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PHARMACOLOGICAL AND TOXICOLOGICAL CHARACTERIZATION OF S1P AS A TREATMENT OPTION IN ANAEMIAS

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LIST OF ABBREVIATIONS

ACD: Anemia of chronic disease

ARD: Anemia of renal disease

BM: Bone marrow

CYP450: Cytochrom P450 enzyme

DIC: Disseminated intravascular coagulopathy

DMSO: Dimethyl sulfoxide

EPO: Erythropoietin

FeLV: Feline leukemia virus

FIV: Feline immunodeficiency virus

FBS: Fetal bovine serum

Hb: Hemoglobin

HDL: High density lipoprotein

HJV: Hemojuvelin

IDA: Iron deficient anemia

IHA: Immune hemolytic anemia

IL-6: Interleukin-6

LDL: Low density lipoprotein

MPS: Mononuclear-phagocytic system

PBS: Phosphate-buffered saline buffer

RBC: Red blood cell

ROS: Reactive oxygen species

S1P: Sphingosin-1-phosphate

IBD: Inflammatory bowel disease

MT-1: Matriptase-1

MT-2: Matriptase-2

TJ: Tight junction

TTSP: Type II transmembrane serine protease

1. INTRODUCTION

The major internal detoxification organ of mammals is the liver, which serves wide spectrum of vital functions. Liver cells are divided into hepatocytes, Kupffer cells, and endothelial cells. Transformed hepatocyte cell lines cannot adequately mimic the phenotype of the liver. However, primary cells were isolated directly from the tissues and have normal cell morphology and are capable of maintaining many of the markers and functions seen *in vivo* (Pan et al. 2009). Therefore primary hepatocyte models are important tools for hepatic toxicology studies.

Matriptase-1 (MT-1) and matriptase-2 (MT-2) belong to the type II transmembrane serine protease family (TTSP) (Hooper et al. 2001), more precisely to the matriptase subfamily (Szabo and Bugge 2008). MT-1 is widely expressed in epithelial tissues, with high levels in the gastrointestinal tract, prostate (Takeuchi et al. 1999), normal and cancerous breast, ovarian and colon tissues (Oberst et al. 2003). MT-1 is required for postnatal survival. According to Oberst et al. (2001), MT-1 has a potential role in cancer invasion and metastasis in some types of cancer. MT-2 is highly expressed in the liver and also in small amounts in the kidney (Hooper et al. 2003). Silvestri et al. (2008) identified the membrane-bound haemojuvelin (HJV) as a substrate for MT-2 *in vitro*. MT-2 can regulate the iron homeostasis (Folgueras et al. 2008). Hepcidin is an iron regulatory hormone the main regulator of systemic iron homeostasis through the interaction with the bonemorphogenetic protein coreceptor HJV. It modulates the iron absorption via duodenal enterocytes (Du et al. 2008; Silvestri et al 2008). Hepcidin negatively regulates the iron export by promoting the degradation of ferroportin, which is an iron exporter located on the surface of intestinal enterocytes, macrophages, and hepatocytes (Nemeth et al. 2004). MT-2 cleaves membrane-bound HJV which is the main activator of hepcidin transcription (Silvestri et al. 2008). Since MT-2 plays an important regulatory role in iron homeostasis, this enzyme may act as a pharmacological target in the treatment of the systemic iron overload (hemochromatosis) or iron-deficiency anaemia (Du et al. 2008).

Sphingosine-1-phosphate (S1P) has been reported as a MT activator. MT-2 regulates the iron homeostasis by the cleavage of membrane-bound HJV, which is a main activator of hepcidin transcription (Silvestri et al. 2008).

1.1. Aim of the study

In previous studies it was shown, that through the activation of MT-1 by S1P intestinal barrier integrity was increased (Pászti-Gere et al. 2016a), therefore it would be interesting to examine other biological effects of S1P. Since S1P is a non-selective regulator of matriptase family, we wanted to investigate how it might or might not influence plasma iron level. The aim of our study was to investigate the possible relationship between S1P and hepcidin production in human and rat primary hepatocyte models. We estimated the cytotoxic properties of S1P and then we also investigated the effect of S1P on baseline extracellular H_2O_2 production. The changes in hepcidin levels in cell-free supernatants were also quantified. In case of human primary hepatocytes we also tested CYP enzyme interaction. By all these measurements preliminary conclusions may be laid for the possible pharmacological treatment of iron deficient anemias with S1P, such as:

- safe applicability
- CYP enzyme activity
- effectiveness in iron regulation.

2. LITERATURE OVERVIEW

2.1. Types of anemias

Practically anemia is the decrease of red blood cells (RBC), packed cell volume (PCV) or hemoglobin (Hb) concentration under reference interval for the given species. Nevertheless, there also can be differences in normal values among different breeds of a given species for example japanese akitas, greyhounds. By the pathogenesis of anemias, we classify them as regenerative, semiregenerative and nonregenerative (Couto 2020). As from practical aspect also can be determined as functional anemia, whereas erythropoietin (EPO) production is not stimulated or there are dysfunctions of the bone marrow, therefore synthesis of RBCs is hindered. Anemias can be caused by blood loss (due to trauma, parasites or hemolysis) or by iron deficiency, where insufficient quantity of hemoglobin is produced due to lack of iron. This latter case has significant importance in iron deficiency of suckling piglets in veterinary field (Goff 2015).

2.1.1. Regenerative anemias

In case of regenerative anemias, the bone marrow is intact, immature (chromatophilic) forms of RBCs and reticulocytes are present in the circulation and their number is in positive correlation with the severity of anemia. Therefore, regenerative anemias are originating from extramedullary factors as blood loss or hemolysis.

Blood loss anemias caused by traumatic injuries (acute blood loss) or blood sucking parasites (chronic blood loss) are resulting in regeneration, identified by reticulocytosis in 48-96 hours. Bone marrow needs time to recognize the need of excess production of RBCs. After the seizing of bleeding resolving of anemia starts in 48-96 hours as the bone marrow realizes the need of RBCs and usually completes within days or weeks, depending on the extent of blood loss. Before this aforementioned 48-96 hours one may recognize it as nonregenerative process (Couto 2020).

Hemolytic anemias can be classified further by their intra- or extravascular origin.

Intravascular hemolysis can be caused by antibodies, RBC infections, drugs, toxins or intense shearing of erythrocytes as for example in disseminated intravascular coagulopathy (DIC).

Extravascular hemolysis occurs when the mononuclear-phagocytic system (MPS) eliminates the damaged or infected RBCs in the spleen, liver and bone marrow. The triggers for the MPS are usually intracellular inclusions. Immune-mediated hemolytic anemia (IHA) is also an extravascular, acquired cause of hemolysis (Feldman et al. 2000).

2.1.2. Nonregenerative anemias

Though anemia of chronic disease (ACD) is considered as the most common nonregenerative anemia it usually has a mild form. Concerning the fact that develops as consequence of primary chronic disorder such as chronic inflammation, degenerative or neoplastic conditions, ACD is usually not evaluated itself alone. ACDs affecting erythropoietic processes (such as neoplastic disease of the bone marrow) shall be distinguished from iron deficient anemia (IDA). Serum ferritin measurement gives clear picture in humans where ferritin concentration of the sera is low in IDA and high in ACD. However, in small animals it is still not a clear-cut. In such cases, histopathological evaluation of bone marrow iron storage is important (Mills 2012; Couto 2020; Feldman et al. 2000).

Anemia of renal disease (ARD) in dogs and cats with chronic kidney disease anemia is common, since kidneys are the main site of EPO production. It is usually normocytic and normochromic anemia with few or no reticulocytes. The improvement of renal function may result is slight RBC increase and also human recombinant EPO administration had been used successfully to treat anemia in dogs and cats with chronic renal failure. However, after the initial 3-4 weeks of treatment, foreign EPO triggered antibody response in 50% of treated patients and nullified the beneficial effect of it.

Endocrine type of anemias are usually mild and in small animals it is an incidental finding in case of hypothyroidism or hypoadrenocorticism of dogs (Couto 2020).

Different disorders of bone marrow (such as neoplasia, hypoplasia dysplastic disorder or toxic effect on bone marrow) may result in anemia or other cytopenias. In case of neoplasia of inflammation there is a so called 'crowding out' of normal precursors of erythropoiesis by the neoplastic of inflammatory cells (myelophthitis). In case of reduced amount or complete absence of the normal factors it is considered as hypoplasia or aplasia. Dogs and cats with bone marrow are pancytopenic in such cases (Reece 2015; Mills 2012).

2.1.3. Semiregenerative anemias

Iron deficient anemia is caused by impaired Hb synthesis. Erythrocyte maturation is dependent upon obtaining a critical Hb concentration. Maturing erythroid precursors undergo additional cell divisions during iron-deficient states. These results in small erythrocytes, termed microcytosis. However, erythrocytes with low Hb concentrations are produced when microcyte formation can no longer compensate iron deficiency. Classic hematological picture with iron deficiency anemia is hypochromic anemia. Microcytes and hypochromasia may also be detectable on blood smear examination as erythrocytes are abnormally small and paler-staining. Additional hematologic changes may include evidence of erythrocyte mechanical fragmentation for example schistocytes and reactive thrombocytosis (Boes-Durham 2017). The Hb-deficient erythroid precursors are undergoing additional mitoses while attempting to achieve ideal cytoplasmic hemoglobin levels, thereby exaggerating the microcytosis (Naigamwalla et al. 2012).

IDA may be classified into 3 stages: storage iron deficiency, iron deficient erythropoiesis, and iron deficient anemia. Initially during blood loss, iron stores are preferentially utilized for

accelerated erythropoiesis. After depletion of body iron stores, heme synthesis, erythropoiesis and production of other iron-containing proteins (such as myoglobin) become limited. Anemia is enhanced as iron-deficient erythrocytes have a shortened survival as it was shown, that hypochromic and microcytic erythrocytes, resulting from absolute iron deficiency, have a shortened survival due to ineffective production coupled with accelerated reticuloendothelial cell sequestration once released into the circulation (Anderson et al. 1999).

Practically, as RBCs become more fragile that results in mild hemolysis, worsening the anemia. Initially, reticulocytosis is present due to increased production and release of reticulocytes secondary to anemia. As iron stores are depleted and the iron deficiency becomes more severe, the absolute reticulocyte count becomes inadequate for the degree of anemia. While normocytic normochromic erythrocytes contain approximately 1/3 Hb, animals with iron deficiency anemia demonstrate progressive decreases in mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and mean corpuscular volume. Early stage iron deficiency may not be suspected as the anemia may be initially normocytic and normochromic (Naigamwalla et al. 2012).

Iron deficiency may result from two origin, either dietary intake does not meet the body's requirement or there is chronic external (nonresorptive) blood loss.

Dietary iron requirement for adult animals in lower than in youngs due to their rapid growing rate, this also stands for humans, as for children with higher dietary iron needs (Naigamwalla et al. 2012; Metallinos-Katsaras et al 2016). Inadequate dietary iron intake does not occur in dogs and cats fed with complete balanced commercial pet foods, but may occur with home-cooked and vegetarian diets without appropriate iron supplementation. High iron content is found in meat products (such as liver, heart, and muscle) due to their blood content (Naigamwalla et al. 2012). In human concerns iron deficiency is considered to be one of the most common nutritional deficiencies in the world. Consequently, vegetarians are affected, however vegetarians of the western world, who consume a variety of foods have a better iron status than those in developing countries living on limited diet (Craig 1994). Studies have also suggested that family income (financial) status may influence dietary iron intake, therefore low-income family children prone to develop IDA (Coad 2014; Giger 2005).

During acute blood loss, body iron stores are generally sufficient for accelerated erythropoiesis and subsequent iron uptake is adequate for restoring iron homeostasis IDA

develops only after weeks to months of chronic or recurrent blood loss in both juvenile and adult animals.

Chronic external blood loss include ectoparasitism, endoparasitism, hematuria, epistaxis, hemorrhagic skin pathology, coagulopathy, thrombocytopenia, thrombocytopathia, and gastrointestinal hemorrhage will result in the exhaustion of iron storages, leading to IDA (Naigamwalla et al. 2012).

In human aspects conditions must be mentioned as pregnancy or premenopausal women having usually low iron status because of iron loss in menstrual blood or foetal development (Coad-Pedley 2014).

Also iatrogenic causes must be mentioned, such as surgical resection of the entire duodenum, that will result in malabsorption of iron or excessive phlebotomies of blood donor animals, as a regular blood donation of 450 mL removes approximately 200 mg of iron from the body and also be induced by repeated phlebotomies for diagnostic and monitoring purposes in smaller animals (Naigamwalla et al. 2012).

2.2. Iron deficient anemias of different species

2.2.1. Food producing animals

Iron deficiency results in hypochromic microcytic anemia due to failure of synthesis of hemoglobin. The light color of meat in meat producing animals is due to low muscle myoglobin levels as a result of restricted dietary iron. Anemic animals are dull, have poor feed intake and weight gain. Another important aspect of iron deficiency is greater morbidity in youngs and mortality associated with depressed immune responses. Increased morbidity in youngs may be observed before there is an effect of iron deficiency on hematocrit. Iron deficiency in adult animals is not common. In part this is because their requirement is reduced, but also because iron is ubiquitous in the environment and, in the case of herbivores soil contamination of forages (and soil ingested by animals on pasture) generally ensures that iron needs of the adult will be met or exceeded. Iron deficiency has huge significance in suckling piglets and also frequent in calves. Rarely found in horses, dogs and cats where iron deficiency due to inflammation is more common (Goff 2015).

All suckling animals are iron−deficient for a time, but this is generally critical only in case of pigs. The most important factors in the high incidence of anemia in piglets is the rapidity with which they grow during early postnatal life and the low iron content in sow's milk (Svobota – Drábek 2005).

Piglets are born with almost no iron stores in their liver and because of their extreme growing rate piglets must retain between 7 and 16 mg of iron each day. They double their bodyweight in the first week of their lives and their blood plasma increases 30% (Goff 2015; Ullrey et al. 1959). The iron concentration of the sow's milk can be increased only to a limited extent by iron supplementation of the sows' feed. The received amount of iron by piglets from milk can cover their iron requirements and prevent them from developing anemia. The amount of iron taken up by piglets from colostrum and milk shows high variability between sows. Milk consumption of a piglets varies between 0.5—1 L per day. Iron content in sows' milk varies from 0.2—4 mg/L and the piglet can only absorb 60—90 % of milk iron. Besides low foetal iron reserves, blood loss at farrowing can also contribute to an overall iron deficit. Bleeding from cord is associated with greater or smaller blood loss due to a deficient contractibility of the umbilical cord. It was written that by vitamin C supplementation of a sow's feed prevented the further occurrence of this disorder. Direct blood loss during teeth clipping, tail docking and castration may also occur. One of the specialties in pigs compared with uniparous animals is, that within one single litter, foetuses with low iron reserves and with normal iron reserves also can be present. The different iron supply of foetuses results also in a significant variability in values of Hb in blood plasma of piglets immediately after birth. Also the iron levels in blood plasma of the piglets immediately after parturition show variability between 5 and 35 mmol/L (Svobota – Drábek 2005).

Significantly, a positive effect on foetal iron reserves and Hb concentration in piglets after birth was achieved by iron−dextran injection of the sows between the 40th and 60th days of pregnancy. However, the intramuscular injection of iron-dextran preparations to sows during late pregnancy does elevate the Hb levels of piglets during the first few weeks of life but not sufficiently enough to prevent anaemia (Svobota – Drábek 2005; Ullrey et al. 1959). This is especially true for piglets raised in closed systems. Pigs raised on pasture, free range, root around in the soil and often can ingest enough iron to meet their needs. Iron deficient piglets will have blood hemoglobin below 10 g/dL. As a consequence hypoxia may cause

hyperventillation is piglets (Goff 2015; Ullrey et al. 1959). First sign of IDA are roughness of hair coat and losing pigmentation of mucus membranes. The skin is wrinkled, animals exhibit apathy, dropping of the head and ears, lack of appetite and reduced weight gain. In severe cases dyspnea, increase heart and respiratory rates, systolic murmurs may occur due to reduced blood viscosity, later piglets die suddenly due to anoxia. Affected piglets have higher prevalence for subcutaneous edema in the neck, shoulder and limb region and they show higher susceptibility to infectious diseases for example pneumonia, influenza and gastrointestinal disorders. It is because the dietary Fe level is directly related to antibody (Victor –Mary 2012).

Concerning ruminants, we also have to mention IDA, where fast growing individuals are only ingesting milk. As it was mentioned before, milk is poor in iron, therefore subclinical IDA may occure transiently in calves and kids (Watson–Canfield 2000). The occurance of developing severe IDA seems more unlike in species where youngs are able to eat solid food in their early age (Harvey 2000). IDA is inevitable in the dietary practice of pale meat production of veal calves, but these animals may also develop anorexia and will have poor growing rate (Watson – Canfield 2000; Harvey 2000). However IDA is debated as a significant problem in ruminants, cobalt deficiency can case severe dietary problems in pasture grazing animals (Watson–Canfield 2000).

Despite of the physiological and morphological differences of birds RBCs (such as nucleated RBC, short lifespan, low PCV), anemias of poultry and exotic birds are originating from the same pathomechanisms as ACD, infectious, dietary, metabolic or neoplastic diseases. Hemolytic anemias are less common but bloodparasites or toxicoses such as ochratoxin-A still can result in hemolysis. There are no reports on IHA of birds. (Huff et al. 1978; Morryssey 2000).

2.2.2. Pet animals

We already discussed the different types of anemias occcuring in mammalians in the chapter of anemias. However a few special conditions concerning pet animals also have to be mentioned.

In case of IDA of dogs and cats RBCs are found microcytic hypochromic. It must be mentioned that dog breeds such as akita, shiba inu or shar-pei have physiologic microcytosis, while other disorders, like portosystemic shunt also can result in it.

IDA of adult cats is rare, however it often appears in heavy parasitic infection of kittens. Concerning dogs, chronic bloodloss is the primary cause of IDA, most frequent caused by gastrointestinal bleeding, ulceration, neoplasia or heavy parasitic infection (also endo- and ectoparasites) (Couto 2020).

In the case of cats feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) are the most common infectious diseases. FeLV can cause tumors (mainly lymphoma), bone marrow suppression for example anemia, and lead to secondary infectious diseases caused by bone marrow suppression by the virus. Mostly FeLV infected cats are presented to the veterinarian not for tumors but for anemia or immunosuppression. Clinical signs associated with FeLV can be classified as tumors, immunosuppression, hematologic disorders, immune-mediated diseases, and other syndromes like neuropathy, reproductive disorders, fading kitten syndrome. Approximately 10% of FeLV-associated anemias are regenerative most FeLV-associated anemias, however, are non-regenerative and are caused by bone marrow suppression of the virus, infecting hemopoetic stem cells. Therefore FeLV and FIV testing is inevitable in case of serious nonregenerativ anemia of a cat. Also BM disorders should be excluded at evaluation by aspiration or biopsy. *In vitro* exposure of normal feline bone marrow to some strains of FeLV caused suppression of erythrogenesis (Hartmann 2012; Couto 2000).

In case of small pet animals we have to mention ferrets, rabbits and pet rodents. In domestic ferrets origin of different anemias are exactly the same as in dogs and cats. Though IHA had not been reported, but t should be taken into consideration when no cause of blood loss or decreased production can be found. BM suppression may be frequent, caused by nephopathies, neoplasia. It must be mentioned that since ferrets are induced ovulators hyperestrogenism in females, adrenocortical disorders and Aleutian disease causing chronic inflammation in both sexes also can result in BM suppression (Morrysey 2000; Malka et al 2010).

Rabbits with infectious hemorrhagic disease must be mentioned and also reproductive disorders as endometriosis or neoplasia of the uterus can result in anemia. ACD and ARD are both frequent in rabbits, guinea pigs and chinchillas (Morrysey 2000).

There are no information of other pet species like reptiles and amphibians but there is an increasing interest in case of fish where folate deficiency seems to be in the background of macrocytic changes (Watson – Canfield 2000)

2.3. Iron metabolism

Tight homeostasis of iron is essential in organisms, since excessive iron accumulation in hepatocytes can cause pathologic damage, termed hemochromatosis, where increased fibrosis and cirrhosis may occur. In contrast, iron deficiency leads to depletion of body iron stores, leading to iron deficient anemia and other metabolic dysfunctions (Naigamwalla et al. 2012).

Iron is vital to many metabolic functions in the form of heme, including oxygen transport in hemoglobin. It is also a component of multiple enzymes, including cytochromes, necessary for energy generation and drug metabolism. Iron in the organism exists in either a reduced ferrous (Fe^{2+}) or an oxidative ferric (Fe^{3+}) form. Usually organic iron complexes are found in Fe^{3+} form, after oral consumption, the hydrochloric acid content of the stomach it is reduced to Fe^{2+} (Goff 2015; Jain 1993; Naigamwalla et al. 2012). Subsequently, free iron is toxic to cells because it catalyzes the formation of reactive oxygen species (ROS) via the Fenton reaction (Zachary 2017). In the organism the majority of functional iron is contained in Hb, smaller quantities found in myoglobin and cytochromes. On contrary the largest non-functional iron stores fond in the liver, what is the site of production of iron transport and storage proteins, as ferritin or hemosiderin (Jain 1993; Harvey 2000). Ferritin is a globular iron storage protein present in all tissues but particularly in the liver, spleen, and bone marrow, where binds free iron and stores it in a nontoxic form, available for when there is need. It is mainly an intracellular protein, but serum concentrations correlate with iron stores (Zachary 2017). Ferritin is both diffuse and soluble, it is the primary storage protein of iron (Naigamwalla et al. 2012; Knovich-Storey 2009). Hemosiderin has similar structure, but has relative more iron compared to protein and is insoluble. Iron is also stored in reticuloendothelial cells of the bone marrow and spleen, but not commonly stored in bone marrow of cats (Naigamwalla et al. 2012).

Dietary iron is absorbed mainly in the duodenal region of the small intestines. In case of carnivores iron supply is provided by the heme content of the prey animals RBCs. After

digestion, the heme-type iron is bound to the iron transport protein of the enterocytes and transported across the apical membrane (Goff 2015; Naigamwalla et al. 2012).

Absorption of iron can be sorted into three phases; first the absorption of iron through brushborder of the enterocytes by divalent metal transporter. Next step is the storage of Fe^{2+} in form of ferritin, then the exiting of iron ions through basolateral membrane by the help of ferroportin. After exiting enterocytes and entering blood stream Fe^{3+} will bound to transferrin in the plasma, that will transport the iron to the sites of storage (liver, spleen, bone marrow) or to the site of hemopoesis (Goff 2015). In the bone marrow, erythroid forms, including reticulocytes, have surface membrane receptors for transferrin. Plasma transferrin binds to these receptors, becomes internalized by endocytosis, then releases its iron, and transferrin is returned to the plasma (Goff 2015). It is also noted that most of the proteins responsible for the uptake and release are still unknown (Naigamwalla et al. 2012; Knovich-Storey 2009).

Storage of iron is tightly regulated to provide adequate iron for cellular needs without developing toxicity as a consequence of excess iron. The organism lacks a mechanism to excrete excessive iron in short time, therefore homeostasis is tightly controlled by limiting enteric iron uptake through impaired release from enterocytes. Iron efflux is regulated by hepcidin. Hepcidin is a peptide hormone secreted by the hepatocytes of the liver to response to iron loading and inflammation. Previous studies had shown that the main signalling factor for hepcidin production is the saturation of transferrin in the plasma (Nemeth et al. 2004).

In case of adequate or high iron stoage, hepcidin is released. Hepcidin binds to intestinal ferroportin resulting in internalization and destruction of ferroportin-hepcidin comlex. This reduction in the amount of ferroportin causes absorbed dietary iron to remain in the enterocytes. Iron trapped in enterocytes will leave the body by the normal shedding of enterocytes. In case of low iron stores, hepcidin production and secretion will be suppressed, resulting in increasing iron efflux from enterocytes into the blood. There are several factors influencing hepcidin level, those will be discussed in a separate chapter (Naigamwalla et al. 2012; Weiss 2010).

2.3.1. Influencing factors of hepcidin level

The iron metabolism is strictly regulated by homeostasis to prevent iron toxicosis. However, there are some factors that may influence regulation of absorption and metabolism (Weiss

2010). The general role of hepcidin and its' regulatory mechanism on iron has been discussed above under the chapter of iron metabolism. However, hepcidin production itself is influenced by some factors, thereby also plasma iron level (Figure 1.).

High plasma iron level will increase hepcidin level, which will block the absorption from duodenum and the release of stored iron. Consequently, plasma iron level will decrease (Nemeth et al. 2004). Infections and inflammatory disorders will also elevate hepcidin production via inflammatory cytokines as interleukin-6 (IL-6) or interleukin-8. As response, iron deficiency and anemia are developed as protective mechanism of the organism. Iron is an essential element also for the circulating infective microbes, therefore the downregulation of iron plasma level hinters the survival of pathogens.

Erythropoiesis or oxidative stress needs high level of iron. In case of RBC formation either a result of bleeding or administration of EPO, hepatocytes will decrease hepcidin production, therefore absorption from the gastrointestinal tract increased (Pászti-Gere et al. 2016; Nemeth et al. 2004; Papanikolaou 2005).

Inflammatory bowel disease (IBD) is a special case, where anemia as a severe extraintestinal side effect is observable, either caused by intestinal hemorrhages or because of the malabsorption of dietary iron. The influence of inflammation and erythropoietic factors on hepcidin level and the consecutive anemia may suggest the therapeutic approaches by the normalization of hepcidin level for patients suffering from IBD (Dudkowiak et al. 2013).

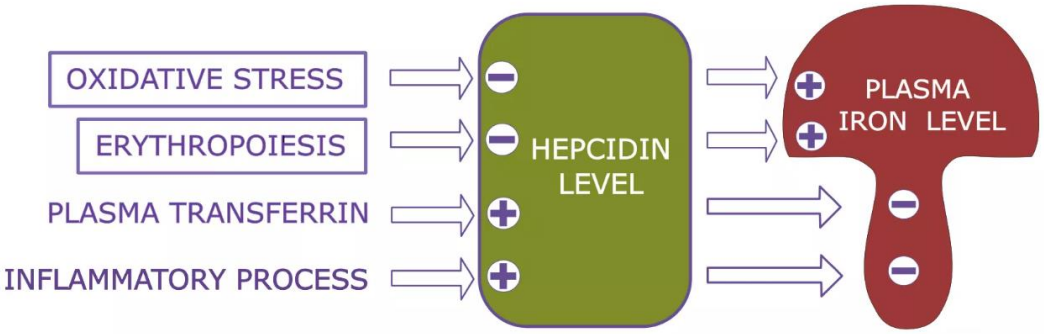


Figure 1. Influencing factors of hepcidin and its effects on plasma iron level. Erythropoiesis and oxidative stress increases plasma iron concentration by decreasing the level of hepcidin. Plasma transferrin and inflammatory processes will decrease plasma iron by elevating hepcidin level.

2.3.2. Role of matriptase

MT-1 (also known as epithin) and MT-2 (also known as TMPRSS6) belong to the type II transmembrane serine protease family, more precisely to the matriptase subfamily (Szabó – Bugge 2008; Hooper et al. 2003).

These enzymes can be anchored on the membrane as in case of stromal cells or can span the membrane of the cell. Transmembrane serine proteases usually found in the epithelial cells and carcinoma cells consist of C-terminal extracellular serine protease domain, thus being able to interact with other cell surface proteins, soluble proteins, proteins on neighboring cells in order to generate biologically active proteins. These proteases also contain an intracellular N-terminal through which they can also influence intracellular signaling mechanics.

MT-1 is widely expressed in epithelial tissues, with high levels in the gastrointestinal tract, prostate (Takeuchi et al. 1999), normal and cancerous breast, ovarian and colon tissues (Oberst et al. 2003). MT-1 is required for postnatal survival. According to Oberst et al. (2001), MT-1 has a potential role in cancer invasion and metastasis in some types of cancer.

MT-2 has been found in high levels in the liver and in small amounts in the kidney (Hooper et al. 2003) and structurally similar to MT-1 (Silvestri et al. 2008) identified the membrane-bound hemojuvelin as a substrate for MT-2 in vitro. MT-2 can regulate the iron homeostasis (Folgueras et al. 2008). Dysfunction of MT-2 can be involved in iron regulatory disorders via altered regulation of hepcidin expression. In mice experiment after chemical inactivation of the MT-2 serine protease region abnormal hair growth, IDA and extremely high plasma hepcidin concentration was observed (Folgueras et al. 2008; Du et al. 2008). In case of human iron resistant IDA (IRIDA) the domain of the serine protease of TMPRSS6 is damaged. In these patients hypochromic microcyter anemia was observed with high plasma hepcidin level, decreased RBC number and low transferrin saturation caused by the impaired iron absorption (Finberg et al. 2008). MT-2 cleaves membrane-bound hemojuvelin which is the main activator of hepcidin transcription, thereby inhibits the hepcidin response (Silvestri et al- 2008).

Both MT-1 and MT-2 are anchored to the cell surface (Takeuchi et al. 2000; Hooper et al. 2003) and can undergo autocatalytic activation in vitro (Takeuchi et al. 1999; Ramsay et al. 2009).

2.4. Spingosine- 1- phosphate

S1P is a bioactive sphingolipid metabolite. Most cells can synthetize S1P that is both an intracellular second messenger and extracellular mediator in mammalian cells. S1P regulates a wide variety of biological responses, for example angiogenesis, vascular maturation, cardiac development and immunity, and is important for cell proliferation and cytoskeletal reorganization, in various cell types (Yatomi et al. 2001).

There are five G-protein-coupled S1P receptors, those enable regulation of numerous downstream signaling pathways. The role of S1P depends on the relative expression of these receptors (Spiegel-Milstien, 2003). A novel biological function of S1P was also determined as an activator of matriptase in epithelial cells. It was also demonstrated that bioactive phospholipids can activate cell surface proteases (Benaud et al. 2002).

The cellular levels of S1P are tightly controlled by various enzymes involved in the phosphorylation / dephosphorylation of sphingosine and also the degradation of S1P. The enzymes responsible for phosphorylation / dephosphorylation are sphingosine kinase 1 and 2 and S1P phosphatase 1 and 2. The irreversible degradation of S1P is carried out by S1P lyase (Pyne et al. 2000). The concentration of S1P in the mammalian plasma varies from 0.2 to 0.9 μM which is significantly higher than in the tissues (Murata et al. 2000). Various effects of S1P stimulation and modulation had already been studied on different cells of organ systems for example vascular endothelium of the lung or gastrointestinal epithelia cell lines (Pászti Gere et al. 2016; Dudek et al. 2004). It was shown that plasma or serum S1P is not always fully active to stimulate the lipid receptors. Thus, the effective or active concentration of S1P in plasma or serum appears to be close to or lower than the dissociation constant (K_d) value under physiological conditions. Plasma components, including lipoproteins such as LDL and HDL, may trap S1P and thereby decrease the activity of S1P to stimulate its cell-surface receptors (Murata et al. 2000). This may also indicate that therapeutic administration of S1P enables the modulation of signaling pathways.

2.5. Matriptase inhibitors

Several selective matriptase inhibitors were developed including the sulfonylated 3-amidinophenylalanine derivatives, the substrate-analogue ketobenzothiazole derivatives

originated from the known P4-P1 Arg-Gln-Ala-Arg substrate sequence at the autocatalytic activation site of matriptase.

Another selective small molecule matriptase inhibitor, the arginal derivative CVS-3983 could suppress the growth of androgen independent prostate tumor xenografts in vivo.

Within the series of the 3-amidinophenylalanine analogues, the dibasic derivative MI-432 possessing an N-terminal dichloro-substituted biphenyl-3-sulfonyl group and a C-terminal aminoethylpiperidide moiety was one of the most potent matriptase inhibitors. Based on a modeled complex of MI-432 in matriptase it was found that the N-terminal biphenyl group of MI-432 fits into the S3/4 pocket of matriptase making a close edge to face contact to the aromatic side chain of the characteristic matriptase residue Phe99 (Hammami et al. 2012; Pászti-Gere et al. 2015).

2.6. Therapeutic prospects of matriptase modulation

As discussed above MT-1 and MT-2 enzymes play an important role in mediating the signal transduction between the cells and their surroundings. The members of the protease family may have conserved evolutionary functions based on that matriptase orthologs that are present in several vertebrate genomes. The possibility of modulation of these enzymes reveal several therapeutic aspects in the future.

2.6.1. Inhibition

MT-1 was tested in *in vivo* and *in vitro* experiments. When the lack of MT-1 caused tissue dysfunctions and could be lethal to null mutant mice. The dysregulation of MT-1 could also enhance the metastasis formation of certain tumors by cleaving the extracellular matrix, and it could also stimulate the matrix metalloproteases, which may lead to osteoarthritis. The MT-1 facilitated the penetration of the human and avian influenza virus into the cells by cleaving the viral precursor of hemagglutinin (Baron et al. 2013; Harten – Klenk 2008; Whittaker – Straus 2019). There are studies concerning severe acute respiratory syndrome coronavirus (SARS-CoV), where it is was found that the inhibition of MT-1, results in decrease of viral replication. Meyer and his colleagues worked with influenza viruses treated with sulfonylated 3-amidinophenylalanine derivatives and found that in the given concentration the viral replication severely decreased (Meyer 2013). It was also show in several experiments that

metastatic spread of different types of tumors such as prostatic cancer MT-1 inhibition may decrease the shed of cells (Vaarala et al. 2001). The other member of the family is the MT-2, which is expressed mainly in the liver and has the most considerable effect on the iron homeostasis. MT-2 regulates the iron absorption inhibits the hepcidin transcription via cleaving the membrane bound HJV. Therefore MT-2 inhibitors may be potential drug candidates for treatment hemochromatosis in veterinary and also human medicine (Pászti-Gere et al. 2019).

2.6.2. Activation

S1P has been reported as a MT activator. MT-2 regulates the iron homeostasis by the cleavage of membrane-bound haemojuvelin, which is a main activator of hepcidin transcription (Silvestri et al. 2008). Hepcidin inhibits the entry of iron into the plasma and its absorption from enterocytes. It was also observed that mice with a TMPRSS6 –/– defect or the absence of matriptase-2 proteolytic activity have iron deficiency anaemia, upregulation in hepcidin transcription, and reduced protein levels of ferroportin on enterocytes (Du et al., 2008; Folgueras et al., 2008). Thus, stimulation of MT-2 activation could be an alternative treatment of iron deficiency anaemia and disturbed iron metabolism.

2.7. Characterization of human and rat primary hepatocytes

Hepatic metabolism is a major pathway to improve or inhibit effect of some chemical compounds. Several researches aim at developing new in vitro powerful methods to predict the liver xenobiotic metabolism and toxicity (Hewitt et al. 2007). Studies have shown that primary hepatocytes consistently remained the leading models for *in vitro* liver toxicity testing. Primary rat hepatocytes were isolated directly from the tissues and therefore have a normal cell morphology and are capable of maintaining many of the markers and functions seen *in vivo* (Pan et al. 2009). The rat primary hepatocyte and human primary hepatocyte model is an important tool for hepatic toxicology studies. However primary cells have a limited lifetime such as 2–4 days (Soldatow et al. 2013; Hewitt et al. 2007).

Primary hepatocytes once isolated and placed in chemically defined culture conditions they will show typical hepatic biochemical functions as far as hepatic drug-metabolizing enzyme system. This is an integrated form providing an *in vitro* model that is a very useful tool for

evaluating drug metabolism and drug hepatotoxicity (Hewitt et al. 2007) and in our experiment also the regulation of transport processes of hepatocytes were studied.

In our research, we used cryopreserved human primary hepatocytes and cryopreserved rat primary hepatocytes. After slow thawing at 37 °C in hepatocyte maintenance medium, the cell had to be inserted on membrane insert plates and treated with antibiotics. After 6 hours we added fetal bovine serum (FBS). Maintenance medium without FBS was then used and replaced every 24 hours. Our cells were incubated at 37 °C, 5% CO_2 for 72 hours.

The major advantage of cryopreserved hepatocytes is that the experiment can be repeated using same conditions therefore the data is reproducible (Hewitt et al. 2007). Besides the maintenance of normal cellular physiology and intercellular contacts *in vitro* has particular importance for optimal phenotypic gene expression and response to drugs and other xenobiotics (LeCluyse 2001) in our case CYP450 enzyme interaction of human primary hepatocytes.

3. MATERIALS AND METHODS

3.1. Cell lines and culture conditions

Cryopreserved primary rat hepatocytes, isolated from male Sprague-Dawley rats, and cryopreserved human primary hepatocytes were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Cryopreserved cells were thawed at 37 °C, pipetted into hepatocyte thaw medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and centrifuged with 100 × g for 10 minutes. Hepatocytes were seeded on membrane insert plate (Costar Transwell permeable supports, 0.4 μm polyester membrane 24 mm insert, 6-well plate, tissue culture treated, Merck, Germany) and 96-well plate (Merck, Darmstadt, Germany). The seeding density was 0.9–1.1 × 106 cells/ml, in 2 ml apical medium. Before seeding the cells, viability was checked by Trypan blue staining (1:8 dilutions, 1 minute, Merck, Germany). The maintenance medium was Williams E medium (Thermo Fisher Scientific, USA) supplemented with 10,000 IU/ml penicillin, 10 mg/ml streptomycin, 2 mM glutamine, 0.2 IU/ml insulin, and 0.22% bicarbonate. FBS 10% was added to the medium only in the first 6 hours after thawing.

The maintenance medium without FBS was then used and replaced every 24 hours. The cells were incubated at 37 °C, 5% CO_2.

3.2. Exposure of primary hepatocytes to S1P

S1P was purchased from Bio-Techne (Minneapolis, Minnesota, United States). S1P was dissolved in dimethyl sulfoxide (DMSO) and the concentration of S1P stock solution was 1 mg/ml. After the first 6 hours, S1P was added to the medium apically at 50, 200 or 1000 ng/ml in case of rat and 500, 1000, 2000 ng/ml in case of human hepatocytes. The maintenance medium with S1P was changed every 24 hours. The treatment time was 72 hours for both human and rat hepatocytes.

3.3. MTS assay for cell viability

The hepatocytes were seeded in a 96-well plate and incubated with S1P 50, 200 or 1000 ng/ml in case of rat and 500, 1000, 2000 ng/ml in case of human hepatocytes for 72 hours. The control wells were incubated with maintenance medium only. After the medium was removed, 100 µl maintenance medium was added with 20 µl CellTiter96 Aqueous One Solution (Promega, Bioscience, Hungary) in every well. This reagent contained the tetrazolium compound MTS (Cory et al., 1991). The assay protocol is based on the reduction of the MTS tetrazolium compound by viable cells to generate a coloured formazan dye that is soluble in cell culture media. This conversion is reported to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The cells were incubated with the dye for 2 hours at 37°C, 5% CO_2. The absorbance of formazan was measured by EZ Read Biochrom 400 microplate reader at 490 nm wavelength (Biochrom Ltd., UK). Treatment was performed for both human and rat hepatocytes.

3.4. Amplex Red assay for extracellular H_2O_2 level measurement

Amplex Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, USA) was used to measure the extracellular concentration of H_2O_2 in cell-free supernatant from the apical compartment. Fluorescence ROS measurement was based on the fact that Amplex Red reagent reacts with H_2O_2 and horseradish peroxidase and it is converted into fluorescent resorufin.

Primary rat cells were treated with 50, 200 or 1000 ng/ ml and primary human cells with 500, 1000, 2000 ng/ml S1P for 72 hours. The samples were collected every 24 hours; 50 µl cell-free supernatant and 50 µl Amplex Red working solution was used to determine the H_2O_2 concentration. After 30 minutes of incubation at room temperature the samples were measured with Victor X2 2030 fluorometer at 560 nm excitation and 590 nm emission wavelength.

3.5. Hepcidin sandwich ELISA for determination of hepcidin level

To determine the concentration of hepcidin in the apical compartment from the cell-free supernatant, we used rat hepcidin ELISA kit from Elabscience Biotechnology (Houston, Texas, USA) and human hepcidin ELISA kit from Cloud-Clone (Katy, Texas, USA) After 24, 48 and 72 hours of S1P treatment at 50, 200 or 1000 ng/ml concentrations, the apical cell-free supernatants were collected. The assay procedure was performed according to the manufacturer's guidelines. The absorbance values were detected with EZ Read Biochrom 400 microplate reader at 450 nm wavelength (Biochrom Ltd, UK).

3.6. Chemiluminescent investigation of human hepatocytes on CYP3A4 enzyme activity

To investigate the drug interaction potential of S1P we examined CYP3A4 enzyme activity. Only human primary hepatocytes were treated for CYP enzyme activity, with 500, 1000, 2000 ng/ml S1P concentrations. For control enzyme induction dexamethasone (50 µM) and for inhibition ketoconazol (25 µM) was used. The cells were incubated for 48 hours. After incubation the treatment medium was removed and the cells were washed with maintenance medium. Luciferin-IPA reagent was added (50 µl/well) to the cells. After 60 minutes incubation 25 µl of supernatant was measured on special 96-well, white background plate. Luciferin Detection Reagent (Promega Corporation, USA) was added (25 µl/well). We investigated with luminometry (Victor X2 2030 multilabel reader), after 20 minutes incubation.

3.7. Immunofluorescent investigation of cell cultures

For investigation of matriptase activity modulation on the tight junction (TJ) assembly we used occludin distribution via immunfluorescent staining. Localization of occludin was examined in untreated control and activator-treated rat and human primary hepatocytes. Our inserts were

fixed in methanol for 5 minutes, followed by BSA (5%, SigmaAldrich, St Luis, MO) protein block for 20 minutes. Sections were incubated for 1 hour in humid chamber at room temperature with anti-St-14 N terminal rabbit polyclonal primary antibody (1:200, Sigma-Aldrich, St. Louis, MO) or anti-occludin polyclonal primary antibody (1:200, Sigma-Aldrich, St. Louis, MO). The antibodies were previously diluted in 5% BSA solution. After antibody Alexa546 (orange red) anti-rabbit Ig-s 1:200 diluted in PBS was used for 1 hour. Sialic acid residues in cell membrane were stained with wheat germ agglutinin (WGA, 1:200 diluted in phosphate-buffered saline buffer (PBS), WGA Alexa Fluor 488, Invitrogen-Molecular Probes) for 10 minutes. Cell nuclei were stained in blue using 4', 6-diamidino-2-phenylindole (DAPI) (1:500 diluted in PBS, Invitrogen-Molecular Probes) for additional 10 minutes. Between incubations the slides were washed in PBS for 3x2 minutes. Membranes were attached on glass slides using fluorescent mounting medium (DAKO, Glostrup, Denmark). The non-treated control and matriptase activated samples were analysed using Olympus IX73 inverted microscope with 100 WHg fluorescent accessory (Unicam Magyarország Kft., Budapest, Hungary) with LUCIA Cytogenetics 2.5 software.

3.8. Statistical analysis

Differences between groups were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test, where the data were of normal distribution and the homogeneity of variances was confirmed. Statistical significance was set at $p<0.05$ (***$p< 0.001$, **$p< 0.01$, *$p< 0.05$). For statistical evaluation the R 2.14.2 software package was applied (2012, Vienna, Austria). The results are expressed as means ± SDs.

4. RESULTS

4.1. The effect of S1P on cell viability

We used MTS assay to quantify the viability of primary rat and human hepatocytes. Cells were seeded in a 96-well plate and incubated with S1P 50, 200 or 1000 ng/ml in case of rat and 500, 1000, 2000 ng/ml in case of human hepatocytes for 72 hours. Control wells were incubated with maintenance medium. It was confirmed that even the highest concentration of

S1P (at 1000 ng/ ml) did not cause significant cell death after 24-hours (p=0.96887) and 72-h (p=0.1960) treatment in rat cells (Figure 2). No significant differences were found between control and S1P- treated cells after 24 and 72 hours of incubation. It was found that S1P administration for 72 hours even at 1000 ng/ml was safe in the case of the rat primary hepatocyte model. The same was confirmed in case of human hepatocytes, even the highest concentration of S1P (at 2000 ng/ ml) did not cause significant cell death after 72 hours (p=0.1960) (Figure 3.). We can assess that any of the used concentrations of S1P is safe to apply.

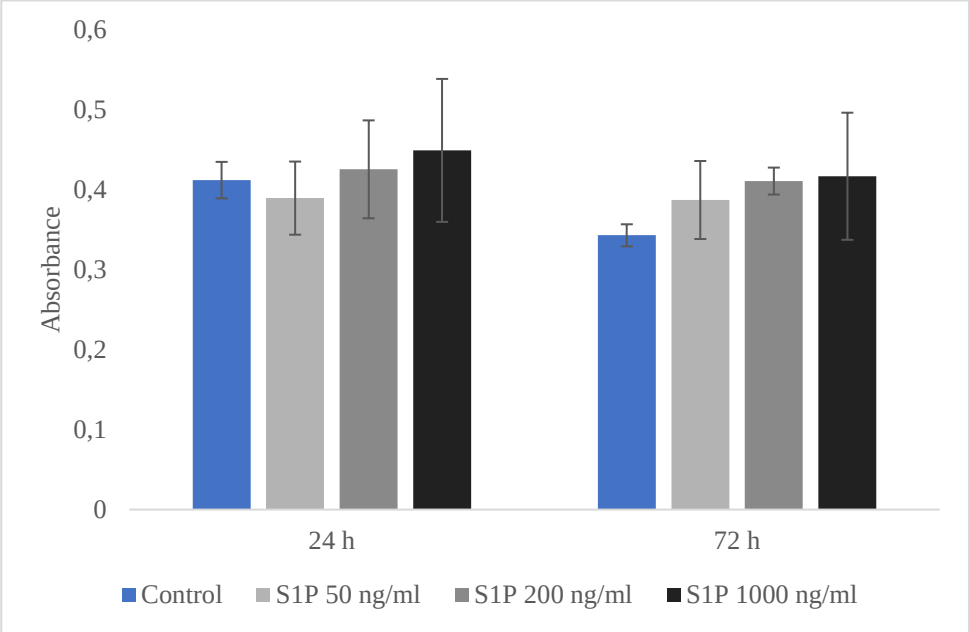


Figure 2. Assessment of cell viability after 24 and 72 hours of incubation of rat primary hepatocytes with S1P at 50, 200 and 1000 ng/ml. The results represent average absorbance values of produced MTS formazan ± standard deviations. The results are representative of three independent experiments (n=3, p>0.05).

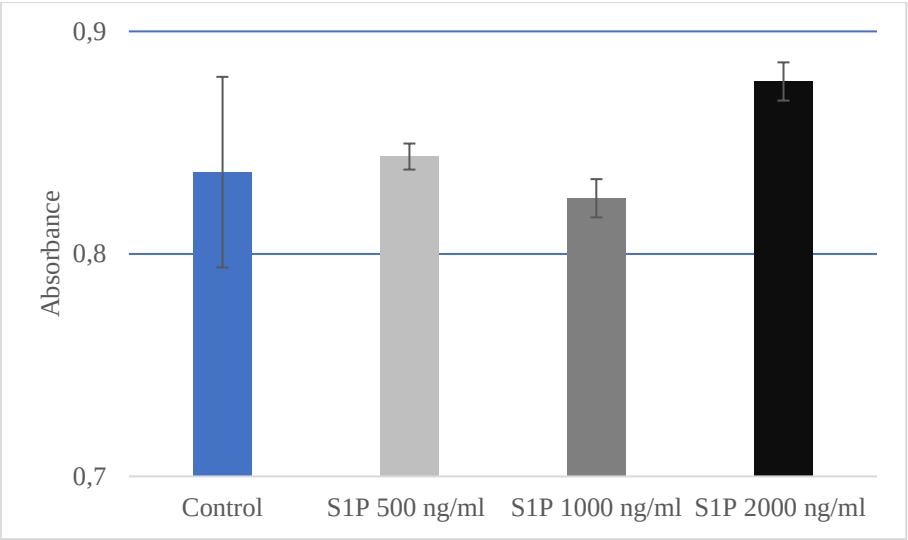


Figure 3. Assessment of cell viability after 72 hours of incubation of human primary hepatocytes with S1P at 500, 1000 and 2000 ng/ml. The results represent average absorbance values of produced MTS formazan ± standard deviations. No significant differences were found between control and S1P- treated cells after 72 hours (n=3, $p>0.05$).

4.2. The assessment of H_2O_2 measurement

We used Amplex Red working solution was used to determine the extracellular H_2O_2 concentrations. Primary rat cells were treated with 50, 200 or 1000 ng/ ml and primary human cells with 500, 1000, 2000 ng/ml S1P for 72 hours. We found that S1P did not cause a significant increase in the extracellular H_2O_2 levels at any applied concentrations within the investigated time period ($p>0.05$ in each case) in any of our examined species. There were no significant differences found between the control and the S1P-treated groups nor in rat neither in human primary hepatocytes (Figure 4. and 5.).

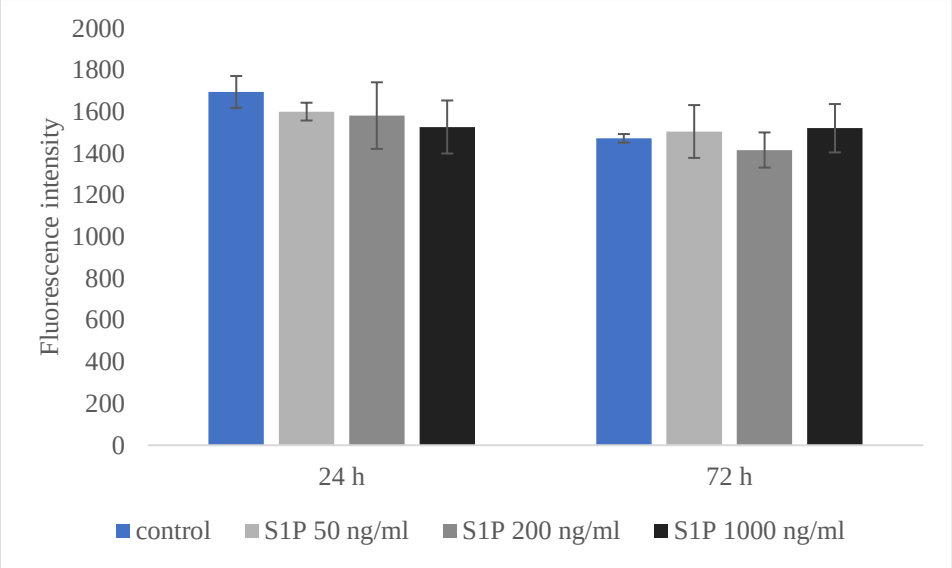


Figure 4. The effects of S1P at 50, 200 and 1000 ng/ml on extracellular H_2O_2 production of rat primary hepatocytes. Cells were exposed to S1P for 24 and 72 hours. The values represent the means of resorufin fluorescence intensities ± standard deviations (n=3, p>0.05).

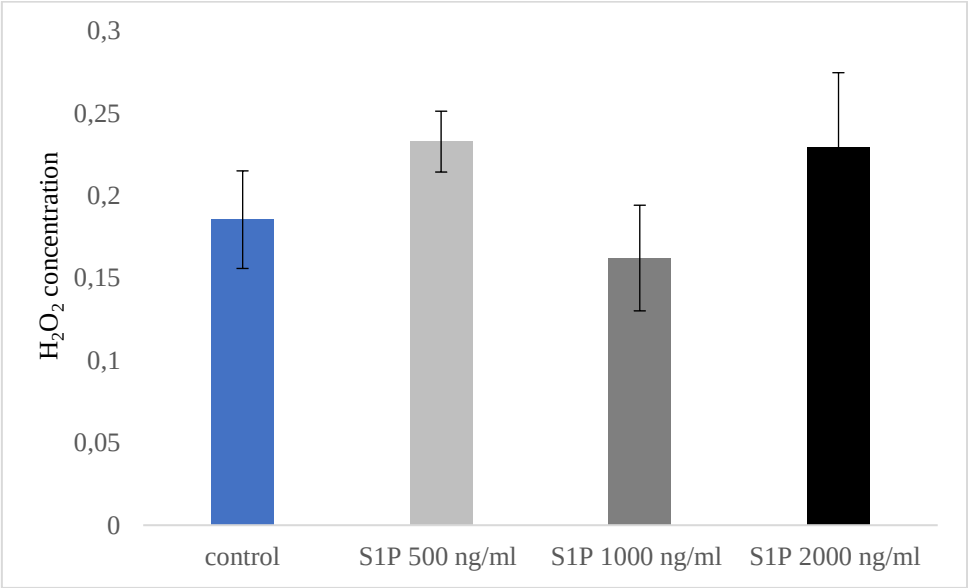


Figure 5. The effects of S1P at 500, 1000 and 2000 ng/ml on extracellular H_2O_2 production of human primary hepatocytes. Cells were exposed to S1P for 72 hours. The values represent the means of resorufin fluorescence intensities ± standard deviations (n=3, p>0.05).

4.3. S1P-influenced changes in hepcidin levels

We used rat hepcidin ELISA kit and human hepcidin ELISA kit to determine changes in hepcidin levels. After 24, 48 and 72 hours of S1P treatment at 50, 200 or 1000 ng/ml concentrations and the apical cell-free supernatants were collected for the measurement. It was found that the hepcidin levels in cell-free supernatants were significantly decreased compared to control samples after 24 hours of incubation at each concentration of S1P (at 50 ng/ml and 200 ng/ml: $p<0.001$, at 1000 ng/ml: $p<0.01$ in rat cells). Hepcidin production was significantly reduced when cells were treated with S1P at 200 ng/ml ($p=0.0206$) and 1000 ng/ml ($p<0.001$) after 48 hours of incubation (at 50 ng/ml: $p=0.8656$). After 72-hours administration of S1P, there were significant differences in hepcidin levels between the treated and the control samples (in the case of S1P at 50 ng/ml: $p=0.00124$, in the case of S1P at 200 ng/ ml and at 1000 ng/ml: $p<0.001$ in case of rat cells and also in human cells at all concentrations ($p<0.001$) (Figure 6. and 7.).

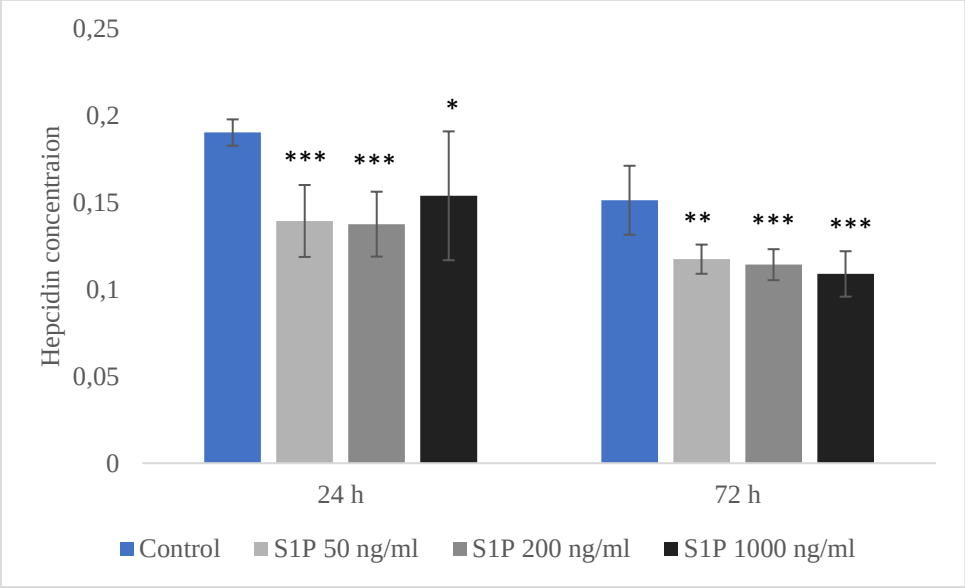


Figure 6. The effects of S1P at 50, 200 and 1000 ng/ml on hepcidin level of rat primary hepatocytes. Significant differences were found between the control and the S1P-treated groups (n=3, $p>0.05$).

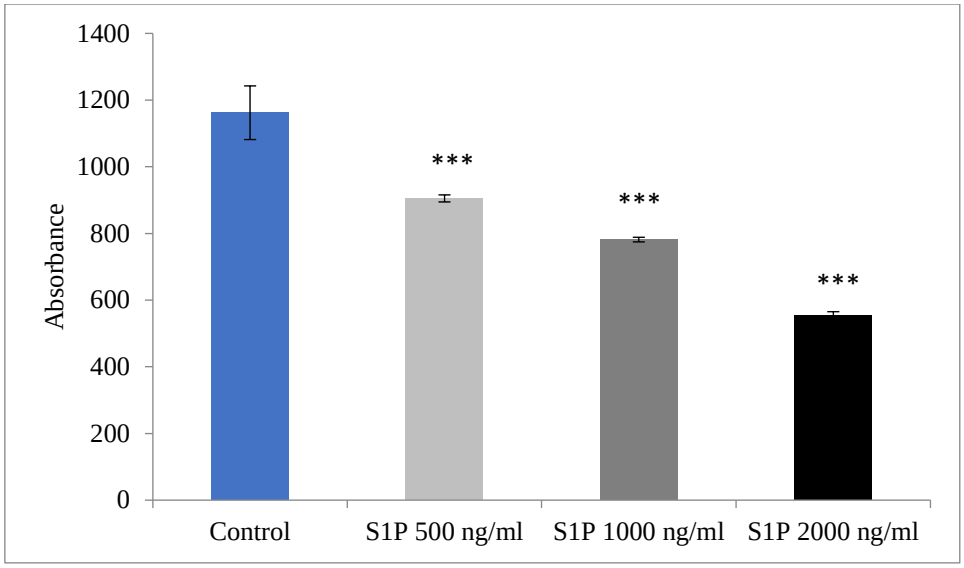


Figure 7. The effects of S1P at 500, 1000 and 2000 ng/ml on hepcidin level of human primary hepatocytes. Significant differences were found between the control and the S1P-treated groups after 72 hours (n=3, p>0.05).

4.4. CYP chemiluminescent investigation

We used Luciferin Detection for the luminometric determination of CYP activity of human primary hepatocytes with 500, 1000, 2000 ng/ml S1P concentrations. The cells were incubated for 48 hours. It was found that the concentration of 500, 1000 ng/ml did not significantly alter CYP enzyme activity, therefore it can be applied. In the highest treated concentration (2000 ng/ml) inhibition of CYP activity was found ($p<0.05$) (Figure 8.).

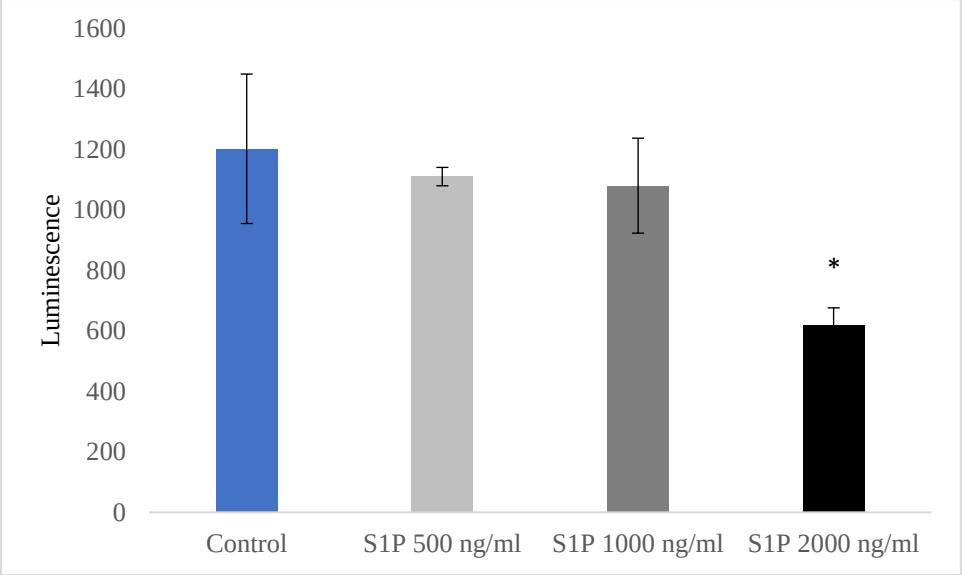


Figure 8. The effects of S1P at 500, 1000 and 2000 ng/ml on CYP3A4 enzyme activity of human primary hepatocytes. There were significant differences between the control and the highest concentration of S1P ($p<0.05$, $n=3$).

4.5 Immunofluorescent measurement of primary rat hepatocytes

To investigate the effect of matriptase activity modulation on TJ assembly, localizaton of occludin was examined in untreated control and activator-treated cells using immunofluorescence staining and was checked after a 48 hours incubation time. In the control cells, occludin localised at the cell membrane and were present in the TJs in polarised monolayers. In the activator treated cell cultures, there was a higher rate of occludin accumulation observed in TJ strands to that detected in control groups based on more intensive immunofluorescence staining pattern (Figure 9.). Immunofluorescent staining of occludin (red) in control, S1P treated were examined after 48 hours incubation time. In control cells there is clear accumulation of occludin along cell membranes. In cells treated with matriptase activator, S1P, there is a more intense fluorescence along the cell-cell borders indicating an increased presence of occludin in the TJs.

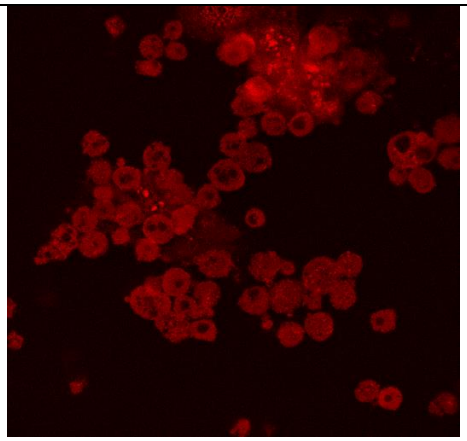
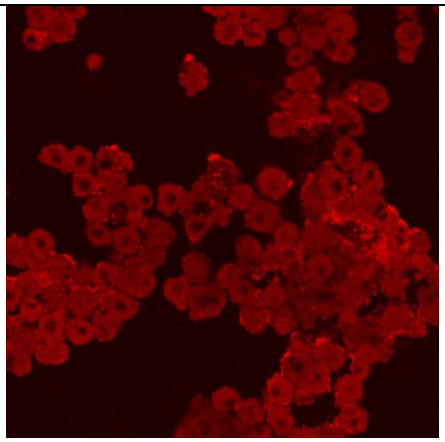
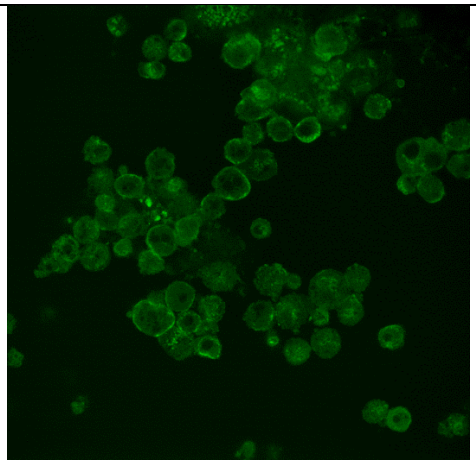
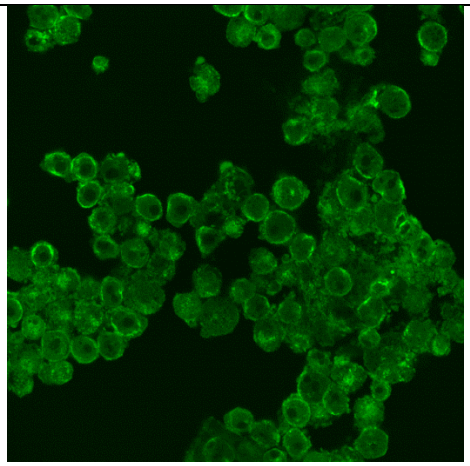
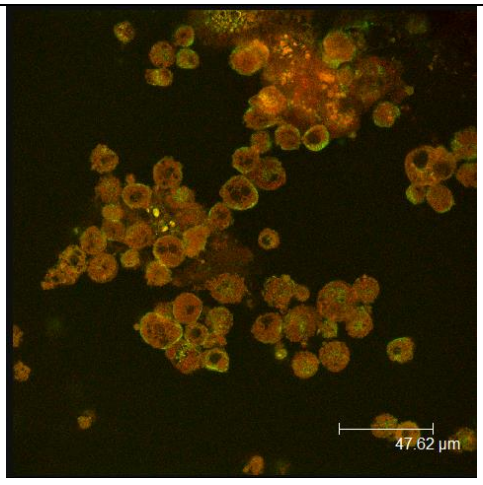
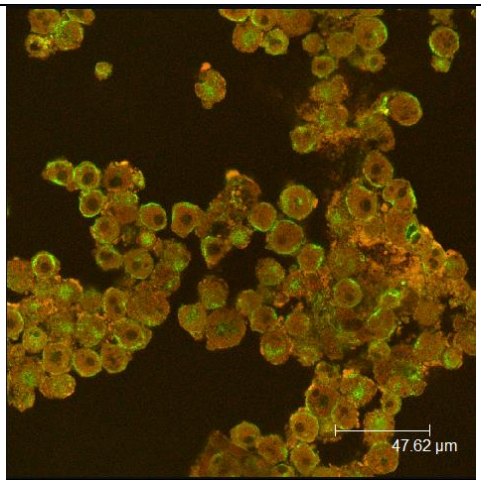
	Control	S1P 1000ng/ml
Occludin		
WGA		
Merge		

Figure 9. Confocal laser scanning microscopy of the control and S1P 1000 ng/ml treated primary rat hepatocytes. In control cells there is clear accumulation of occludin along cell membranes. In cells treated with S1P, there is a more intense fluorescence along the cell-cell borders indicating an increased presence of occludin in TJs.

5. DISCUSSION

Transmembrane serine proteases mainly found in epithelial and carcinoma cells consist of C-terminal extracellular serine protease domain, therefore able to interact with other cell surface proteins, soluble proteins, proteins on neighboring cells in order to generate biologically active proteins. These proteases also contain an intracellular N-terminal through which they can also influence intracellular signaling mechanics (Szabó – Bugge 2008).

TTSP matriptase subfamily have significant role in various physiological and pathological processes. MT-1 is widely expressed in epithelial tissues, with high levels in the gastrointestinal tract, prostate (Takeuchi et al. 1999), in normal and cancerous breast, ovarian and colon tissues (Oberst et al. 2003). MT-1 is also required for postnatal survival. According to Oberst et al. (2001), MT-1 has a potential role in cancer invasion and metastasis in some types of cancer.

MT-2 is found in high levels in the liver and in small amounts in the kidney (Hooper et al. 2003) and able to regulate the iron homeostasis (Folgueras et al. 2008). Therefore, dysfunction of MT-2 can be involved in iron regulatory disorders via altered regulation of hepcidin expression. In mice experiment after chemical inactivation of the MT-2 serine protease region abnormal hair growth, IDA and extremely high plasma hepcidin concentration was observed (Folgueras et al. 2008; Du et al. 2008). In case of human iron resistant IDA hypochromic microcyter anemia was observed with high plasma hepcidin level, decreased RBC number and low transferrin saturation (Finberg et al. 2008).

S1P was tested on IPEC-J2 non-tumorigenic cell line (Pászti-Gere et al., 2016). IPEC-J2 was isolated from the jejunum of a neonatal, non-suckled piglet. They found that main advantages of IPEC-J2 cells were that they possess enterocyte-like microvilli and TJs (Schierack et al., 2006). Occludin, claudin-1, -3, -4, -5, -7, and -8 were also detected (Zakrzewski et al., 2013) in IPEC-J2 cells. Therefore IPEC-J2 cell line more closely mimics *in vivo* conditions than other, tumorigenic cell lines (Vergauwen, 2015). In this study it was found that S1P at 200 ng/ml concentration was not cytotoxic and did not cause an elevation in extracellular hydrogen peroxide levels. S1P had strong barrier protective effect, since the transepithelial electrical resistance was elevated and a higher amount of occludin appeared in cell membranes (Pászti-Gere et al. 2016a). In our study performed on rat and human primary hepatocytes it was found

that administration of S1P for 72 hours even at 1000 ng/ml was safe in the case of the rat primary hepatocyte model. The same was confirmed in case of human hepatocytes, even the highest concentration of S1P (2000 ng/ ml) did not cause significant cell death after 72 hours.

In case of IPEC-J2 cells it was also shown, that no extracellular hydrogene peroxidase levels were significantly elevated nor in the examined 50 ng/ml, 100 ng/ml neither in the largest dose of 200 ng/ml for 48 hours. Fluorescent ROS measurement was performed by using Amplex Red reagent (Pászti-Gere et al. 2016). In our study we also used Amplex Red method for investigation extracellular H_2O_2 concentrations influenced by S1P treatment. We found that S1P did not cause a significant increase in the extracellular H_2O_2 levels at any applied concentrations within the investigated 72 hours in any of our examined species. There were no significant differences found between the control and the S1P-treated groups nor in rat neither in human primary hepatocytes even with the highest tested concentrations, 1000 ng/ ml in primary rat cells and 2000 ng/ml in case of primary human cells.

MT-2 regulates iron homeostasis via cleavage of membrane-bound HJV, which is the main activator of hepcidin transcription (Silvestri et al. 2008). Hepcidin inhibits the transport of iron into the plasma and the absorption from enterocytes. It was also observed that mice with a TMPRSS6 –/– defect or then absence of MT-2 proteolytic activity have iron deficiency anaemia, upregulation in hepcidin transcription, and reduced protein levels of ferroportin on enterocytes (Du et al., 2008; Folgueras et al., 2008). Therefore, stimulation of MT-2 could be an alternative treatment of iron deficiency anaemia. Our aim in the study was to examine the effect of S1P on hepcidin levels, via activation of MT-2. We used sandwich ELISA for rat and human hepcidin level measurements. We found that hepcidin levels significantly decreased in rat and also in human primary hepatocytes exposed to S1P after an incubation time of 24 hours.

Concerning CYP enzyme what plays role in xenobiotic metabolism, rabbit primary hepatocytes were examined after treatment with a CYP inducer (phenobarbital) and 2 different inhibitors (alphanaphthoflavone and ketoconazole). It was shown that regarding CYP3A6, both induction and ketoconazole-mediated inhibition were highly effective. Luciferrin based assey was performed to show drug interactions of CYP enzymes with inducers and inhibitors (Csikó et. al 2017.). There were no relevant experiments found concerning CYP interaction with S1P on hepatocytes before. However, we used similar cell culture and for controlling S1P

effect, we used the same inhibitor (ketoconazole) and for induction we used deaxamethasone for control measures of S1P. We found that only in the highest concentration of S1P (2000 ng/ml) inhibiton of CYP activity was observed.

To investigate the effect of matriptase activity modulation on the cell TJ assembly, localizaton of occludin was examined in activator-treated IPEC-J2 cells, using immunofluorescence staining and was checked after a 48 hours incubation. In the treated cell cultures (exposed to S1P at 200 ng/mL), there was a higher rate of occludin accumulation observed in TJ strands to that detected in control groups based on more intensive immunofluorescence staining pattern (Pászti-Gere et al. 2015). In our research we also found, that after treatment with S1P, there is a more intense fluorescence along the cell-cell borders indicating an increased presence of occludin in the TJs, compared to the control cells.

In our research tried to establish correlations between modulation of MT-2 by S1P and physiological function alterations of hepatocytes, so that S1P may have a good prognosis in medical treatment of anemias.

6. ABSTRACT

Sphingosine-1-phosphate (S1P) has been reported as a nonselective matriptase activator. Matriptase-1 (MT-1) and matriptase-2 (MT-2) belong to the type II transmembrane serine protease family. However, MT-1 is widely expressed in epithelial tissues, MT-2 is mainly expressed in the liver and also in small amounts in the kidney. MT-2 can regulate the iron homeostasis via modulation of hepcidin level. The aim of our study was to reveal if S1P can influence hepcidin production on primary human and rat hepatocytes after 72 hours of incubation via modulating the MT-2. Investigations were done on how S1P can affect the viability and the redox status of primary hepatocytes of human and rat primary hepatocytes. Also the distribution of the occludin, as a tight junction protein on primary rat hepatocytes was detected. In the case of human cells, cytochrome P450 3A4 (CYP3A4) enzyme interaction was also measured. Primary rat hepatocytes were treated with S1P at 50, 200 és 1000 ng/ml concentrations, while human hepatocytes were incubated with 500, 1000, 2000 ng/ml S1P. Cell-free supernatants were collected every 24 hours. Cell viability was tested by a colorimetric method on both cells, the hepcidin levels in the cell-free supernatants were examined by hepcidin sandwich ELISA to determine the effect of S1P on the hepcidin-modulating ability of MT-2. In order to estimate the extent of S1P generated oxidative stress, extracellular H_2O_2 measurements were performed by the use of fluorescent method. Based on the findings, S1P treatment did not cause cell death for 72 hours at the highest concentrations nor in rat neither in human primary hepatocytes at any applied concentrations within the investigated time period. S1P did not influence the extracellular H_2O_2 production for 72 hours in any of our examined species. The distribution of occludin did not alter after treating the primary rat hepatocytes with S1P. The hepcidin levels were significantly suppressed after 72 hours in hepatocytes exposed to S1P treatment in both species. In the case of human primary hepatocytes we observed inhibition of CYP3A4 enzyme activity only in the highest concentration. These findings might presume that S1P might be a drug candidate in treatment of iron deficient anaemias, as any of the used concentrations were safe to apply and the hepcidin level significantly altered after the treatment, however drug-drug interaction might appear at the highest applied concentration.

7. ÖSSZEFOGLALÁS

A szfingozin-1-foszfát (S1P) nem-szelektív matriptáz enzim aktivátor, mely a legtöbb sejttípusban megtalálható a szervezetben. A matriptáz-1 (MT-1) és matriptáz-2 (MT-2) enzimek a kettes típusú transzmembrán szerin-proteáz család tagjai. Míg az MT-1 számos epiteliális szövetben megtalálható, az MT-2 legfőképp a májban, valamit kisebb mennyiségben a vesékben is megtalálható. Az MT-2 a hepcidin szint szabályozásán keresztül befolyásolja a szervezet vasháztartását. A kutatásunk célja volt meghatározni, van-e szignifikáns hatása az S1P enzimnek a hepcidin szintjére humán és patkány primer májsejteken 72 órás inkubációs időt követően az MT-2 szabályozásán keresztül. A kísérlet során vizsgáltuk, hogyan befolyásolja az S1P a vizsgált humán és patkány primer hepatociták életképességét, valamit redox státuszukat, illetve, hogy patkány hepatocitákon befolyással van-e az okkludin, mint sejtkapcsoló struktúra eloszlására. A humán primer hepatociták esetében citokróm P450 3A4 (CYP3A4) enzim változást is vizsgáltunk. A patkány primer hepatocitákat 50, 200 és 1000 ng/ml koncertrációkkal, a human primer hepatocitákat 500, 1000, 2000 ng/ml koncertrációkkal kezeltük. A 72 órás inkubációs időben 24 óránként vettünk mintát a sejtek felülúszójából. A sejtek életképességét kolorometriás módszerrel detektáltuk, a felülúszók hepcidin szintjét szendvics-ELISA módszerrel mértük, hogy meghatározzuk a S1P okozta hepcidin szint változást az MT2 modulációján keresztül. Az S1P által okozott lehetséges oxidatív stressz meghatározásához extracelluláris H_2O_2 szintet mértünk, fluoreszcens módszer segítségével. Megállapításra került, hogy az S1P kezelés nem okozott sejthalált a vizsgált 72 órában, a legnagyobb alkalmazott dózisban sem, sem a humán sem a patkány primer májsejtek esetében. Az S1P szignifikánsan nem befolyásolta az extracelluláris H_2O_2 termelődést a vizsgált 72 órában egyik fajban sem. Patkány hepatocitákon végzett immunfluoreszcens festést követően nem detektáltunk változást az okkludin eloszlásában. A hepcidin szint mindkét vizsgát fajban szignifikánsan csökkent 72 óra után S1P kezelés hatására. A humán primer hepatociták esetében CYP3A4 enzim gátlása csak a legmagasabb alkalmazott dózisban (2000 ng/ml) volt detektálható. Mivel az S1P minden alkalmazott koncentrációban biztonságosnak bizonyult és szignifikánsan befolyásolta a hepcidin szintjét, az eredmények arra mutatnak, hogy az S1P egy potenciális gyógyszerjelölt lehet a vashiányos anémiák kezelése terén, azonban 2000 ng/ml alkalmazott dózis esetén fennáll az esélye az egyes gyógyszerek között interakciók kialakulásának.

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- *a nyílt hozzáférés támogatása.*

Témavezetői nyilatkozat

Alulírott Pomothy Judit Mercédesz és dr. Czimmermann Ágnes Eszter, mint témavezetők nyilatkozzuk, hogy Paréj Zsuzsanna állatorvostan-hallgató „PHARMACOLOGICAL AND TOXICOLOGICAL CHARACTERIZATION OF S1P AS A TREATMENT OPTION IN ANAEMIAS" c. dolgozata részt vehet az Állatorvostudományi Egyetem 2020. évi Tudományos Diákköri Konferenciáján.

Budapest, 2020. 10.24.

Pomothy Judit Mercédesz

témavezetők

DECLARATION

I hereby declare that the thesis entitled PHARMACOLOGICAL AND TOXICOLOGICAL CHARACTERIZATION OF S1P AS A TREATMENT OPTION IN ANAEMIAS
is identical in terms of content and formal requirements to the TDK research paper submitted in 2020.

Date: Budapest, 11 day 11 month 2021 year

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Zsuzsanna Paréj

Student name and signature