Molecular mechanism of action of nonsteroidal ant-inflammatory drugs and flavonoids in terms of their potential use in the treatment of lysosomal storage diseases

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Lysosomal storage diseases (LSDs) are a group of rare metabolic disorders caused by mutations in genes that encode proteins involved in the lysosome function. Because of the crucial function in recycling of intra- and extra-cellular macromolecules any impairment of lysosomal activity can lead to accumulation of different undegraded substrates. The pathological effect is caused by the genetic defect diagnosed with a prevalence of 1:7500 live births and in the most cases this mutation impairs enzymatic activity of a lysosomal hydrolase. Since 1963 when the first LSD has been identified (Pompe disease), to date about 70 different LSDs have been described. The classification and diagnosis of these diseases are based on the nature of the primary stored material within lysosomes. Thus, we can distinguish e.g. mucopolysaccharidoses, sphingolipidoses or mucolipidoses. The undigested substrates progressively impair the function of tissues, organs and eventually the whole body, which in turn leads to the premature death. The vast majority of symptoms are observed up to age of two, thus LSDs mostly affect children. However, due to the variability of different genetic and environmental factors, some of the LSDs may have a late onset. The age of onset and clinical symptoms usually correlate with the type of mutation, which in some cases can lead to the production of enzyme with residual activity. Unfortunately, very often there is no direct correlation between genotype and phenotype, which makes difficulty in accurate diagnosis. Currently, the vast majority of LSDs are incurable or available therapies are insufficient, especially when it comes to symptoms from central nervous systems (CNS), which are observed in about 80% of all LSDs. Among the diseases with a lysosomal defect in degradation of macromolecules mucopolysaccharidoses (MPSs) are one of the largest group. Depending on mutation in one of eleven genes coding for glycosaminoglycans (GAGs) degradation proteins, seven different subtypes of MPS are distinguished, called MPS type I, II, III, IV, VI, VII and IX. In Poland, a combined birth prevalence of MPSs is 1.8:100000 live births and MPS III, also called Sanfilippo disease, accounts for about 48% of all cases. The primary GAGs accumulation within cells leads to progressive pathophysiology in MPSs patients. Different classes of GAGs are stored depending on the subtype of the disease. Among them dermatan sulfate, heparan sulfate, chondroitin sulfate, keratan sulfate or hyaluronan can be distinguished and in some

cases, the accumulation of more than one GAG is observed. The common feature of macromolecules mentioned above is a chemical structure, which is determined by the repeating disaccharide units forming long and unbranched anionic chains. With exception of hyaluronan, all GAGs are sulfated and covalently attached to the proteins forming structures called proteoglycans.

Currently, we can distinguish a few different therapeutic approaches existing to treat LSDs. The most common strategy is based on delivery of the active, exogenously produced enzyme to the patient with lack of sufficient hydrolase activity. To this end, native protein is injected intravenously to the patient and this concept is called enzyme replacement therapy (ERT). Other clinically available method for the delivery of recombinant enzyme is a hematopoietic stem cell transplantation (HSCT). Unfortunately, these concepts are not effective treatments for LSDs with neurological manifestation due to the fact that enzyme cannot cross the blood-brain barrier (BBB). Among a few different therapeutic strategies for example, gene therapy or direct delivery of the enzyme in the cerebrospinal fluid, completely different approach can be distinguished. In this case, the dynamic balance between synthesis and degradation of particular macromolecules is reached through the inhibition in the biosynthesis of the accumulating substrate. To this end, a small inhibitory molecule prone to penetrate BBB is employed. Together, with the presence of about 3% of the residual activity of particular hydrolase, this approach allows restoring balance in the metabolism of macromolecules which leads to the lack of stored material within lysosomes. Implementation of this strategy is called substrate reduction therapy (SRT) and the efficiency of this rationale has been observed using genistein in Sanfilippo disease and miglustat (Zavesca®) in Gaucher disease.

Nonsteroidal anti-inflammatory drugs (NSAIDs) constitute a large and diverse group of medicines consumed every day by about 30 million people worldwide. NSAIDs are used in order to relieve pain and fever as well as to reduce inflammation. The beneficial mechanism of action of NSAIDs is achieved owing to the inhibition of prostaglandins (PGs) biosynthesis. In the human body PGs act as paracrine factors involved in inflammatory response and regulation of a broad spectrum of physiological functions. The effect of action of NSAIDs is inhibition the activity of the cyclooxygenase (COX, PGHS) which is the crucial enzyme in the biosynthesis of prostanoids, including besides PGs also prostacyclin and thromboxane. Currently, two main COX isoenzymes are distinguished, the constitutively expressed (COX-1) and the inducible (COX-2). The selectivity of NSAIDs against particular isoenzyme has allowed to distinguish different classes of these drugs according to the relative inhibition of COX isoenzymes. An example of nonselective COX-1 inhibitor is a indomethacin (2-[1-(4-

chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid), and an example of preferential COX-2 inhibitor is a nimesulide (N-(4-nitro-2-phenoxyphenyl)methanesulfonamide). However, due to the complexity of this group of medicines, there is no universal classification of NSAIDs so far.

Among flavonoids, organic compounds present in plants as secondary metabolites, genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is an example of very promising substance as a novel therapy for MPSs. Many investigations have revealed that due to the pleiotropic effects genistein can be used to modulate pivotal mechanisms in human cells for instance, cell cycle, metabolism of macromolecules or biogenesis and activity of lysosomes. Because patients suffering from different types of MPSs have many defects in cellular processes mentioned above, this isoflavone is considered as a novel therapeutic, especially in a combination therapy.

The objective of my PhD thesis was to describe the mechanism of action of selected NSAIDs and their mixtures with genistein in terms of their modulatory effect on cellular processes with emphasis on application in the treatment of MPSs. In my research, I worked on *in vitro* model by using human dermal fibroblasts, adult (HDFa) and skin fibroblasts from patients suffering from MPS types I, II, IIIA, IIIB, and MPSVI. Selected NSAIDs were indomethacin and nimesulide according to the literature data concerning their influence on GAGs metabolism. Moreover, at the beginning of my work acetaminophen N-(4-hydroxyphenyl)acetamide), the analgesic drug with the inhibitory activity against COX-1 splice variant called COX-3, was implemented. Due to the recent investigations and data about the inhibitory effect on GAGs synthesis and cellular metabolism in cultures of skin fibroblasts, genistein was chosen as a flavonoid component.

The first step of research was the selection of concentrations of tested drugs and assessment of their cytotoxic and antiproliferative activity in cultured skin fibroblasts. The final concentrations were chosen based on the literature data and information corresponding to the clinically achievable values for NSAIDs and acetaminophen. Genistein was used in concentration of 100 μM in accordance with the previous investigations [1, 2]. All tested compounds were dissolved in 100% dimethyl sulfoxide (DMSO) in order to achieve the final concentration of 0,05% or 0,1% for the mixtures. The cytotoxic effect of tested drugs and their mixtures with isoflavone was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) which depends upon a mitochondrial activity. To this end, cells were treated with different concentrations of selected drugs or their mixtures with genistein for 24 h, 48 h and 7 days. This study demonstrated that 24 h and 48 h treatment with

tested compounds had a little cytotoxic effect on skin fibroblasts [3]. The concentrations lethal to 25, 50 and 75% of cells (LC_{25}, LC_{50} and LC_{75}, respectively) were estimated after 7 days of treatment and revealed that indomethacin had a noticeably higher impact on cell growth compared to nimesulide [4]. However, the highest cytotoxic effect, confirmed by light microscopy observations, was observed for the mixture of tested NSAIDs and genistein [3].

In order to assess the effectiveness of modulation of GAGs synthesis by investigated substances the experiments based on measurement of ^{35}S or ^{3}H uptake were made. Both of isotopes are crucial for the formation of GAGs chains and their proper functions. For instance, sulfur as a SO_4^{2-} group, while hydrogen is a component of sugar monomers forming GAGs backbone. According to the results from cytotoxic assays and the literature data, three different concentrations of each drug were implemented for further experiments. Assessment of the modulation of GAGs synthesis was based on HDFa cells and three different lines of MPS fibroblasts (MPS type IIIA, IIIB and VI) after 72 h and for HDFa cells additionally after 7 days of treatment. Based on obtained results, it was shown that both indomethacin and nimesulide revealed the ability to inhibit GAGs biosynthesis in all tested cell cultures. The applied final concentrations with the most inhibitory effects were shown for indomethacin of 2 and 10 μM, while for nimesulide of 5 and 25 μM [3]. In case of acetaminophen, there was no inhibitory effect, hence this drug was excluded from the subsequent experiments. Experiments with genistein demonstrated its inhibitory potential in terms of GAGs synthesis, thus indicating the application of this isoflavone as a candidate for SRT [2]. Moreover, it is well known that the beneficial effect of action of genistein is associated with the modulation of genes coding for enzymes involved in degradation of GAGs and with the inhibition of different signaling pathways. These promising results concerning genistein resulted in the implementation of this compound in the mixtures with NSAIDs. To this end, HDFa cells and MPS IIIA fibroblasts were treated for 72 h with appropriate concentrations of both, particular drug and isoflavone. The most pronounced impairment of GAGs synthesis was observed after 72 h exposure to the mixture of 25 μM nimesulide and 100 μM genistein, results in an additive effect. For HDFa cells the inhibition of GAGs production was at the level of around 48%, and for MPS fibroblasts the decrease was observed at the level of around 40% [3]. Interestingly, effects of mixtures of NSAIDs and genistein were more noticeable compared to the results when only genistein was applied [2].

The next purpose of my dissertation was to establish, which cellular mechanisms are involved in modulation of observed changes in phenotype of cells treated with selected drugs or their mixtures with isoflavone. Taking into account literature data about general mechanism

of action of NSAIDs and their influence on the activity of epidermal growth factor receptor (EGFR), this pathway was the first target in my research. Furthermore, it was observed previously that EGFR has a crucial function in the modulation of GAG production and the inhibition of this receptor by using flavonoids resulted in reduction of stored material within lysosomes. Experiments concerning EGFR activity were conducted using the MUSE® Cell Analyzer which detects signals from fluorescent reagent and due to the stable phenotype, HDFa cells were used in experimental procedures. For this, cells were treated with 100 ng/ml of epidermal growth factor (EGF) in order to stimulate EGFR phosphorylation, and simultaneously with 10 μM indomethacin or 25 μM nimesulide. Samples treated with 100 ng/ml EGF and 0.05% DMSO were used as a control. After 24 h, the efficiency of phosphorylation inhibition of EGFR was assessed using phospho-specific antibodies. Results revealed, that indomethacin, nimesulide as well as their mixtures with genistein were able to modulate EGFR activity [3]. The most significant effect was observed for cells treated with nimesulide and inhibition was even more effective than that of genistein or mixture. The next part of my work was to define more precisely, which other cellular pathways can be involved in the properties of NSAIDs and compounds' in terms of GAGs metabolism. To this end, in further experiments the activity of phosphatidylinositol-3-kinase (PI3K) was investigated by using the analogous procedure with MUSE® Cell Analyzer. One might suppose that because PI3K signaling pathway, besides being involved in pivotal cellular functions, as a downstream target of EGFR can be influenced by the activity of this receptor. The PI3K activity was assessed in HDFa cells after treatment with the same experimental conditions implemented previously for EGFR activity assays. In this case, additional controls were applied by using Jurkat T cells with constantly phosphorylated PI3K and wortmannin as an inhibitor of this pathway. The results of conducted analysis allowed to estimate the percentage of cells with activated or inhibited PI3K pathway and revealed that both, indomethacin and nimesulide has the ability to decrease phosphorylation of PI3K [3]. The most significant inhibition was observed in the case of cells treated with 25 μM nimesulide which was in accordance with the results obtained for EGFR experiments. However, this time mixture of indomethacin and genistein had a higher inhibitory effect on PI3K compared to drug used alone.

In order to investigate effects of indomethacin and nimesulide on gene expression in HDFa cells, DNA microarrays Illumina's Human HT-12 v4 Expression BeadChips targeting about 25000 annotated genes, were implemented. Analyses were performed on fibroblasts after 24 h and 48 h treatment with indomethacin of 2 and 10 μM or nimesulide of 5 and 25 μM. Microarray results were interpreted by calculation the signal ratio for samples treated with

NSAIDs and for vehicle (0.05% DMSO). The estimated ratios were significant when the values were below or equal 0.7 and ≥ 1.3. The preliminary analysis revealed, that transcriptome profile after treatment at selected experimental conditions was slightly changed, due to the fact that concentrations of NSAIDs used in experiments were relatively low and close to clinically relevant molar doses [3, 4]. For more detailed insights into transcriptomic changes as a result of NSAIDs action, analyses were performed based on Gene Set Enrichment Analysis (GSEA) and GOrilla (Gene Ontology enRIchment anaLysis and visuaLizAtion tool). Due to the lack of statistically relevant changes after treatment with a lower concentration of both drugs, and after 48 h treatment time period, these results were not analysed. However, study done in case of higher concentrations of NSAIDs revealed, that after 24 h treatment genes involved in regulation of metabolism pathways are significantly modulated [3]. Moreover, this analysis indicated the influence of applied conditions on expression of genes responsible for signal transduction pathways for instance, Wnt (Wingless-related integration site), TCR (T cell receptor) or PI3K and the vast majority of significantly modulated genes were observed after treatment with nimesulide [3]. Because of the lack of tolerable intensity signals in the microarray experiments, the activity of some interesting genes were not defined. In this case, the microarray results obtained for 100 μM genistein in the previous study were implemented, in order to use them in further analysis [2].

The most of studies concerning the effects of NSAIDs on human cells were performed with cancer-derived cell lines. Only a few reports have been done in order to investigate the influence of NSAIDs on non-transformed cells. Furthermore, the concentrations used in *in vitro* experiments are predominantly much higher than concentrations accessible for *in vivo* procedures. Thus, in my research I put emphasis on quantitative changes in HDFa transcriptome after treatment with clinically achievable concentrations of indomethacin and nimesulide. This issue is a crucial assessment of the action of tested drugs in terms of their safety and potential side effects. Conducted analysis revealed a total of 3803 of significantly modulated genes with the highest number of up-regulated genes [4]. Despite the fact the total number of differentially expressed genes were relatively high, it is worth to mention that the level of noticed changes was slight in terms of transcriptomic analysis. Importantly, only a few genes were altered more than twice and 24 h of treatment with 25 μM nimesulide had the most prominent influence on the transcriptome. Among the transcripts which levels were affected most significantly, about 20% accounted for genes with uncertain function suggesting that better understanding mechanisms of action of NSAIDs is still needed. Interestingly, by monitoring particular mRNA levels in HDFa cells at particular studied conditions, genes involved in cell cycle progression

and DNA metabolism were identified for example, *SPHAR, RECQL4* and *NEK7* [4]. Thus, the next step of my research was to evaluate whether NSAIDs can influence cell cycle in HDFa fibroblasts. Moreover, fibroblasts derived from patients suffering from MPS type I and II were included in my experiments. The evaluation of cell cycle progression was done using fibroblasts exposed to different concentrations of tested compound for the 24 h period of time. Fluorescence signals from propidium iodide (PI) were detected by using the MUSE® Cell Analyzer in order to determine the percentage of cells in different phases of cell cycle. Conducted experiments showed that addition of selected drugs to HDFa fibroblasts culture results in any significant changes in the population of cells present in the specific stage when compared to the control [4]. Analysis made for MPS fibroblasts gave similar results with the exception of MPS type I cells where some smallish but statistically significant alterations were noticed. However, the biological impact of these changes seems to be irrelevant in terms of cell cycle progression. Experiments described above revealed, that selected concentrations of NSAIDs have no influence on HDFa and MPS fibroblasts during the cell cycle which might be very important information in particular for studies concerning MPS patients.

In order to validate microarray experiments and more precisely determine the changes in mRNA level of particular genes, the real-time qRT-PCR method was implemented. The first objective of my analysis was to investigate the expression patterns of genes involved in GAGs metabolism, as well as lysosomal function [3]. Additionally, the aim of real-time qRT-PCR experiments was to establish whether genistein and nimesulide act in similar fashion in terms of GAGs metabolism and activity of EGFR [1, 2, 3]. For this, gene expression levels were also analysed for samples treated with the mixture of 100 μM genistein and 25 μM nimesulide. The fold change (FC) of gene expression levels between treated and non-treated cells was established by using three references genes, including *ACTB, SDHA* and *YWHAZ*. The constant levels of housekeeping genes listed above were assessed independently in real-time qRT-PCR experiments. The results of transcripts with altered expression were considered as modulated when the FC value was below or equal 0.7 or ≥ 1.3. Among 14 analysed genes with a crucial function in terms of LSDs pathology, almost a half of them (*ACP5, AGA, ASAH1, CTSK, HEXA* and *MANBA*) were more significantly regulated in samples treated with the mixture of genistein and nimesulide compared to samples treated with genistein only [3]. These observations might suggest the beneficial effect of nimesulide on expression of genes responsible for significant molecular processes affected in LSD. Moreover, an additive effect of the mixture of isoflavone and genistein assessed in EGFR analysis support the assumption about the valuable application of nimesulide. Results obtained for genes coding for EGFR, mTOR (mammalian target of

rapamycin, mechanistic target of rapamycin kinase) and TFEB (transcription factor EB) were found to be unchanged for both indomethacin and nimesulide treatment in comparison to genistein which is a well-known modulator of these genes. Thus, it is tempting to speculate that genistein modulates these genes independently. Importantly, the mixture of isoflavone and tested NSAIDs resulted in activation of *mTOR* and *TFEB* genes however, the level of mRNA for EGFR was unchanged. These observation confirmed the complex relationships of transcriptome response when pleiotropic substances are used.

In order to accomplish scientific purposes comprising my dissertation, the mechanism of action of selected NSAIDs and their mixture with genistein was proposed. The results obtained with the human fibroblasts model allowed to learn about a putative modulatory properties of tested substances in terms of a potential use in the treatment of LSDs. Moreover, current findings enabled to better understand the nature of flavonoids, substances which have been extensively investigated in the same culture model. It was described for the first time, that NSAIDs can be considered as an agents with the potential in the treatment of LSDs, in particular MPSs. Proposed mechanism of action of tested drugs comprising inhibition of GAGs synthesis and modulation of EGFR and PI3K pathways makes a novel input concerning the activity of NSAIDs. Observations for the mixture of tested drugs with genistein resulted in an additive effect, revealed a promising potential in a future studies of this isoflavone in terms of treatment LSDs. Furthermore, conducted investigations emphasize the safety profile of NSAIDs regarding the effects on the transcriptome of non-transformed cell line. In conclusion, all findings comprising my PhD thesis allowed to expand the knowledge concerning mechanisms of action of NSAIDs and their potential application in the modulation of metabolic pathways with the mixture of different compounds.