Investigation of the effects of MIF tautomerase inhibitors on inflammatory macrophage activation and experimental colitis

PhD Thesis

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1. Literature Review

1.1. General characterisation of inflammatory bowel diseases

Inflammatory bowel diseases (IBD) encompass two main clinical entities: Crohn's disease (CD) and ulcerative colitis (UC). The prevalence and incidence of both conditions are increasing worldwide. In the United States alone, it is estimated that 2.4 to 2.7 million individuals are affected by IBD.

Crohn's disease may involve any segment of the gastrointestinal tract, from the mouth to the anus, although it most commonly affects the terminal ileum. It is characterised by transmural inflammation, which extends through all layers of the intestinal wall, from the mucosa to the serosa. The disease manifests with sharply demarcated lesions and causes structural damage to the intestinal mucosa. The clinical presentation varies widely but typically includes recurrent diarrhoea, crampy abdominal pain, and fever that lasts for days to weeks.

In contrast, ulcerative colitis is a relapsing inflammatory disease restricted to the colon and characterised by mucosal ulceration. Except in severe cases such as toxic megacolon, inflammation is generally limited to the superficial layers of the bowel wall, namely the mucosa and submucosa.

IBD is a multifactorial disorder with an incompletely understood aetiology. In addition to genetic predisposition, various environmental factors—such as diet, infections, smoking, and appendectomy—are thought to contribute to disease development. The interplay between genetic and environmental influences often leads to disruption of the intestinal epithelial barrier. When barrier integrity is compromised, luminal bacteria and microbial products can translocate into the bowel wall, triggering uncontrolled immune activation and the release of cytokines. Without adequate anti-inflammatory treatment, acute mucosal inflammation may progress to chronic intestinal inflammation. Nonetheless, it remains unclear whether epithelial barrier dysfunction is a primary cause or a consequence of the inflammatory process.

1.2. Therapies Used in Inflammatory Bowel Diseases

A variety of therapeutic strategies are currently employed in the management of IBD. In most cases, pharmacological interventions are effective in achieving clinical improvement; however, surgical treatment may be required in certain situations. The main therapeutic goals include symptom relief, induction and maintenance of remission, prevention of relapse,

restoration of intestinal function, and, in some cases, temporary mucosal healing. To date, no curative (etiological) therapy exists for IBD, and permanent resolution of the disease remains unachievable. Accurate localisation of inflammatory lesions is essential, as it guides the choice between systemic and localised therapeutic approaches. Current pharmacological treatments primarily include immunosuppressive agents, corticosteroids, and antibiotics. Although effective, the long-term use of these drugs is limited by their adverse effect profile, which may include fever (pyrexia), seizures, and hypertension.

1.3. The Role of Mitochondria in Inflammatory Bowel Diseases

Mitochondrial biogenesis, along with the stability of mitochondrial function and structure, plays a crucial role in maintaining cellular metabolic balance. Accordingly, proper mitochondrial function is essential for sustaining the physiological integrity of the intestinal epithelium. Mitochondrial dysfunction impairs cellular bioenergetics, disrupts intercellular communication, and interferes with physical interactions between mitochondria and other organelles (e.g., the endoplasmic reticulum [ER]). These disruptions contribute to the generation of reactive oxygen species (ROS) and the activation of immune cells, which together compromise the intestinal barrier, increase permeability, and promote intestinal inflammation.

The process of oxidative phosphorylation (OXPHOS) occurs within mitochondria, which serve as central hubs for major metabolic pathways and efficient ATP production. During catabolic metabolism, NADH and $FADH_2$ act as electron carriers, transferring electrons from the citric acid cycle to the electron transport chain (ETC). Most electrons are passed through the mitochondrial respiratory complexes to cytochrome c oxidase (Complex IV), where they reduce molecular oxygen (O_2) to form water. However, a small proportion (approximately 2%) of electrons, primarily from Complexes I and III, escape the ETC and react with molecular oxygen to generate superoxide anions. These reactive oxygen species are subsequently dismutated either spontaneously or enzymatically by mitochondrial manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H_2O_2). If damaged mitochondria are not efficiently repaired or eliminated, and mitochondrial ROS production increases, this can result in the accumulation of mitochondrial components such as formylated peptides. These molecules function as damage-associated molecular patterns (DAMPs), triggering immune cell activation and amplifying the inflammatory response.

1.4. The Role of Macrophages in Inflammatory Bowel Diseases

In both ulcerative colitis and Crohn's disease, a defective immune response to microbiota-derived antigens in the gut has been observed. Under physiological conditions, the intestinal microbiota does not elicit a pro-inflammatory immune response—a phenomenon referred to as immune tolerance. However, in inflammatory bowel diseases, this tolerance is disrupted. Effective immune defence relies on the coordinated function of both innate and adaptive immunity, which depends on intact epithelial integrity, a protective mucus layer, and normal peristaltic activity. In healthy individuals, monocytes continuously migrate to the intestinal mucosa, where they differentiate into mature macrophages exhibiting an M2-like phenotype. These M2-like macrophages are associated with anti-inflammatory activity, characterized by the secretion of cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), as well as tightly regulated expression of Toll-like receptors (TLRs). In IBD, however, this differentiation into the M2 phenotype is impaired. Instead, chemokines such as CCL2, CCL7, and CCL8 recruit circulating monocytes to sites of inflammation, where they differentiate into pro-inflammatory M1 macrophages. These M1-polarized macrophages produce pro-inflammatory cytokines, including IL-1, IL-6, IL-18, and tumour necrosis factor-alpha (TNF-α), which directly or indirectly damage intestinal epithelial cells, leading to cellular injury or necrosis. The excessive secretion of these inflammatory mediators is a hallmark feature of IBD pathogenesis.

1.5. Macrophage Metabolism During Inflammation

In macrophages, key immunometabolic pathways include glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, fatty acid synthesis, and amino acid metabolism. M1 polarisation involves a comprehensive metabolic reprogramming that enables macrophages to meet pro-inflammatory functional demands.

Upon stimulation with lipopolysaccharide (LPS) or interferon-γ (IFN-γ), macrophages undergo a metabolic shift known as Warburg metabolism. This phenomenon is characterised by a switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, resulting in increased lactate production despite adequate oxygen availability. During this metabolic reprogramming, macrophages become highly dependent on glucose uptake for ATP and lactate production, while mitochondrial OXPHOS is suppressed. Interestingly, despite reduced TCA cycle activity, mitochondrial succinate oxidation is enhanced via succinate dehydrogenase. This

process contributes to increased ROS production and reinforces M1 polarisation. Macrophages adopting Warburg-type metabolism become locked in a pro-inflammatory M1 state and are resistant to repolarisation, even upon stimulation with IL-4, a potent M2-polarising cytokine. Proper mitochondrial function appears to be essential for facilitating the transition from M1 to M2 phenotype.

Altered lipid metabolism has also been observed in patients with inflammatory bowel disease, and inflammatory lipid mediators are under investigation as potential disease biomarkers. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) may serve as a critical link between lipid signalling and innate immune regulation. Notably, activation of PPAR-γ in macrophages confers protection against experimental colitis in mice, whereas macrophage-specific PPAR-γ deficiency exacerbates colonic inflammation. These findings suggest that lipid metabolism plays a pivotal role in macrophage polarisation, and PPAR-γ represents a promising therapeutic target in IBD.

Disturbances in amino acid metabolism have also been associated with disease activity in IBD. Patients often exhibit impaired glutamine metabolism, evidenced by reduced glutamine uptake, low plasma glutamine levels, and decreased mucosal glutaminase activity. Arginine levels are similarly depleted during intestinal inflammation due to increased nitric oxide (NO) production from L-arginine. Since NO synthesis is upregulated in inflammation, the demand for arginine exceeds its availability. This depletion is concerning, as arginine exerts anti-inflammatory effects in macrophages through multiple signalling pathways.

1.6. MIF

Macrophage migration inhibitory factor (MIF), first identified over four decades ago, was among the earliest cytokines recognized as being secreted by T lymphocytes. The human MIF protein consists of 114 amino acids in its monomeric form; however, its biologically active form is now known to be a homotrimer. Although macrophages are major producers of MIF, the cytokine is also expressed in a wide range of other cell types, including lymphocytes, monocytes, endothelial cells, and fibroblasts. MIF exhibits diverse biological activities. In macrophages, it enhances cellular adhesion, promotes phagocytosis, and inhibits cell migration. It plays a central role in both innate and adaptive immune responses and has been implicated in tumour progression and angiogenesis. MIF promotes the production of multiple pro-inflammatory cytokines and inflammatory mediators in macrophages, such as TNF-α, IFN-γ,

IL-1β, IL-2, IL-6, IL-8, nitric oxide, and cyclooxygenase-2 (COX-2). As an endocrine mediator, MIF is regulated by the hypothalamic–pituitary–adrenal (HPA) axis and is expressed at all levels of this axis, including the hypothalamus, pituitary gland, and adrenal cortex.

Interestingly, MIF also exhibits tautomerase enzymatic activity, catalysing the tautomerization of D-dopachrome—a synthetic compound not naturally occurring in biological systems—as well as the keto-enol tautomerization of phenylpyruvate and hydroxyphenylpyruvate. This enzymatic activity includes both ketonase and enolase subfunctions. Although its physiological substrates have not been definitively identified, catecholamine metabolites have been proposed as potential candidates. It is hypothesized that this tautomerase activity contributes to MIF's biological effects, although the underlying mechanisms remain incompletely understood.

1.7. The Role of MIF in Inflammation

MIF is released in response to mitogens, inflammatory stimuli (e.g., lipopolysaccharide), specific antigens, and cytokines such as TNF-α and IFN-γ. Once secreted, biologically active MIF exerts its effects both extracellularly-via receptor-mediated signalling, and intracellularly through the activation of signalling proteins. During acute inflammation, MIF promotes the production of pro-inflammatory cytokines (e.g., TNF, IL-6), NO, and other inflammatory mediators. Notably, MIF also acts as an endogenous antagonist of the immunosuppressive effects of glucocorticoids. In vitro studies have demonstrated that MIF can counteract glucocorticoid-mediated inhibition of several immune functions, including the production of TNF, IL-1, IL-6, and IL-8 by peripheral blood mononuclear cells, the activity of cytosolic phospholipase A2 (PLA2), arachidonic acid release in fibroblasts, and T-cell proliferation. Similar to glucocorticoids, MIF levels increase in response to inflammation, infection, and stress. MIF plays a key role in the pathogenesis of both acute and chronic inflammatory diseases, such as sepsis and pulmonary disorders like chronic pneumonia and acute respiratory distress syndrome (ARDS). Elevated MIF levels have also been observed in patients with inflammatory bowel diseases.

1.8. Inhibition of MIF

The protective effects of MIF inhibition have been demonstrated in various inflammatory diseases. One proposed mechanism by which MIF promotes inflammation is its antagonism of

the immunosuppressive actions of glucocorticoids. As a result, MIF inhibition has emerged as a promising therapeutic strategy for treating inflammatory and autoimmune disorders.

Research has revealed a direct link between the cytokine activity of MIF and its tautomerase enzymatic function. Based on this, it is hypothesized that inhibition of MIF tautomerase activity may exert beneficial effects on various inflammatory processes. Small-molecule modulators of MIF tautomerase activity can influence MIF's cytokine function by altering its conformation and/or its interactions with other proteins. Numerous natural MIF tautomerase inhibitors have been identified; examples include caffeic acid and curcumin. Synthetic inhibitors include the paracetamol metabolite NAPQI and ISO-1. Among these, ISO-1 is the most commonly used reference inhibitor of MIF tautomerase activity. Belonging to the isoxazoline class, ISO-1 inhibits MIF tautomerase activity in a dose-dependent manner by binding at the same site as the substrate, p-hydroxyphenylpyruvate, i.e., the tautomerase active site. Consequently, ISO-1 serves as a reference inhibitor in the testing and validation of small-molecule MIF tautomerase inhibitors.

Despite this progress, potent MIF inhibitors with favourable pharmacokinetic and pharmacodynamic properties remain scarce, and only a limited number have been thoroughly evaluated in relevant disease models.

1.9. KRP-6 and TE-11

One of the compounds used in my work, E-3-(2-methoxybenzylidene)chroman-4-one (KRP-6), strongly inhibited the ketonase activity of MIF (IC_{50} = 4.31 ± 1.34 μmol/L), while it did not significantly affect the enolase activity (IC_{50} = 1260 ± 159 μmol/L); thus, it can be considered a selective ketonase inhibitor. The other compound, (E)-2-(pyridin-2-ylmethylene)-3,4-dihydronaphthalen-1(2H)-one (TE-11), effectively inhibited both the ketonase (IC_{50} = 5.63 μmol/L) and enolase (IC_{50} = 28.58 μmol/L) enzymatic activities of MIF. These compounds, which share structural similarity with naturally occurring MIF-inhibitory flavonoids, have been confirmed by our research group to exhibit inhibitory effects on MIF tautomerase enzymatic activity.

2. Aims

The inhibitory effect of MIF has been shown to exert beneficial effects in various inflammatory diseases. Therefore, in our work, we investigated the effects of the compounds KRP-6 and TE-11 in a TNBS-induced experimental colitis model, as well as in IFN-γ and LPS+IFN-γ-induced M1 macrophages.

The following research questions were addressed in my thesis:

1. Previous results demonstrated that TE-11 was one of the most effective MIF inhibitors synthesised and tested by our research group in an LPS-induced inflammatory macrophage model, and it exhibited protective effects in a mouse model of septic shock. Therefore, we examined the effect of TE-11 treatment on the symptoms induced by TNBS, as well as on colonic ulceration in a mouse model of Crohn's disease.
2. How does TE-11 affect the production of inflammatory cytokines in the colon?
3. KRP-6 was identified as one of the most selective ketonase inhibitors among all tested compounds. Hence, we investigated whether KRP-6 and the highly potent but non-selective TE-11 reduce neutrophil and eosinophil migration and whether they influence MIF-induced apoptosis?
4. What is the effect of KRP-6 and TE-11 on ROS, nitrite, and inflammatory cytokine production in IFN-γ and LPS+IFN-γ-stimulated macrophages?
5. How do KRP-6 and TE-11 influence the energy metabolism of inflammatory macrophages?
6. Does KRP-6 affect the expression of PARP enzymes in LPS+IFN-γ-stimulated macrophages?

The primary objective of my thesis is to contribute to the expanding scientific evidence supporting the therapeutic potential of MIF tautomerase inhibition through the use of animal models and cell culture systems.

3. Results

3.1. Investigating the effect of TE-11 in the TNBS-induced colitis mouse model

3.1.1. Macroscopic evaluation of the colon

We investigated the effects of TE-11 in a TNBS-induced mouse model of colitis. Compared to the control group, TNBS administration caused significant colonic tissue damage, characterized by haemorrhagic ulcers and elevated macroscopic scores. During macroscopic evaluation, parameters such as colon length shortening, colon weight, wall thickness, consistency, and presence of blood in the stool, ulcer number and size, and intra-abdominal adhesions were assessed. Administration of TE-11 at a higher dose (10 mg/kg) significantly attenuated tissue damage by reducing ulcer size, leading to a lower composite inflammation score compared to the TNBS group.

3.1.2. Inflammatory cytokine levels

TNBS induced inflammatory cytokine production in the colon. Specifically, TNBS significantly increased IL-6 production, whereas treatment with TE-11 reduced IL-6 levels by approximately 50%. TNBS treatment also elevated IL-1β production in the tissue; however, TE-11 administration resulted in a significant decrease in IL-1β levels.

3.2. Investigation of the effects of KRP-6 and TE-11 on polymorphonuclear leukocyte (PMNL) and eosinophil migration and apoptosis

3.2.1. Results of chemotaxis assays

MIF is highly expressed during chronic inflammation and regulates leukocyte migration by binding to various cell surface receptors, including CXCR2, CXCR4, and CD74. To investigate its effects on leukocyte migration, we utilized human peripheral blood-derived neutrophils and eosinophils. Chemotaxis was induced using different chemotactic agents: neutrophil migration was stimulated with either MIF or IL-8, whereas eosinophil migration was triggered by MIF or CCL11. Both ISO-1 and KRP-6 significantly reduced MIF-induced neutrophil and eosinophil migration. In contrast, neither compound affected IL-8-induced neutrophil chemotaxis. However, KRP-6 inhibited CCL11-induced eosinophil migration,

whereas ISO-1 had no such effect. TE-11 was able to reduce neutrophil migration induced by both MIF and IL-8, as well as eosinophil migration stimulated by MIF and CCL11.

3.2.2. Results of cell viability assay

Since MIF has been shown to inhibit neutrophil apoptosis through both direct and indirect mechanisms, we examined the effects of KRP-6 and TE-11 on apoptosis in neutrophils and eosinophils. In our first study, MIF was found to reduce both early and late apoptosis in neutrophils, but it did not affect either form of apoptosis in eosinophils. Specifically, early apoptosis decreased by 40%, and late apoptosis by 47%. KRP-6 effectively countered this anti-apoptotic effect of MIF, reducing one of its pro-inflammatory properties in neutrophils. Conversely, in our second study focusing on TE-11, MIF was observed to decrease late apoptosis and necrosis in both neutrophils and eosinophils. Pre-treatment with TE-11 significantly increased the number of apoptotic and necrotic cells among neutrophils and eosinophils compared to MIF alone. Notably, in this second experiment, the anti-apoptotic and anti-necrotic effects of MIF on eosinophils may be due to an increase in serum concentration from 1% to 3% fetal bovine serum (FBS) in the culture medium, based on previous findings.

3.3. Investigation of the effects of KRP-6 and TE-11 in an inflammatory macrophage model

3.3.1. Results of ROS measurement

We investigated the effects of KRP-6 and TE-11 on ROS production in RAW264.7 macrophages stimulated with IFN-γ or a combination of IFN-γ and LPS following 24-hour treatment. After adding DHR123, fluorescence was measured immediately (background) and then after 2 hours; the difference between these values was calculated to determine the actual amount of ROS produced. In the IFN-γ stimulated model, ROS production in the untreated group was approximately 73% of that in the IFN-γ treated group. Treatment with KRP-6 reduced the elevated ROS production. In the model stimulated with both IFN-γ and LPS, ROS production in the untreated group was about 44% of that in the IFN-γ plus LPS-treated group. TE-11 treatment similarly decreased the elevated ROS levels.

3.3.2. Results of nitrite measurement

Nitric oxide synthesis is a key feature of macrophage activation. Due to its high reactivity, NO rapidly converts to nitrite, which accumulates in the culture medium and can be detected using the Griess reagent. The nitrite concentration serves as an indirect measure of NO production. After 24 hours of treatment with IFN-γ or IFN-γ combined with LPS, nitrite production was measured in RAW264.7 macrophages. We investigated whether nitrite levels differ between cells treated with IFN-γ versus IFN-γ+KRP-6 and IFN-γ+LPS versus IFN-γ+LPS+TE-11. KRP-6 treatment had no significant effect on the elevated nitrite production, as the observed 18% difference was not statistically significant. In contrast, TE-11 treatment significantly reduced the increased nitrite levels in a statistically significant manner.

3.3.3. Results of HIF-1α measurement

LPS+IFN-γ treatment induced HIF-1α mRNA transcription and protein expression in RAW264.7 cells. While LPS+IFN-γ significantly increased HIF-1α mRNA levels, this induction was markedly attenuated by TE-11 treatment. Similarly, the elevated HIF-1α protein expression triggered by LPS+IFN-γ was also reduced upon TE-11 administration.

3.3.4. Effect of TE-11 on inflammatory mRNA expression

LPS+IFN-γ treatment induced the mRNA expression of several inflammatory genes in macrophages. The levels of CCL2, IL-6, TNF-α, iNOS, and SOD2 were elevated compared to the DMSO-treated (VEH) group. In contrast, TE-11 reduced the mRNA levels of CCL2 and IL-6 but did not affect the transcription of TNF-α or iNOS. Notably, TE-11 further increased the expression of the SOD2 mRNA in LPS+IFN-γ-activated cells. In our model, no change was observed in Nrf1 mRNA expression.

3.3.5. Results of inflammatory cytokine level measurement

Macrophage-derived cytokine production plays a central role in inflammatory processes; therefore, we measured the levels of CCL2, IL-6, and TNF-α using ELISA assays. We aimed to determine whether treatment with KRP-6 or TE-11 alters inflammatory cytokine production in macrophages activated with IFN-γ alone or in combination with LPS. Stimulation with IFN-γ alone significantly increased TNF-α secretion compared to untreated controls, whereas KRP-6 treatment did not produce a significant reduction in TNF-α levels. In the LPS+IFN-γ-stimulated

model, the production of TNF-α, CCL2, and IL-6 was markedly elevated. TE-11 treatment significantly reduced the levels of CCL2 and IL-6 in this model; however, TNF-α production remained unaffected.

3.3.6. Results of extracellular acidification rate (ECAR) measurement

M1 macrophages shift their metabolism from oxidative phosphorylation toward aerobic glycolysis. Accordingly, we measured the extracellular acidification rate (ECAR) in LPS+IFN-γ-treated RAW264.7 cells. This rate essentially reflects lactate production, which indicates the cells' fermentative ATP production (i.e., aerobic glycolysis) activity. In cells treated with IFN-γ and LPS, the basal acidification rate increased, suggesting a metabolic shift toward glycolysis. However, treatment with KRP-6 and TE-11 significantly decreased the ECAR. Furthermore, oligomycin treatment increased the ECAR in VEH-treated cells, indicating that OXPHOS plays a key role in ATP production in our macrophage model. The inhibition of the F_OF1-ATP synthase by oligomycin reduces mitochondrial ATP generation, which the cell compensates for by enhancing glycolytic flux. FCCP, rotenone, and antimycin A did not further alter ECAR in any of the treatment groups.

3.3.7. Results of mitochondrial function analysis

Upon addition of oligomycin, which inhibits the F_O subunit of the F_OF1-ATP synthase, oxygen consumption decreases. FCCP, a mitochondrial uncoupler, disrupts the proton gradient across the inner mitochondrial membrane, thereby uncoupling the electron transport chain from ATP synthesis. This collapse of the proton gradient accelerates electron flow through the ETC, leading to an increase in oxygen consumption at complex IV (CIV). Antimycin A and rotenone inhibit mitochondrial oxygen consumption by targeting complexes I and III of the ETC, respectively. By subtracting the non-mitochondrial oxygen consumption from the basal oxygen consumption, the basal respiration can be determined, which consists of oxygen used for ATP production and proton leak. Maximal respiratory capacity is defined as the oxygen consumption measured following FCCP treatment, while spare respiratory capacity is calculated as the difference between maximal and basal respiration. Basal respiration values were derived from the data from measurements taken prior to oligomycin administration.

In the first phase of the measurement, the basal respiration of the cells was determined. Treatment with LPS+IFN-γ resulted in an approximately 80% reduction in basal respiration. In

the presence of KRP-6, this decrease was 62%, while TE-11 treatment resulted in a reduction of only 29%. No statistically significant difference was observed between the VEH and TE-11 groups. Following the addition of oligomycin, ATP production was calculated. LPS+IFN-γ treatment led to a 95% reduction in ATP production compared to control cells. In the presence of KRP-6, ATP production decreased by 67%, while TE-11 treatment caused a 24% reduction. LPS+IFN-γ also impaired mitochondrial coupling efficiency by 75%, which was restored to control levels by both KRP-6 and TE-11. Upon FCCP addition, the proton gradient collapsed, leading to increased mitochondrial oxygen consumption, which allowed the determination of maximal respiration. LPS+IFN-γ treatment reduced maximal respiration by 93% relative to controls. In the presence of KRP-6, the reduction was less pronounced (72%), while TE-11 reduced maximal respiration by only 48%. Spare respiratory capacity was reduced by 99% following LPS+IFN-γ treatment. KRP-6 treatment did not influence this parameter, as the observed ~78% difference was not statistically significant. In contrast, TE-11 treatment resulted in a 67% reduction. Proton leak was calculated by subtracting the non-mitochondrial oxygen consumption (measured after the addition of rotenone and antimycin A) from the oxygen consumption measured after oligomycin treatment. In our study, LPS+IFN-γ treatment resulted in approximately 54% proton leak, which was not affected by either KRP-6 or TE-11 treatment.

3.3.8. Effect of KRP-6 on PARP enzymes

Previous results suggest that activated macrophages reduce PARP-1 mRNA expression. Therefore, we investigated the mRNA expression of PARP-1, -2, and -3 in RAW264.7 macrophages treated with LPS and IFN-γ. Our results demonstrated that while PARP-1 and PARP-2 mRNA expression decreased in LPS+IFN-γ-induced macrophages, the synthesis of PARP-3 mRNA remained unchanged. In contrast, KRP-6 treatment significantly increased the expression of PARP-1 and PARP-2.

4. Summary

In this study, I investigated the effects of two compounds from a novel family of macrophage migration inhibitory factor inhibitors: KRP-6 and TE-11. TE-11 was evaluated in a TNBS-induced experimental colitis model. In addition, the effects of both TE-11 and KRP-6 were examined *in vitro* on classical M1 macrophage polarization, as well as leukocyte and eosinophil migration.

In the first part of this study, I focused on the effects of KRP-6 in IFN-γ-stimulated macrophages. Our results demonstrated that KRP-6 inhibited ROS production and reduced glycolytic activity while enhancing mitochondrial energy production. Furthermore, KRP-6 upregulated the transcription of the PARP1 and PARP2 genes, suggesting a potential role for MIF in the regulation of these PARP isoforms. Determining whether these effects are mediated via MIF receptor pathways or through its tautomerase activity lies beyond the scope of this thesis. Nonetheless, our findings indicate that KRP-6 — a highly selective ketonase inhibitor — significantly suppresses macrophage activation and leukocyte migration, underscoring its potential as a pharmacological target for the treatment of chronic inflammatory and autoimmune diseases Since the anti-inflammatory effects of KRP-6 have, to date, been examined only in cell culture models, further in vivo studies are required before clinical application. In addition, clarifying the distinct biological roles of receptor-mediated activation versus tautomerase inhibition and identifying processes dependent solely on ketonase or enolase activities would be highly informative. Since proline-1 mutant MIFs completely lack tautomerase activity, these genetic models are inadequate for dissecting the two enzymatic functions independently. Therefore, selective inhibitors such as KRP-6 represent valuable tools for advancing our understanding of MIF's complex biological roles.

The second part of this study focused on TE-11, a novel inhibitor of MIF tautomerase activity. TE-11 attenuated inflammatory macrophage activation and prevented the metabolic shift from oxidative phosphorylation to aerobic glycolysis. It also effectively inhibited leukocyte and eosinophil migration and alleviated disease symptoms in a murine model of experimental colitis that resembles Crohn's disease. TE-11 treatment resulted in fewer macroscopic lesions and reduced tissue levels of the pro-inflammatory cytokines IL-6 and IL-1β. Overall, our findings suggest that MIF tautomerase activity—likely acting through the MIF/HIF-1α axis—regulates glycolytic processes, and that its inhibition can attenuate the metabolic reprogramming associated with M1 macrophage activation.

Given that leukocyte migration and metabolic reprogramming contribute significantly to the pathogenesis of inflammatory bowel disease, MIF represents a promising therapeutic target for modulating these key processes. Based on our results, TE-11 may serve as a potential drug candidate for the future treatment of IBD.

8. List of publications

Publication related to the thesis

Vámos E, Kálmán N, Sturm EM, et al. Highly Selective MIF Ketonase Inhibitor KRP-6 Diminishes M1 Macrophage Polarization and Metabolic Reprogramming. *Antioxidants (Basel)*. 2023;12(10):1790. Published 2023 Sep 22. **IF:6.000**

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First-author conference posters related to the thesis

Eszter Vámos, Viola Bagóné Vántus, Dominika Kovács, Péter Deák, Ferenc Gallyas Jr., Balázs Radnai "*Effect of MIF tautomerase inhibitors on macrophage activation and mitochondrial function*", Hungarian Molecular Life Sciences 2023 Conference, Eger, Hungary, 24-26 March 2023

Eszter Vámos, Viola Bagóné Vántus, Dominika Kovács, Péter Deák, Ferenc Gallyas Jr., Balázs Radnai "*Effect of KRP-6, a novel MIF tautomerase inhibitor on macrophage activation and mitochondrial function*", Annual Meeting of the Hungarian Biochemical Society 2022 Conference, Pécs, Hungary, 25-27 August 2022

Vámos Eszter, Bagóné Vántus Viola, Kovács Dominika, Deák Péter, Kőszegi Balázs, Kálmán Nikoletta, Vass Ibolya, Ifj. Gallyas Ferenc, Radnai Balázs "*A MIF TAUTOMERÁZ INHIBITOR KRP-6 GÁTOLJA A GYULLADÁSOS MAKROFÁG AKTIVÁCIÓT ÉS VÉDI A MITOKONDRIÁLIS ENERGIATERMELÉST. "51.* Membrán-Transzport Konferencia Sümeg, Magyarország 2022. május 17-20

Additional first-author conference posters

Eszter Vámos, Viola Bagóné Vántus, Ferenc Gallyas Jr., Balázs Radnai "*The effect of trimethylamine, a metabolite of gut microbiota on the metabolic reprogramming of M1*

macrophages", Annual Meeting of the Hungarian Biochemical Society 2024 Conference, Budapest, Hungary, 29-31 August 2024

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