<i>703; 876</i>
Duodenum	Median	744	1084^a	676^b
	<i>25p; 75p</i>	<i>694; 798</i>	<i>1015; 1229</i>	<i>536; 897</i>
Jejunum	Median	762	1077^a	754^b
	<i>25p; 75p</i>	<i>683; 824</i>	<i>960; 1267</i>	<i>679; 805</i>
Ileum	Median	827	1012^a	781^b
	<i>25p; 75p</i>	<i>742; 906</i>	<i>972; 1084</i>	<i>694; 864</i>

Colon	Median 25 ^p ; 75 ^p	946 837; 1034	984 754; 1069	977 862; 1048
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Table 4. The effects of Ket and Ket-Tris treatment on changes in red blood cell velocity (RBCV) [$\mu\text{m/s}$]. ^a $p < 0.05$ between groups vs the control group; ^b $p < 0.05$ between the Ket-treated group vs the Ket-Tris-treated group.

4.3.5. Changes in biochemical factors

24 h after colitis induction, significant elevation was demonstrated in the level of MPO and XOR activities, MDA level and Cyt c level relative to the control group values (Figure 15). In the Ket-treated colitis group, significantly decreased XOR activity, MDA and Cyt c levels were observed. The Ket-Tris treatment effectively reduced all of the observed parameters.

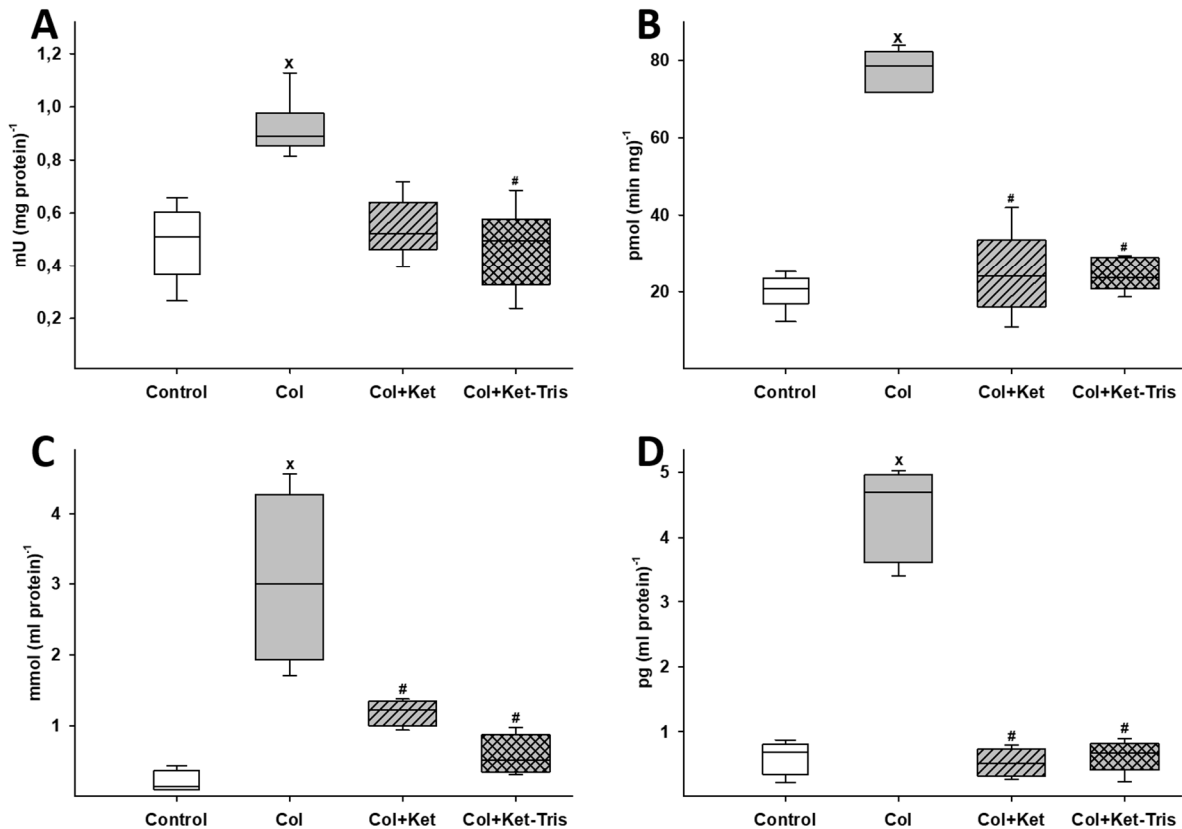


Figure 15. Changes in colonic MPO activity (A) and XOR activity (B), levels of MDA (C) and Cyt c (D) in the control (empty box), colitis (grey empty box), Ket-treated colitis (striped grey box) and Ket-Tris-treated colitis (checked grey box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^x $p < 0.05$ between groups vs the control group, [#] $p < 0.05$ between the non-treated colitis group vs the Ket- or Ket-Tris-treated group.

4.3.6. In vivo detection of mucosal damage in the colon and serosal microcirculation with in vivo microscopy

After TNBS enemas, severe mucosal injury was present 24 h after TNBS induction. Fluorescent dye leakage with oedema formation and the complete loss of the epithelium were commonly observed. In the control (vehicle-treated) group, the normal structure of capillaries and the crypts covered by epithelial cells were visualized. Ket and Ket-Tris treatments prevented structural changes in the microvasculature and the loss of epithelium of the inflamed colonic mucosa. Figure 16A shows the score for *in vivo* histological examination. The RBCV in the colonic subserosa was significantly raised in the colitis group as compared to the control group. Ket and Ket-Tris treatment decreased the elevated RBCV by the end of the observation period (Figure 16B).

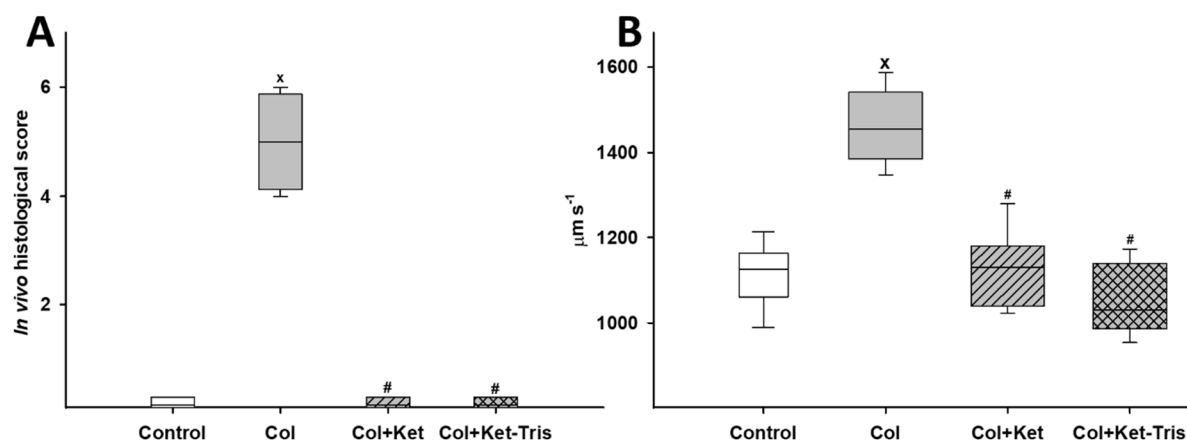


Figure 16. Changes in mucosal *in vivo* histological score (A) and serosal red blood cell velocity (RBCV) (B) in the control (empty box), colitis (grey empty box), Ket-treated colitis (striped, grey box) and Ket-Tris-treated colitis (checked grey box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^x $p < 0.05$ between groups vs the control group, [#] $p < 0.05$ between the non-treated colitis group vs the Ket- or Ket-Tris-treated group.

5. DISCUSSION

The physiological barrier of the mucosa is vital for the body's homeostasis (Foitzik et al., 1999), and numerous studies have been conducted to reduce the incidence of NSAID-induced haemorrhagic GI damage. Ulcerations and bleeding are major side effects of NSAID therapies in the upper GI tract and it is well recognised that early events in the pathogenesis involve microvascular leakage and leukocyte accumulation in the mucosa (Vallace et al., 1990).

NSAIDs may also alter gastric mucosal cell functions (Alino et al., 2008; Pizzuto et al., 1997), and paradoxically stimulate Ca^{2+} -dependent TNF- α release by activated macrophages, which has direct cytotoxic effects (Fiorucci et al., 1998).

A substantial body of literature has addressed strategies such as increasing the bioavailability (Kumar et al., 2022) or combining NSAIDs with other analgesics or adjuvant agents (Pergolizzi et al., 2023). Current ulcer-prevention management typically relies on “modified release” enteric coating formulations or additional acid secretion inhibitors and antacids, though effective multimodal therapy can be challenging, especially in non-compliant patients (Evans et al., 1983, Scheen et al., 1983). Co-therapy of NSAIDs with other compounds has also been explored. Molecular modifications offer another avenue. Several modified compounds have been examined in experimental settings over the past decades, with some, such as the 1,3,4-oxadiazole derivative of diclofenac, demonstrating significantly reduced ulcerogenic effects (Bhandari et al., 2008, Ilango et al., 2015). However, there remains a need for effective chemical modification that inherently lower the ulcerogenic properties.

Our approach with chemical conjugation of the amino-alcohol Tris with ASA or Ket, falls into this category. The main goal was to synthesize a novel compound with NSAID-like therapeutic efficacy but reduced GI tract-damaging side effects. To this end, we have developed a technique to combine ASA or Ket and the amino-alcohol precursors and the data show that the ASA- or Ket-Tris conjugates work effectively as anti-inflammatory compounds, with much less damage to the gastric mucosa as compared to the original ASA or Ket. Notably, the possibility of a chemical reaction occurring between NSAIDs and their Tris conjugates has never been previously investigated.

As the next step, we characterized ASA-induced haemorrhagic mucosal injury through a combination of in vitro assays and in vivo tests in rodents and evaluated the effects of newly developed ASA-Mono, ASA-Bis, and ASA-Tris conjugates within the same models. Intragastric administration of ASA disrupted the mucosal barrier and caused notable deterioration of both the serosal and mucosal microcirculation. Intravital histology confirmed the progressive endothelial injury along with a severe loss of epithelial integrity. The critical involvement of PMN leukocytes in ASA’s harmful effects was also confirmed, as indicated by elevated MPO levels showing PMN anchoring in the inflamed gastric tissue. These damaging alterations were further associated with an increase in XOR activity, a primary enzyme responsible for ROS production. Elevated tissue levels of MDA, NOx, and Cyt c release were

observed, alongside increased plasma TNF- α concentrations and Cyt c release from the liver, illustrating the spread of inflammatory signals and the systemic dimension of a localized injury.

In contrast, the ASA-Tris conjugate did not induce PMN accumulation, inflammatory enzyme activation, or cytokine release. Notably, ASA-Tris did not elevate gastric MDA levels, and Cyt c release was undetectable in both gastric and hepatic tissues. *In vivo* histology further corroborated the absence of mucosal damage following ASA-Tris administration. The ASA-Tris conjugate effectively prevented increases in inflammatory markers and weight loss and preserved the microvascular structure.

In our experiments, we observed distinct responses across different ASA conjugates. ASA-Mono treatment elevated MPO and XOR activity as well as plasma TNF- α levels, although body weight remained stable, and microcirculation showed only a moderate decline. ASA-Bis proved somewhat more effective, showing no mucosal injury and resulting in lower TNF- α release compared to ASA. While microvascular leakage was consistently present in both ASA-Mono and ASA-Bis groups, it was absent after ASA-Tris treatment. Overall, RBCV changes correlated with microvascular damage: the lowest values were recorded with ASA treatment, followed by moderate RBCV decreases in the ASA-Mono and ASA-Bis groups, while RBCV remained at control levels with ASA-Tris treatment.

For ASA-Mono, MDA levels were unaffected, but Cyt c levels were elevated. In contrast, ASA-Bis and ASA-Tris maintained MDA and Cyt c levels within normal ranges. To provide a clearer overview, we summarized the effects of ASA, ASA-Mono, ASA-Bis, and ASA-Tris in a simplified numerical format, which underscores the relationship between reduced GI toxicity and the specific molecular structures of these conjugates (see Supplement I).

We assessed platelet responses using validated, standardized methods (Boeer et al., 2010). As anticipated, ASA treatment reduced COX-dependent aggregation, collagen-induced aggregation, and ADP-induced platelet activation. By contrast, ASA-Tris reduced COX-dependent aggregation to a lesser extent and did not significantly affect collagen- or ADP-induced platelet activation, suggesting ASA-Tris may not function as a COX inhibitor. This points to an alternative anti-inflammatory mechanism distinct from ASA's action.

ROS are key contributors to ASA-induced acute ulceration (Fiorucci et al., 1998). ROS can arise from mitochondrial sources, activated leukocytes, or cellular oxidases, leading to lipid peroxidation that compromises biomembrane integrity and disrupts cellular homeostasis (DeCuyper and Joniau, 1980; Slater et al., 1984). Lipid peroxidation, indicated by MDA levels,

damages membrane phospholipids and can lead to Cyt c release from mitochondria. This release initiates apoptosis via caspase activation (Linkermann et al., 2014).

In our study, increased MDA and Cyt c levels following ASA administration confirmed ROS-related mitochondrial damage, while ASA-Tris did not induce such effects. Additionally, ASA-Tris administration did not elevate NOx levels, further underscoring the absence of oxidative and nitrosative stress. These findings suggest that ASA-Tris protects cellular energy states, preserving cell function and reducing mucosal injury.

The precise mechanism underlying ASA-Tris's action, however, remains unclear. The masked carboxyl group in ASA-Tris may minimize gastric irritation, with effectiveness seemingly proportional to the number of hydroxyl groups in each ASA conjugate (Mono, Bis, Tris). The structure of ASA-Tris possibly promotes a redox control mechanism; for instance, the hydroxyl groups may support antioxidant activity akin to flavonoids, which are more potent with multiple hydroxyl groups.

ASA-Tris might also operate through a redox-buffering mechanism. Ethanol, for example, increases NADH, which enhances reduced glutathione levels, shifting the cellular redox state toward a healthier, reduced condition (Watson et al., 2011). Finally, ASA-Tris's antioxidant properties may be due to radical scavenging; the molecule's hydroxyl groups might provide protective hydrogen atoms, thus sparing cellular biomembranes from peroxidation.

The lack of a clear understanding of the etiology and pathomechanisms of IBD underscores the need to explore new treatment options that could modulate disease outcomes. Currently, the role of NSAIDs in IBD therapy remains ambiguous, as COX inhibitors can either worsen or alleviate disease activity (Takeuchi et al., 2006; Ding et al., 2014). Hemorrhagic GI side effects further complicate their use. Nevertheless, NSAIDs are considered valuable for IBD patients, particularly those with concurrent musculoskeletal disorders like peripheral arthritis or ankylosing spondylitis (Jose and Heyman, 2008). Given this, we tested the bioactivity of novel compounds in a well-established rodent model of IBD.

In our next protocol the primary objective was to evaluate the comparable therapeutic potential of ASA-Tris, mesalamine, and ASA in TNBS-induced colitis, a model known to produce varying degrees of mucosal damage across GI tract regions. Following TNBS enema administration, we observed significant weight loss and severe mucosal damage in the colon, along with increased local inflammatory enzyme activity and elevated plasma TNF- α levels.

Intravital CLSEM imaging and *in vivo* histology revealed significant epithelial loss and endothelial injury in the colonic mucosa.

In IBD, intramural perfusion of the colon is typically impaired (Laroux and Grisham, 2001), and microcirculatory dysfunction is exacerbated by increased cell-cell interactions (Granger, 1999). ROS-generating enzymes, like XOR, are activated and the endothelial dysfunction disrupts the balance between vasoconstrictive and vasodilatory agents (Palmer et al., 1987; Masaki, 1989; Hatoum et al., 2003). Additionally, PMN leukocyte infiltration into the villus microcirculation exacerbates microvascular damage (Granger and Kubes, 1994), increasing capillary permeability and leading to edema formation (Granger et al., 1988). These hallmark signs of IBD pathology were all observed in our study, along with increased XOR activity and tissue MPO levels. Furthermore, elevated local NO_x concentrations indicated oxido-nitrosative stress within the intestinal tissue. The *in vivo* histological investigation provided evidence that ASA treatment does not entirely prevent the evolution of TNBS-induced injury in the large intestine. The capillary permeability remained higher and, simultaneously, increased XOR activity and NO_x levels were detected. ASA-Tris, however, effectively reduced the weight loss, decreased the level of inflammatory markers in the colon, and mucosal destruction was also diminished. The *in vivo* microscopic observation of the colonic mucosal surface provided evidence for an intact microvascular structure, and the effects of the ASA-Tris conjugate were similar to the efficacy of mesalamine in the colon on the third day of colitis.

The effects of the treatments were compared and evaluated on the gastric mucosa as well. Mesalamine and ASA-Tris administration did not cause gastric damage and they did not change the level of inflammatory markers in the stomach, but the administration of ASA elevated the oxidative and nitrosative stress markers; and besides this, tissue injury was observed. The ASA therapy-induced damage of the gastric mucosal surface was characterized by the loss of epithelium and accompanied by visible signs of capillary injury.

The exact molecular background governing the process of IBD is still poorly understood, but the overproduction of inflammatory cytokines, including TNF- α is viewed as fundamental. TNF- α is thought to be largely responsible for the spreading of local reactions to remote organs, and the TNF- α blockade has the theoretical advantage of abrogating the systemic inflammatory response at an earlier stage and in a specific fashion. In this study the TNBS colitis elevated the plasma TNF- α level significantly and, once again this increase was moderated by ASA-Tris treatment, which is as efficient as mesalamine treatment.

As we mentioned above, the pathways leading to mucosal destruction are still not well understood, but IBD is characterized by an energy deficiency state of the colonic epithelium (Kameyama et al. 1984). It was also shown that ASA administration may also inhibit mitochondrial respiration and the production of ATP in a concentration-dependent manner (Spenny and Bhowan 1977). Previously, it was also demonstrated that COX inhibition opens mitochondrial permeability transition pores, which is responsible for Cyt c release, a marker of mitochondrial inner membrane damage (Gugliucci et al. 2002). In the current study, ASA treatment led to mitochondrial dysfunction involving depressed mitochondrial electron transport and lower OxPhos capacity. The decreased electron flux was accompanied by the inhibition of the activities of respiratory chain enzymes, especially Cyt c oxidase (Complex IV). The *in vitro* experimental data demonstrated the direct effects of ASA-Tris on isolated liver mitochondria. The respiratory complex-specific H₂O₂ production was not affected by the compound, but the ASA-Tris conjugate reduced the disturbances associated with electron transport and increased the efficacy of oxygen consumption. Our results therefore suggest that ASA-Tris is not a radical scavenger directly; rather the favourable effects are probably linked to its protective potential against oxidative biomembrane damage.

The effects of Ket and its chemically modified Ket-Tris counterpart were also analysed respectively on GI mucosal changes in a cross-sectional study via *in vivo* tests in rodents. Ket gavage caused significant endothelial and epithelial injury in the rat stomach, and the matching microcirculatory dysfunction was accompanied by elevated MPO levels, indicating PMN accumulation in the gastric mucosa. Enhanced activity of XOR, elevated tissue MDA and NOx levels were also present. Meanwhile, Cyt c release demonstrated the presence of nitroxidative stress, and the higher circulating plasma TNF- α concentrations showed the spread of local inflammatory signals.

It was found that Ket-Tris conjugate applied in the same way did not result in the development of such pro-inflammatory processes, did not increase MPO and XOR activity, MDA, Cyt c and NOx, and left plasma TNF- α levels unchanged. Further, RBCV was kept at control level, and endothelial injury of GI mucosa was not observed. In conclusion, these data provided evidence that conjugation with Tris reduces the ulcerogenic characteristic of Ket; the newly developed compound has significantly lower mucosa-damaging potential as compared to the original NSAID.

Another goal of this protocol was to test the therapeutic potential of Ket-Tris in a health condition where the intestines are inflamed. To this end, TNBS-induced experimental colitis

was used, where various degrees of mucosal damage are expected in different regions of the GI tract. In this experiment, TNBS enema caused severe mucosal damage with higher inflammatory enzyme activities in the colon with hyperaemic microcirculation in the serosa, and the activation of ROS-generating XOR, but Ket-Tris conjugate prevented those changes, so the results confirmed the specific effect of Tris in the protection of the GI tract from NSAID-induced damage. It is conceivable that Tris conjugation results in upkept cell viability and preserved mucosal morphology as compared to the more harmful effect of the original NSAID.

Limitations

The studies of the thesis present several limitations that highlight areas for further research. First, a deeper investigation into the biochemical interactions between ASA-Tris and its potential targets, particularly the mechanisms influencing ROS generation, is needed. Additionally, knowledge gaps remain regarding the metabolism and tissue penetration of ASA- and Ket-Tris derivatives.

We used a TNBS colitis model characterized by acute, erosive epithelial injuries. To better understand the influence of these compounds on mucosal repair and inflammatory responses, future research should incorporate chronic models that engage the adaptive immune system more fully. Despite these limitations, our findings indicate that ASA- and Ket-Tris conjugates could offer a promising alternative to traditional NSAID therapies, positioning them as strong candidates for further evaluation in IBD treatment.

Moreover, the relatively short follow-up period limits insights into the long-term roles of Tris conjugates in mucosal defence and repair. Future studies should explore intracellular and molecular pathways involved in mucosal protection, focusing on gene expression, transcriptional changes, and impacts on the mucus-bicarbonate barrier and COX pathway markers. Such investigations could reveal important therapeutic mechanisms of ASA- and Ket-Tris derivatives and advance our understanding of their therapeutic potential.

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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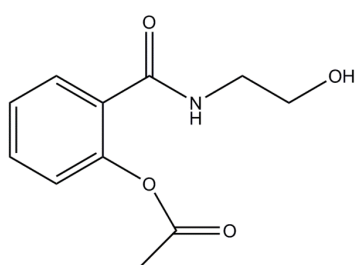
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Supplement I.

The chemical synthesis of ASA-Mono, ASA-Bis and ASA-Tris conjugates

ASA-Mono

3.6 g acetyl salicylic acid was dissolved in 150 ml abs. THF and cooled to -15°C. 2.6 ml of isobutylchloroformate and 2.79 ml triethylamine were added under stirring. After 25 minutes of stirring at -15°C, 1.2 ml of ethanolamine was added and the reaction mixture was stirred for an additional hour at 0°C. The stirring was continued overnight. The reaction mixture was then filtered, evaporated and the resulting oily material was dissolved in ethyl acetate and extracted with water. After evaporation of the solvent, the remaining oil was identified as compound below (1.6 g, 35.7 %. Ms 224.1).



HPLC:

solvents: A 0.1 % TFA, B 0.1 % TFA, 80 % AcN

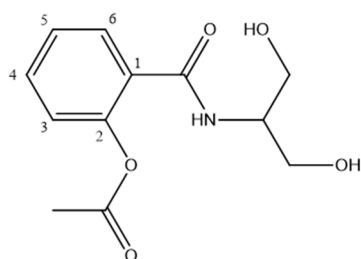
column: Phenomenex Luna 10 C 18 100Å

gradient 5-95 % B in 30 min, flow 1.2 ml/ min, detection at 220

nm Rt 9.75 min

ASA-Bis

3.6 g acetyl salicylic acid was dissolved in 150 ml abs. THF and cooled to -15°C. 2.6 ml of isobutylchloroformate and 2.79 ml triethylamine were added under stirring. After 25 minutes of stirring at -15°C, 1.2 ml of 2-amino-1,3-propanediol was added and the reaction mixture was stirred for an additional hour at 0°C. The stirring was continued overnight. The reaction mixture was then filtered, evaporated and the resulting oily material was dissolved in ethyl acetate and extracted with water. After evaporation of the solvent, the remaining oil was identified as compound below (1.5 g, 30 %. Ms 254.0).



HPLC:

solvents: A 0.1 % TFA, B 0.1 % TFA, 80 % AcN

column: Phenomenex Luna 10 C 18 100Å

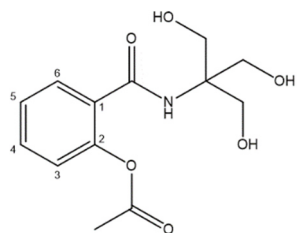
gradient 20-40 % B in 20 min, flow 1.2 ml/ min, detection at

220 nm Rt 13.88 min

¹H NMR (600 MHz, DMSO): δ= 2.25 (s, 3H, CH₃), 3.44-3.53 (m, 4H, 2 x CH₂-OH), 3.84-3.90 (m, 1H, CH), 4.69 (bs, 2H, OH), 7.18 (d, J = 7.67 Hz, H-3), 7.33 (t, J = 7.67 Hz, H-5), 7.51, t = 7.67 Hz, H-4), 7.66 (d, J = 7.67 Hz, H-6); ¹³C NMR (150 MHz, DMSO): δ=21.2 (CH₃), 52.8 (CH), 60.4 (CH₂-OH), 123.7 (C-3), 126.3 (C-5), 129.6 (C-1), 129.9 (C-6), 131.8 (C-4), 148.3 (C-2), 165.2 (C=O-NH), 169.3 (O-C=O).

ASA-Tris

2.88 g acetyl salicylic acid was dissolved in 150 ml abs THF and cooled to -15°C . Under stirring 2.1 ml isobutylchloroformate and 2.23 ml triethylamine were added. After 25 min stirring at -15°C 1.94 g of tris hydroxymethyl amino methane was added and was stirred for additional one hour at 0°C . The stirring was continued at RT overnight. The reactions mixture was filtered, evaporated and the resulting crystalline material was washed with diethylether-hexane resulting the pure product (2.34 g, 52 %; Ms 284.1)



HPLC:

solvents: A 0.1 % TFA, B 0.1 % TFA, 80 % AcN

column: Phenomenex Luna 10 C 18 100Å

gradient 5-80 % B in 25 min, flow 1.2 ml/ min, detection at 220 nm

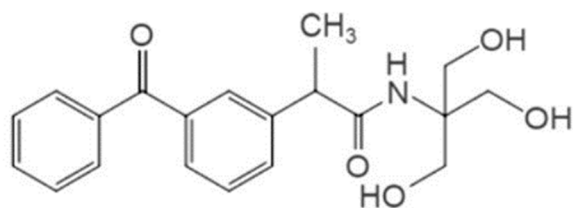
Rt 10.08 min

^1H NMR (600 MHz, DMSO): δ = 2.29 (s, 3H, CH_3), 3.65 (d, J = 4.90 Hz, 6H, 3 x $\text{CH}_2\text{-OH}$), 4.77 (t, J = 4.90 Hz, 3H, $\text{CH}_2\text{-OH}$), 7.19 (d, J = 7.53 Hz, 1H, H-3), 7.26 (s, 1H, NH), 7.34 (t, J = 7.53 Hz, 1H, H-5), 7.52 (t, J = 7.53 Hz, 1H, H-4), 7.69 (d, J = 7.53 Hz, 1H, H-6); ^{13}C NMR (150 MHz, DMSO): δ =21.3 (CH_3), 60.6 ($\text{CH}_2\text{-OH}$), 63.0 ($\text{C-CH}_2\text{-OH}$), 123.8 (C-3), 126.4 (C-5), 129.7 (C-1), 130.2 (C-6), 131.9 (C-4), 147.9 (C-2), 165.6 (C=O-NH), 169.4 (O-C=O).

Supplement II.

Ket-Tris

Ket (5.09 g) was dissolved in absolute tetrahydrofuran (200 ml) and cooled to -15°C . Isobutyl chloroformate (2.62 ml) and triethylamine (2.79 ml) were added while stirring. After stirring at -15°C for 25 min, Tris (2.42 g) was added and the mixture was stirred for an additional 1 h at 0°C and then at room temperature overnight. The reaction mixtures were filtered and evaporated, and the resulting crystalline materials were washed with diethyl ether-hexane, resulting in the pure products (2.9 g, 40.56 %, Ms 358.06).



HPLC:

solvent: A 0.1 % TFA, B 0.1 % TFA, 80 % can;

column: Phenomenex Luna 10 C 18 100Å;

gradient: 20-100 % B in 20 min, flow 1.2 ml/

min, detection at 220 nm Rt 10.38 min

^1H NMR (600 MHz, DMSO): δ =1.34 (d, J = 7.01 Hz, 3H, CH-CH₃), 3.50 (dd, J = 5.87, 10.91 Hz, 3H, CH₂-OH), 3.55 (dd, J = 5.87, 10.91 Hz, 3H, CH₂-OH), 3.88 (q, J = 7.04 Hz, 1H, CH-CH₃), 4.71 (t, J = 5.66 Hz, 3H, CH₂-OH), 7.36 (s, 1H, NH), 7.49-7.76 (m, 9H, H-2, H-4, H-5, H-6, Ar); ^{13}C NMR (150 MHz, DMSO): δ =19.3 (CH-CH₃), 45.3 (CH₂), 61.0 (CH₂-OH), 62.6 (C-CH₂-OH), 126.5 (C-5), 129.0 (Ar-C-2, Ar-C-4, Ar-C-6), 130.2 (C-6, Ar-C-3, Ar-C-5), 132.2 (C-2), 133.2 (C-4), 137.3 (C-3), 137.5 (Ar), 143.2 (C-1), 174.8 (C=O-NH), 196.2 (C=O).

ANNEX I.

ANNEX II.

ANNEX III.