„Molecular Therapies for Mucopolysaccharidosis in Mouse Models”
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Mucopolysaccharidoses (MPS) belong to the group of hereditary metabolic diseases where, as a result of a mutation in a gene encoding an enzyme responsible for the breakdown of compounds from the mucopolysaccharide group (glycosaminoglycans, GAG), these compounds accumulate in the cell [1,2]. The process of their degradation in a healthy organism is a sequence reaction of several enzymes, however, when one of them does not function properly, the reaction stops and GAG(s) accumulate(s) in the cells. Depending on which enzyme is inactive, there are 13 types and subtypes of MPS. Many symptoms are common to all or most types and subtypes of MPS, but the most severe are those related to the central nervous system (CNS) and the skeletal system [3]. In both cases, the currently available therapies are not able to overcome the symptoms of the CNS and those related to the skeletal system.

The most commonly used MPS therapy is enzyme replacement therapy (ERT), which uses the active form of the missing enzyme. However, the supply of the missing enzyme is not sufficient in the case of MPS types whose symptoms are expressed in CNS (such as MPS III), because the enzyme does not cross the blood-brain barrier [4,5]. There are also other limitations associated with ERT, including a short half-life of the enzyme, high cost of the therapy, limited impact on avascular tissues and organs, weekly infusions for lifetime [6,7]. Moreover, even though ERT for non-neuronopathic MPS IVA has been approved, it has a limited effect on skeletal abnormalities, which is one of the main symptoms of the disease [8]. Therefore, research into alternative therapeutic approaches for these types of MPS is highly desirable.

One alternative strategy is accelerating the degradation of GAG(s) through autophagy, a lysosomal process that breaks down unnecessary or abnormal macromolecules [9]. Recent discoveries suggested GAGs can be targeted by autophagy, potentially offering a new treatment for diseases involving polysaccharide storage [10]. A key question is how autophagy can degrade GAGs when a crucial enzyme is impaired. While complete enzyme absence is challenging, many MPS cases have some residual enzyme activity. Enhancing autophagy could boost this residual activity, improving GAG degradation. Additionally, non-specific hydrolases, though low in abundance, might aid GAG removal if autophagy is stimulated. Even if full GAG clearance is not possible due to enzyme deficiencies, degrading secondary storage materials can still benefit patients, as necessary enzymes are present in MPS cell lysosomes [11-Article no.1].

The potential drug for MPS IIIB should also cross the blood-brain barrier and be safe in long-term therapy. It seems that one of the polyphenols, resveratrol, may meet these requirements. Grapes, peanuts, mulberries and black currants are particularly rich in resveratrol. It has multiple biological functions, such as anti-inflammatory, antioxidant and neuroprotective effects. It is a compound that has been widely studied and activates the process of macromolecule degradation through several mechanisms [12]. Due to its pleiotropic mechanism of autophagy induction, ability to cross the blood-brain barrier, and safety profile, resveratrol is a promising candidate for treating neuronopathic forms of MPS [11-Article no.1]. Resveratrol induces autophagy through multiple pathways, including activation of PTEN, AMPK, FoxOs, and TFEB, as well as inhibition of mTOR kinase and the BcL-2-encoding gene. These mechanisms have been detailed in Rintz et al. 2019 [11-Article no.1]. Thus, I treated MPS IIIB mouse model with resveratrol to improve behavior with reduction in accumulated GAG [13-Article no.2].

A promising alternative therapy for MPS IVA patients is gene therapy. ERT with elosulfase alfa has limited effects on bone growth in MPS IVA (Morquio A syndrome) patients. Hematopoietic stem cell transplantation (HSCT) may offer more benefits, such as improved heart function, bone mineral density, and joint laxity [14], but it also has significant limitations: risk of mortality and morbidity, difficulty in finding donors, post-transplant complications, and limited impact on bone growth [14,15]. Both ERT and HSCT rely on cross-correction via the mannose-6-phosphate receptor pathway, where lysosomal enzyme-producing cells help enzyme-deficient ones [16]. However, this method struggles to penetrate bone cartilage effectively [15]. Thus, there is a high demand for bone-penetrating agents to treat avascular bone lesions and induce ossification in MPS IVA patients.

C-type natriuretic peptide (CNP) has shown a promise in inducing bone growth by activating the natriuretic peptide receptor B (NPR-B) on chondrocytes [17]. Stimulation of the intracellular molecule cGMP production by CNP activates multiple pathways, resulting in bone growth (for details see ref. [18-Article no.3]). Despite its potential, natural CNP has a short half-life, necessitating frequent injections [19]. Thus, I aimed to improve bone pathology in MPS IVA by combining gene therapy of a small peptide to stimulate growth [20-Article no.4]. With bone growth induction, the next step was to combine two transgenes (*GALNS* and *NPPC*) in AAV system to improve bone growth with reducing GAG accumulation in other tissues [21-Article no.5].

The purposes of this PhD thesis were:

1) Determination of the role of resveratrol and the exact molecular mechanism of its action during long-term studies with the MPS IIIB mouse model.

2) Development of an innovative combination gene therapy to improve bone changes in the mouse MPS IVA model.

Objective 1 - Determination of the role of resveratrol and the exact molecular mechanism of its action during long-term studies effects in with the MPS IIIB mouse model.

Both wild-type and MPS IIIB mice were tested in two groups where water or resveratrol was administered every day for the course of 30 weeks. To test the effectiveness of resveratrol several tests were performed. Behavioral tests were performed. To measure hyperactivity of animals locomotor activity test was performed. Open field test was performed to measure anxiety in the mouse model. Biochemical and molecular tests were also performed to assess the effects of resveratrol on cells from various organs, especially the brain and liver, where GAG accumulation is at its highest. Urine GAG concentrations (samples were taken at 5, 10, 20 and 30 weeks of age) with Blyscan test. The effect of resveratrol on specific protein levels in terms of the molecular mechanism of autophagy induction by resveratrol was assessed by Western-blot technique.

Among compounds tested *in vitro* (genistein, capsaicin, curcumin, resveratrol, trehalose, and calcitriol), resveratrol showed the greatest effects, specifically reducing levels of heparan sulfate (HS) the GAG which accumulates in Sanfilippo disease. This suggested that the pathological GAG was degraded after autophagy stimulation. Experiments with the MPS IIIB mouse model confirmed resveratrol-mediated activation of otherwise impaired autophagy, normalizing urinary GAG levels and improving behavior in affected animals. These findings supported the proposal of the use of resveratrol as a potential drug for Sanfilippo disease treatment, warranting further research. Resveratrol has also been proposed for other lysosomal storage diseases due to its many biological functions. However, its mechanism of action can be different in various diseases; for example, it showed antioxidant properties in Batten disease models. Resveratrol-mediated activation of autophagy may involve multiple pathways, including mTOR-independent and protein phosphatase 2A-dependent mechanisms. Additionally, behavioral studies with MPS IIIB mice revealed that hyperactivity was reduced after treatment with resveratrol, as was the anxiety level [13 - Article no.2].

Objective 2- Development of an innovative combination therapy to improve bone changes in the mouse MPS IVA model.

MPS IVA mice were tested for monotherapy and combination therapy to see treatment effectiveness. At 4 weeks of age, mice were intravenously administered with a viral vector that contains in its expression cassette a NPPC gene for CNP peptide that activates mouse growth, the enzyme GALNS, or a combination therapy where the mice were given a mixture of both vectors. From that time animals were measured weekly for the growth effectiveness. To determine the effectiveness of treatment, biochemical and molecular tests were performed, including testing the enzyme activity in the blood, organs and bones. As well as histological slides showing bone pathology and treatment effectiveness. As for the vector copy number determination, I measured the concentration of the CNP peptide in the blood of animals, using the ELISA test. Levels of anti-GALNS antibodies were also measured with ELISA to monitor the immune reaction. The GAG levels was assessed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Additionally, a specialized computer microtomography examination was used to determine the bone density and structure after treatment.

In the MPS IVA mouse model, I observed an accumulation of mono-sulfated keratan sylfate (KS) in bone, a GAG stored in Morquio disease, together with disorganized column structure of chondrocytes. GALNS enzyme activity was not detected in the tissues and plasma of the MPS IVA mouse model. Although I did not observe significant bone growth abnormalities or skeletal dysplasia, there were tendencies towards increased bone volume and higher bone area in MPS IVA mice, compared to wild-type (WT) control. The study also showed that untreated MPS IVA mice had about 200-times higher mono-KS levels in the liver and 100-times higher mono-KS levels in the lung, compared to WT, with a 30% increase in bone. The mouse models reflect human MPS IVA pathology with no enzyme activity, GAG accumulation, and bone histological changes. However, none of the existing mouse models fully replicate the severe skeletal phenotype seen in humans.

I used an AAV8 vector to express the *NPPC* gene, encoding the CNP peptide, which induced bone growth and reduced KS accumulation in bone. CNP has been shown to stimulate bone growth and regulate GAG synthesis during chondrogenesis. The study demonstrated a decreased KS accumulation and increased chondrocyte proliferation in bone after CNP treatment of MPS IVA mice. In summary, using an AAV8 vector to express *NPPC* in MPS IVA mice resulted in a high level CNP secretion, bone growth induction, improved bone pathology, and changes in GAG levels. Further research with larger sample sizes and long-term studies is necessary to validate these findings and to develop effective treatments for skeletal dysplasia in MPS IVA patients [20- Article no.4].

The CNP peptide supports cartilage homeostasis and bone formation. Combining two vectors (one for GALNS and one for CNP production) showed promising results, with sustained GALNS activity and reduced KS accumulation, enhancing bone growth without negative outcomes. I observed high GALNS activity and reduced KS levels in blood and tissues after combination treatment, leading to improved bone pathology. The optimal CNP dose is crucial to avoid excessive growth. Biodistribution analysis confirmed the GALNS presence in bone and liver, while NT-proCNP levels correlated with CNP production and growth induction. Combining high doses of AAV8-hNPPC and AAV9-hGALNS vectors yielded the most significant improvements.

In conclusion, co-expressing *GALNS* and *NPPC* genes, especially in separate vectors, enhanced bone growth and GALNS activity. Defining the optimal CNP dose in more extensive studies is essential to avoid overgrowth and adverse effects, moving towards clinical trials. Implementing a microRNA system to control CNP expression may be considered to prevent excessive growth [21- Article no.5].

In summary, this PhD thesis focuses on addressing the challenge of delivering therapeutic agents to hard-to-reach tissues in mouse models of MPS. I propose to use small molecules capable of crossing biological barriers to effectively target these tissues. Specifically, I investigated the potential of resveratrol to cross the blood-brain barrier (BBB) in the treatment of MPS IIIB, and CNP for targeting avascular chondrocytes in MPS IVA. Both therapies demonstrated a significant potential in improving treatment outcomes for mice suffering from these forms of MPS by enhancing drug delivery to otherwise inaccessible sites within the body. This approach not only aims to improve the efficacy of current treatments but also to pave the way for the development of new therapeutic strategies for MPS and potentially other lysosomal storage disorders.