Critical Review

Gene Expression-targeted Isoflavone Therapy

Alicja Węgrzyn

Laboratory of Molecular Biology (affiliated with the University of Gdańsk), Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Kładki 24, 80-822 Gdańsk, Poland

Summary

Lysosomal storage diseases (LSD) form a group of inherited metabolic disorders caused by dysfunction of one of the lysosomal proteins, resulting in the accumulation of certain compounds. Although these disorders are among first genetic diseases for which specific treatments were proposed, there are still serious unsolved problems that require development of novel therapeutic procedures. An example is neuronopathy, which develops in most of LSD and cannot be treated efficiently by currently approved therapies. Recently, a new potential therapy, called gene expression-targeted isoflavone therapy (GET IT), has been proposed for a group of LSD named mucopolysaccharidoses (MPS), in which storage of incompletely degraded glycosaminoglycans (GAGs) results in severe symptoms of virtually all tissues and organs, including central nervous system. The idea of this therapy is to inhibit synthesis of GAGs by modulating expression of genes coding for enzymes involved in synthesis of these compounds. Such a modulation is possible by using isoflavones, particularly genistein, which interfere with a signal transduction process necessary for stimulation of expression of certain genes. Results of *in vitro* experiments and studies on animal models indicated a high efficiency of GET IT, including correction of behavior of affected mice. However, clinical trials, performed with soy isoflavone extracts, revealed only limited efficacy. This caused a controversy about GET IT as a potential, effective treatment of patients suffering from MPS, especially neuronopathic forms of these diseases. It this critical review, I present possible molecular mechanisms of therapeutic action of isoflavones (particularly genistein) and suggest that efficacy of GET IT might be sufficiently high when using relatively high doses of synthetic genistein (which was employed in experiments on cell cultures and mouse models) rather than low doses of soy isoflavone extracts (which were used in clinical

trials). This proposal can be tested in double-blinded, placebo-controlled clinical trials.   © 2012 IUBMB

INTRODUCTION

Lysosomal storage diseases (LSDs) are inherited metabolic disorders caused by mutations in genes coding for: (i) proteins that hydrolyze compounds that are transported into the lysosomes or appear in the lysosomes as a result of fusion with autophagic vacuoles or endocytic vesicles, (ii) proteins that are needed for the activity of the hydrolases mentioned above, such as the saponins, (iii) proteins that are needed for lysosomal transport (*1, 2*). There are more than 50 different LSDs identified to date. Defects of functions of the lysosomal proteins or their absence cause the accumulation of one or more specific compounds such as glycogen, glycosaminoglycans (GAGs), sphingolipids, cholesterol ester, and others in lysosomes and sometimes in the entire cell. This leads to dysfunctions of cells, tissues, and organs. Attempts are made to treat lysosomal storage diseases by enzyme replacement and substrate reduction therapies, as well as other potential therapies which are being developed (*2–6*).

Mucopolysaccharidoses (MPS) form a group of LSD in which accumulation of GAGs occurs (*3*). There are 11 types and subtypes of MPS, classified depending on the kind of lacking or deficient enzyme and the kind of accumulated GAG(s) (Table 1). All MPS types are severe diseases, with average life span between one and two decades. In most of them (excluding types IV and VI), particularly those in which heparan sulfate (HS) is the sole or one of accumulated GAG(s), neuronopathy develops, which includes mental deterioration, behavioral changes and other severe neurological symptoms (ref. *4* and references therein). MPS are progressive diseases; affected

Table 1

Mucopolysaccharidoses: deficient enzymes, corresponding genes, and stored GAGs

MPS type[a]	Syndrome name (OMIM ID)[b]	Enzyme (UniProt no.)[c]	Gene and its localization	Stored GAG(s)[d]
MPS I	Hurler (607014) Scheie (607016) Hurler-Scheie (607015)	α-L-iduronidase (P35475)	*IDUA*, 4p16.3	HS, DS
MPS II	Hunter (309900)	Iduronate 2-sulfatase (P22304)	*IDS*, Xq28	HS, DS
MPS IIIA	Sanfilippo A (252900)	*N*-sulfoglucosamine sulfohydrolase (Heparan *N*-sulfatase) (P51688)	*SGSH*, 17q25.3	HS
MPS IIIB	Sanfilippo B (252920)	α-*N*-actylglucosaminidase (P54802)	*NAGLU*, 17q21	HS
MPS IIIC	Sanfilippo C (252930)	Acetyl-CoA:α-glycosaminide acetyltransferase (Q68CP4)	*HGSNAT*, 8p11.1	HS
MPS IIID	Sanfilippo D (252940)	*N*-acetylglucosamine 6-sulfatase (P15586)	*GNS*, 12q14	HS
MPS IVA	Morquio A (253000)	*N*-acetylgalactosaminide 6-sulfatase (P34059)	*GALNS*, 6q24.3	KS, 6-CS
MPS IVB	Morquio B (253010)	β-galactosidase (P16278)	*GLB1*, 3p21.33	KS
MPS VI	Maroteaux-Lamy (253200)	*N*-acetylgalactosamine 4-sulfatase (arylsulfatase B) (P15848)	*ARSB*, 5q11–13	DS
MPS VII	Sly (253220)	β-glucuronidase (P08236)	*GUSB*, 7q21.11	HS, DS, 4,6-CS
MPS IX	Natowicz (601492)	Hyaluronoglucosaminidase-1 (hyaluronidase) (Q12794)	*HYAL1*, 3p21.3	HA

[a]The syndroms included in MPS I are recognized solely according to clinical features, as deficiency of the same enzyme occurs in all MPS I patients. The names MPS V and MPS VIII are no longer used (MPS V has been recognized as a clinical variant of MPS I, and MPS VIII has been originally diagnosed in a patient who apparently suffered from two other MPS types simultaneously).

[b]The ID numbers of Online Mendelian Inheritance in Man (OMIM) are provided (see: http://www.ncbi.nlm.nih.gov/omim).

[c]The names of enzymes are provided according to recommendations of International Union of Biochemistry and Molecular Biology (IUBMB), and alternative names are included if frequently used in literature. The numbers are according to UniProt (http://www.uniprot.org).

[d]The abbreviations of glycosaminoglycans (GAGs) are: 4,6-CS; chondroitin-4,6-sulfate; 6-CS, chondroitin-6-sulfate; DS, dermatan sulfate; HA, hyaluronic acid; HS, heparan sulfate; KS, keratan sulfate.

patients are born usually without any obvious symptoms, but they appear within first months or years of life and become successively more and more severe. In most patients, the life span is significantly shortened, with death occurring during the first or second decade.

THERAPEUTIC OPTIONS FOR MPS

Because MPS are caused by a lack or dysfunction of certain enzymes involved in degradation of GAGs, one obvious therapeutic strategy is to provide the lacking enzyme to cells of patients. This idea appeared promising in LSD, because a biochemical signal, a phospho-mannose, for transportation of some lysosomal enzymes to their target localization (lysosomes) has been discovered, and the possibility of uptake of such enzymes by cells from extracellular fluids has been demonstrated (for reviews, see refs. *2* and *3*). This knowledge was used to develop enzyme replacement therapy (ERT), in which a recombinant enzyme is administered intravenously and can be distributed to lysosomes located in various cells (*5*). Another way to deliver an active enzyme to cells of MPS patients is to transplant bone marrow or hematopoietic stem cells, which excrete a portion of produced lysosomal proteins, thus, the enzymes can be recognized by mannose-6-phosphate receptors on MPS cells' membranes and transported to lysosomes (*6*). Transplantation therapy can only be effective when the procedure is performed during first 2 years of life, and only in some MPS types; for example, positive effects were detected in MPS I but not in MPS III patients (*6*).

ERT is considerably more efficacious than bone marrow transplantation; however, improvement in clinical parameters was observed only in tissues other than the central nervous system. This is caused by the fact that a recombinant enzyme, when administered intravenously, cannot cross the blood-brain-barrier (*5, 6*).

Gene therapy is another option for treatment of MPS and is predicted to be the most efficient way to cure genetic diseases (*7*). However, despite many years of studies performed in different laboratories, it is still a therapy under development (*7*).

Because of problems mentioned above, there is still a need for novel therapies, suitable to treat patients suffering from MPS, especially from neurological symptoms. Understanding of molecular mechanisms of the disease led to proposals for alternative methods of treatment of MPS patients (*8*). Among them,

there is substrate reduction therapy (SRT), which decreases the efficiency of synthesis of compounds (GAGs in the case of MPS) that cannot be degraded in cells of affected organism (9). Impairment of GAG synthesis can be achieved by various ways. One example is the use of rhodamine B ([9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene]-diethylammonium chloride). This compound was found to impair GAG production in cultured cells of MPS type IIIA and MPS type VI patients, decreasing GAG storage (10). GAG levels were reduced in tissues of MPS type IIIA mice treated with 1 mg/mL rhodamine B, in which behavioral improvement was also evident (11). However, rhodamine B appears to be toxic for humans, particularly it may be harmful when swallowed or injected, and may cause irritation of skin, eyes and respiratory tract (for a review, see ref. 9). Although it was demonstrated recently that transgenerational exposure to low levels of rhodamine B did not adversely affect litter size and liver function in murine MPS type IIIA model (12), it is not known whether low doses of this compound could be effective in treatment of patients suffering from MPS.

Another possibility to reduce efficiency of GAG synthesis is to decrease levels of enzymes involved in this process. This can be achieved by the use of either siRNA or shRNA, as demonstrated recently (13, 14). However, the problems with efficient delivery of RNA molecules to cells, especially to the central nervous system, and with their stability, must be overcome before siRNA or shRNA can be used for therapies of genetic diseases, including MPS (15, 16).

GENE EXPRESSON-TARGETED ISOFLAVONE THERAPY

Limitations of the use of either rhodamine B or RNAi-based procedures indicated that another form of SRT should be developed to address the problem of efficient treatment of neuronopathic MPS types. As GAGs are composed of monosaccharides (like xylose, galactose at the reducing end of the polymer where it is connected to the serine or its protein, and others) that are used in various biochemical pathways, it is assumed that any chemical analogue of such compounds, blocking activity of certain GAG-synthesizing enzyme, might have severe adverse effects due to interference with other biochemical reactions. Theoretically, inhibitors of glucuronyl C5 epimerase, the enzyme that produces iduronic acid in HS, could be developed as potential safe drugs. However, to my knowledge, no successful studies involving such a strategy were performed to date. Therefore, another option had to be found.

Rationale

The first report suggesting a possibility for development of gene expression-targeted isoflavone therapy (GET IT; although this name was not used at that time) was based on analysis of genetic regulation of GAG turnover (17). As both synthesis and degradation of GAGs depends on activities of many enzymes, it was assumed that any variations in efficiency of expression of
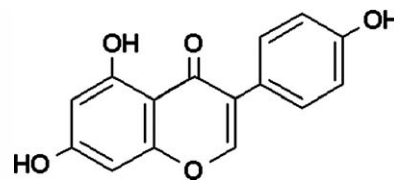

Figure 1. Structure of genistein (4′,5,7-trihydroxyisoflavone or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one).

genes coding for proteins involved in GAG synthesis should influence both severity and dynamics of progression of MPS disease. If so, determination of efficiency of GAG synthesis, in combination with measurement of residual activity of the deficient GAG-degrading enzyme, might provide a basis for prediction of severity and clinical progress of MPS (17) (which was, in fact, confirmed experimentally a few years later, see ref. 18). Apart from this, such a hypothesis led to the idea that inhibition of expression of genes coding for enzymes necessary for GAG synthesis might allow us to achieve a new balance between efficiency of GAG synthesis and degradation, disturbed in cells of MPS patients (17). The question was what compound(s) can both decrease efficiency of expression of such genes and cross the blood-brain-barrier? Analysis, of previous articles on basic mechanisms of regulation of GAG synthesis (in fact, published many years ago), suggested that genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) (Fig. 1), a compound belonging to the group of isoflavones, can be such a molecule. Addition of epidermal growth factor (EGF) to cultures of human cells caused an increase of GAG synthesis efficiency (19), and genistein was described as inhibitor of EGF receptor kinase activity (20). Moreover, genistein was found to be able to cross the blood-brain-barrier in rats with efficiency of about 10% (21). Thus, it was reasonable to test whether genistein can decrease production and storage of GAGs in MPS cells.

Experiments with Cell Cultures

The first experiments on effects of genistein on GAG production and storage were performed in cultured fibroblasts derived from patients suffering from MPS types I, II, IIIA, and IIIB (22). In all tested cultures, this isoflavone significantly impaired GAG synthesis efficiency, with a dose-response correlation. A considerable reduction in storage was determined by both biochemical reactions and electron microscopic analyses. These results were encouraging, and a potential treatment of MPS with genistein has been named GET IT, according to putative mechanism of action of this isoflavone on GAG synthesis (22). Subsequent studies confirmed that genistein-mediated inhibition of EGF receptor autophosphorylation, and resultant inefficient signal transduction, is the main, although not necessary the only one, cause of this phenomenon (23).

Recent reports indicated that genistein is not the only isoflavone able to reduce efficiency of GAG synthesis. Other isoflavones (formononetin, daidzein, biochanin A, and glycitein)

were also effective in decreasing GAG storage in fibroblasts derived from patients suffering from MPS types IIIA and VI (24). Interestingly, combinations of various isoflavones were more effective in reducing GAG storage than any one single isoflavone (24). This unexpected phenomenon of synergistic action of isoflavones might be explained by results of recent experiments, which indicated that other flavonoids, unlike genistein, can decrease efficiency of GAG synthesis in reactions independent on EGF receptor autophosphorylation (25). Therefore, one can propose that there are different mechanisms of inhibition of GAG production by genistein and other isoflavones (or flavonoids), thus, effects of mixtures of these compounds may be additive.

Studies on Animal Models

Results of *in vitro* experiments encouraged researchers to perform studies on animal models. To date, mouse models of MPS types II and IIIB were tested. In studies on MPS type II mice, treated with soy isoflavone extract at doses corresponding to either 5 or 25 mg genistein per 1 kg of body weight daily, several important effects were observed (26). Urinary GAG levels were reduced after 10 weeks of the treatment, as were GAG levels in tissue samples from liver, spleen, kidney, and heart. Importantly, in some animals, decreased GAG deposits in brain were observed after genistein treatment (26).

MPS type IIIB mice were treated with synthetic genistein in both short-term (8 weeks) and long-term (9 months) experiments. The short-term experiment indicated that genistein was safe for animals at doses ranging from 5 to 160 mg per 1 kg of body weight daily (27). A significant reduction in GAG storage in various peripheral tissues was found, and the changes depended on the genistein dose used (27). However, no statistically significant changes in GAG levels in brain were observed after treatment of animals for 8 weeks (27).

Long-term experiment with MPS type IIIB mice was focused on changes in central nervous system. Contrary to the short-term experiment, treatment of these animals with genistein for 9 months at the dose of 160 mg/kg/day resulted in significant (although far from complete) reduction of GAG storage in brain (28). Moreover, secondary symptoms of MPS, such as inflammatory reactions, ganglioside storage, and reduced expression of *VAMP2* were considerably improved in genistein-treated MPS type IIIB mice compared to untreated controls (28). Importantly, nine different behavioral parameters that were dramatically worsened in untreated animals, have been completely corrected (relative to wild-type mice) after 9-month treatment with genistein at 160 mg/kg/day (28). It is surprising that incomplete reduction of GAG storage and other biochemical and histological parameters correlated with complete correction of behavior of mice. But such results are compatible with the course of development of the disease in children, where symptoms are absent for several months or even a few years, despite unquestioned accumulation of GAGs and secondary storage

materials ongoing since birth. This suggests that significant therapeutic effects can be obtained without complete clearance of GAG from patient's cells and tissues.

It is interesting that both animal models used in studies on GET IT for MPS consisted of null mutants (26–28). Therefore, a molecular mechanism for genistien-mediated reduction in GAG storage must not be restricted to residual activity of the deficient enzyme, which in combination with impaired synthesis could lead to a decrease in amount of already accumulated material, as suspected previously for any kind of SRT (9). One may speculate that dilution of already accumulated GAGs and/or actions of unspecific GAG endoglycosidases, like heparanase, may significantly contribute to GAG clearance in MPS cells subjected to GET IT. These mechanisms might act due to either growth and divisions of affected cells or bypassing the inhibited step of GAG degradation. Irrespective of the actual mechanism, experiments on animals indicated that GET IT might be potentially efficacious not only for MPS patients in which residual enzyme activity is detectable, but also for those bearing two null alleles of the affected gene and producing no residual activity of its product.

Clinical Trials

Successes in studies on GET IT with cell cultures and animal models were rapidly followed by pilot clinical trials. This was justified by the fact that genistein is a natural product (present in many plants) and appears to be safe. It is available in soy isoflavone extracts that are widely sold in health-food stores. SRT, including GET IT, has been called a new hope for patients suffering from neuronopathic forms of LSD (29). This increased the pressure for testing this therapy in severe, debilitating and actually deadly diseases, like MPS.

The first pilot clinical study, in which GET IT has been tested, was performed on 10 pediatric patients suffering from MPS types IIIA and IIIB (30). In this open-label trial, a soy isoflavone extract was used at the dose corresponding to 5 mg genistein per 1 kg of body weight daily. After 1 year treatment, following effects were observed: (i) urinary HS (the GAG accumulated in MPS type III) level decreased significantly (from the mean value of 22 to 16 mg per 1 g of creatinine; $P = 0.028$), (ii) hair morphology (a parameter described previously as a potential biomarker in assessment of efficacy of anti-MPS treatment, see refs. 31 and 32) was significantly improved (from the mean value of hair dysmorphology of 1.9 to 0.9; $P = 0.012$), and (iii) scores in a cognitive test were higher than at baseline (the mean score at baseline was 16.5, and it increased to 19.7 after 12 months of the treatment; $P = 0.012$). These results might look promising, nevertheless, it is necessary to note that although statistical significance was achieved in analyses of differences between results measured at baseline and after 12 months of the treatment, the P values calculated in particular tests were not spectacular. Moreover, one should note that clinical improvements were observed in 7, 8 and 8 patients (out of 10 tested), depending on the tested parameters (30).

A 2-year follow-up study, which included eight patients, was performed and its results has been published recently (33). Cognitive functions and behavior were assessed, as symptoms related to functions of central nervous system are the most severe ones in MPS type III. Among eight patients, in which GET IT was used for 3 years (1 year clinical trial and 2-year follow-up) at the genistein dose of 5 mg/kg/day, an improvement of cognitive functions in seven patients and stabilization in one patient were assessed during the first year, while after the third year (2-year follow-up) further improvement was observed in two patients, stabilization in three patients and some deterioration in three patients. Monitoring of general and behavioral symptoms revealed improvement in all patients after the first year of the treatment, further improvement in five patients, and deterioration in three patients during the next 2 years. These results suggest a general stabilization of patients during 3 year GET IT; however, variability in response to this treatment among patients is obvious. One should note that even in patients that deteriorated during the therapy, the progress of the disease was slower than that expected on the basis of our general knowledge on MPS type III disease.

Another open-label clinical trial with MPS type III patients, in which GET IT was used, has been performed with the employment of different tests (34). The soy isoflavone extract at the dose corresponding to 5 mg/kg/day genistein was used, like in studies described in previous paragraphs, however, apart from measuring urinary GAG levels and assessing hair morphology, another test was employed to assess the level of disability. In this trial, a decrease in frequency of infections and gastrointestinal symptoms and improvement in skin texture and hair morphology were noted (34). However, urinary GAG levels did not change significantly and disability was not decreased. This might suggest a low efficacy of the treatment, which is in contrast to conclusions proposed on the basis of other studies (compare refs. 30, 33, and 34). One possible explanation of this discrepancy could be a genetic difference between two populations studied, Polish (30, 33) and Spanish (34). However, in my opinion, another issue may be more important. Namely, it is necessary to analyze carefully methods used for assessment of particular parameters by both groups. For example, it appears that the special disability scale, used by the authors testing Spanish patients, is proper to estimate the level of disability, but it may be of too low sensitivity to detect subtle cognitive and behavioral changes. On the other hand, the modified Brief Assessment Examination (BAE), used by the other group (30, 33), can be employed to detect only a very serious cognitive disability, but it is useful in identification of even small improvement in severely affected patients. Thus, one might expect very different results of studies on MPS patients if these two tests are employed alternatively. This discrepancy highlights general problems with choosing the most appropriate tests to assess efficacy of any treatment of MPS patients, and particularly MPS type III patients.

Different responses of various patients to GET IT, observed in studies described above, may suggest that the employed dose of genistein was not optimal, thus, some patients responded better or worse to the treatment. Therefore, the next small study, in which six MPS III patients were enrolled, was performed with the dose of the soy isoflavone extract corresponding to 10, and then 15 mg genistein per 1 kg of body weight daily (35). Improvement in the urinary GAG level and hair morphology was noted in all patients, and this improvement correlated with the increase in the genistein dose (35).

Simultaneously to the open-label study with increased genistein dose, a double-blinded, placebo-controlled clinical trial, with 10 mg/kg/day genistein has been conducted. In this trial, a cross-over has been performed after 6 months and 1 month wash out, thus, each patient was treated for half a year. Six-month follow-up study was then conducted for one group of patients. Results of this trial indicated a significant decrease of GAG levels in urine and plasma, but no clinical improvement could be detected (36). According to the authors' suggestion (36), perhaps the treatment was too short and the dose was too low to observe significant clinical changes, which might be analogous to the results of short-term studies performed with MPS type IIIB mice (compare refs. 27, 28, 30, 33–36).

All clinical studies described above were performed with patients suffering from MPS type III. However, recent report indicated that GET IT may also be beneficial for other MPS type(s), as improvement in joint mobility has been reported in adult MPS type II patients treated with a soy isoflavone extract (at the dose corresponding to 5 mg genistein/kg/day) for 6 months (37).

As soy isoflavone extracts are easily available in various countries, it is not a surprise that, in the light of a lack of other therapeutic options for MPS type III, many families started to administer such extracts to children suffering from Sanfilippo disease (MPS type III) on their own risk. However, it is important to note that this may be a risky procedure, indeed. This is not due to putative genistein-mediated adverse effects (in fact, no adverse effects of GET IT were observed in experiments on animals and clinical studies performed to date, see refs. 26–28, 30, 33–37), but because of the fact that various soy extracts contain different amounts of isoflavones. In fact, among 7 soy isoflavone products, randomly chosen and purchased in pharmacy shops, only two contained amounts of genistein which were equal or similar to those declared by manufacturers (38). In some products, genistein occurred in amounts between 4 and 200 times lower than those indicated in information sheets (38). Moreover, the products containing lower than expected genistein amounts caused an increase, rather than decrease, in the efficiency of GAG synthesis by MPS type III cells in vitro (38).

The summary of results of studies on animal models and clinical trials, performed to date with GET IT for MPS, is presented in Table 2. Following conclusions can be drawn from these studies. First, GET IT appears to be a safe treatment. Second, genistein may be a compound causing improvement of at

Table 2
Summary of animal studies and clinical trials with the use of GET IT for MPS

Study type, subjects and duration	Genistein dose	Tested parameters	Main results	Reference
Animal study, MPS II mice, duration: 10 weeks	5 or 25 mg/kg/day	GAG levels in various tissues	Reduction in GAG levels in urine, liver, spleen, kidney, heart, and brain (in some animals)	26
Animal study, MPS IIIB mice, duration: 8 weeks	5–160 mg/kg/day	GAG storage in liver and brain; hair morphology	Reduction in GAG levels in liver, but not in brain; improved hair morphology	27
Animal study, MPS IIIB mice, duration: 9 months	160 mg/kg/day	GAG levels, neuroimflammation, secondary storage and VAMP2 expression in brain; behavior	Reduction of GAG levels, neuroimflammation, storage of gangliosides and improved expression of VAMP2 in brain; complete correction of behavior	28
Open label clinical trial, 10 MPS IIIA and IIIB patients, 1 year	5 mg/kg/day	Urinary GAG level; hair morphology; cognitive functions	Decreased urinary GAG levels; improved hair morphology; improved cognitive functions	30
Open label follow-up study, 8 MPS IIIA and IIIB patients, 2 years	5 mg/kg/day	Cognitive functions; general and behavioral symptoms	Improvement, stabilization or deterioration of tested parameters, depending on patient	33
Open label clinical trial, 19 MPS IIIA, IIIB and IIIC patients, 1 year	5 mg/kg/day	Urinary GAG level; hair morphology; disability; general clinical parameters	No decrease in urinary GAG level; stabilization or worsening in disability score; improved hair morphology; less frequent infections and gastrointestinal symptoms	34
Open-label clinical trial, 6 MPS IIIA and IIIB patients, 1 year	10 and then 15 mg/kg/day	Urinary GAG level; hair morphology	Reduced urinary GAG level; improved hair morphology	35
Randomized, double-blinded, placebo- controlled, cross-over clinical trial, 30 MPS IIIA, IIIB and IIIC patients, 19 months	10 mg/kg/day	GAG levels in urine and plasma; behavior; hair morphology	Decreased levels of GAGs in urine and plasma; no significant changes in behavior; no changes in hair morphology	36
Open-label clinical trial, 7 MPS II patients, 6 months	5 mg/kg/day	Joint range of motion	Improved shoulder range of motion; improved motion of elbow and wrist (in some patients); improved motion of lower extremity joints (in some patients)	37

least some biological markers of MPS. Third, GET IT may lead to either stabilization of the disease or even improvement in at least some patients suffering from MPS types II and III. Fourth, there is a huge variability in the response to GET IT among patients. Fifth, doses of genistein significantly higher than those used in clinical studies performed to date may be considerably more effective in GET IT for MPS. Sixth, it is an urgent need for a double-blinded, placebo-controlled clinical trial with synthetic genistein (like in studies on animals) used at high doses (perhaps as high as 100–150 mg/kg/day, which were effective in studies on MPS type IIIB mice and did not cause any adverse effects).
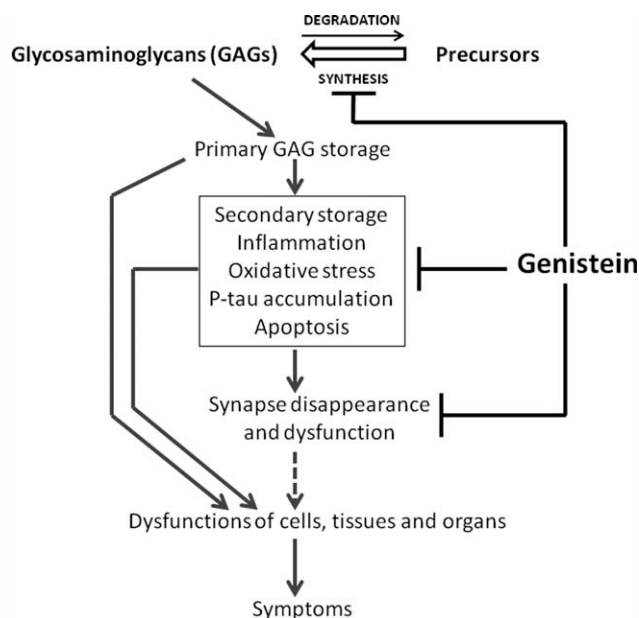
Figure 2. Experimentally confirmed and putative mechanisms of genistein action in GET IT for MPS (the disease in which degradation of glycosaminoglycans, GAGs, is impaired and storage of these compounds is the primary cause of symptoms). Gray arrows indicate stimulation, while blunt-ended black lines indicate inhibition of particular reactions or processes.

Secondary Roles of Genistein in Treatment of MPS

Although it appears that the primary action of genistein in GET IT for MPS is inhibition of GAG synthesis through impairment of EGF-mediated signal transduction (23), one may ask whether it is the sole mechanism of efficacy of this therapy. This question is substantiated especially in the light of various biological activities of genistein, described previously (for reviews see refs. 39–41). Moreover, results of recent studies on pathomechanisms of MPS led to proposals that not only primary GAG storage but also secondary reactions are crucial for development of symptoms characteristic for this disease. These include secondary storage of gangliosides, accumulation of hyperphosphorylated tau protein (P-tau), oxidative stress, inflammation, cytotoxicity, and apoptosis (42–45).

Considering the complex pathomechanism of MPS and a large spectrum of genistein activities, one may assume that this isoflavone can be beneficial in therapy of this disease due to both its primary action as indirect GAG synthesis inhibitor and its secondary, MPS-unspecific, effects. These include decreasing of ganglioside storage (28), neuroprotection against P-tau-induced neurotoxicity (46), attenuation of oxidative stress in brain (47), prevention of neuroinflammation (48), and inhibition of apoptosis in neuronal cells (49).

It might be intriguing that improvement in cognitive functions was observed in some severely affected MPS type III patients subjected to GET IT (30, 33). One might suppose

that already lost cognitive functions could not be restored due to potentially irreversible neurodegeneration, which is apparently not the case, as shown experimentally in studies with animals and patients (28, 30, 33). However, findings that genistein may stimulate expression of genes coding for VAMP2 (28) and synaptophysin (50) may provide a rationale for explaining such effects. These proteins are involved in formation and functioning of synapses, and synthesis of VAMP2 is impaired in MPS type III cells (28). Therefore, I suggest that by facilitating synapse formation and function, genistein is capable of not only protecting but also improving cognitive abilities of MPS patients, even those in which deterioration of brain functions was evident. Mechanisms of genistein-mediated therapeutic action in MPS patients, both confirmed experimentally and putative, are summarized schematically in Fig. 2.

CONCLUDING REMARKS AND FURTHER PERSPECTIVE

In vitro experiments with cell cultures and studies on animal models suggested that GET IT may be a safe and effective therapeutic option for patients suffering from MPS. However, clinical trials, in which soy isoflavone extracts were used, suggested a limited efficacy. Still, GET IT remains a hope for MPS patients, particularly those with severe neurological symptoms, especially in the light of findings that indicated the complete correction of behavior of MPS type IIIB mice. The main difference between highly efficient action of genistein in MPS cell cultures or MPS type IIIB mice and its limited efficacy in clinical trials was the use of high doses (up to 160 mg/kg/day) of synthetic genistein in former studies and low doses (5–15 mg/kg/day) of soy isoflavone extracts in latter studies. Therefore, I suggest that efficacy of GET IT might be potentially improved when using relatively high doses of synthetic genistein. This proposal can be tested in double-blinded, placebo-controlled clinical trials. Moreover, one may speculate that combination of GET IT with other treatments can be optimal for MPS patients due to different mechanisms of action of therapeutic molecules employed in various procedures, and possible cumulative positive effects. For example, combination of GET IT and ERT might lead to rapid clearance of previously accumulated GAGs by a recombinant enzyme in easily reached tissues and organs, together with reduction of storage in those which are hardly accessed by enzyme but reachable by small molecules (*e.g.*, genistein), like brain or bones; at the same time, GET IT-mediated impaired synthesis of GAGs could facilitate clearance of these compounds by ERT even in the former group of tissues and organs.

REFERENCES

1. Swarts, K. (2009) *Genetic Disorders*. Greenhaven Press, Farmington Hills, MI.

2. Barranger, J. A. and Cabrera-Salazar, M. (2007) *Lysosomal Storage Disorders*. Springer, Berlin-Heidelberg.

3. Neufeld, E. F. and Muenzer, J. (2001) Mucopolysaccharidoses. In: *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly. W. S., and Valle, D., eds.). pp. 3421–3452, McGraw-Hill Co., New York.

4. Węgrzyn, G., Jakóbkiewicz-Banecka, J., Narajczyk, M., Wiśniewski, A., Piotrowska, E., et al. (2010) Why are behaviors of children suffering from various neuronopathic types of mucopolysaccharidoses different? *Med. Hypoth.* **75**, 605–609.

5. Wraith, J. E. (2009) Enzyme replacement therapy for the management of the mucopolysaccharidoses. *Int. J. Clin. Pharmacol. Ther.* **47** (Suppl 1), S63–S65.

6. Beck, M. (2010) Therapy for lysosomal storage disorders. *IUBMB Life* **62**, 33–40.

7. Cotrim, A. P. and Baum, B. J. (2008) Gene therapy: some history, applications, problems, and prospects. *Toxicol. Pathol.* **36**, 97–103.

8. Schultz, M. L., Tecedor, L., Chang, M., and Davidson, B. L. (2011) Clarifying lysosomal storage diseases. *Trends Neurosci.* **34**, 401–410.

9. Jakóbkiewicz-Banecka, J., Piotrowska, E., Gabig-Cimińska, M., Borysiewicz, E., Słomińska-Wojewódzka, M., et al. (2011) Substrate reduction therapies for mucopolysaccharidoses. *Curr. Pharm. Biotechnol.* **12**, 1860–1865.

10. Roberts, A. L., Thomas, B. J., Wilkinson, A. S., Fletcher, J. M., and Byers, S. (2006) Inhibition of glycosaminoglycan synthesis using a rhodamine B in a mouse model of mucopolysaccharidosis type IIIA. *Pediatr. Res.* **60**, 309–314.

11. Roberts, A. L., Rees, M. H., Klebe, S. Fletcher, J. M., and Byers, S. (2007) Improvement in behaviour after substrate deprivation therapy with rhodamine B in a mouse model of MPS IIIA. *Mol. Genet. Metab.* **92**, 115–121.

12. Roberts, A. L., Fletcher, J. M., Moore, L., and Byers, S. (2010) Transgenerational exposure to low levels of rhodamine B does not adversely affect litter size or liver function in murine mucopolysaccharidosis type IIIA. *Mol. Genet. Metab.* **101**, 208–213.

13. Dziedzic, D., Węgrzyn, G., and Jakóbkiewicz-Banecka, J. (2010) Impairment of glycosaminoglycan synthesis in mucopolysaccharidosis type IIIA cells by using siRNA: a potential therapeutic approach for Sanfilippo disease. *Eur. J. Hum. Genet.* **18**, 200–205.

14. Kaidonis, X., Liaw, W. C., Roberts, A. D., Ly, M., Anson, D., et al. (2010) Gene silencing of EXTL2 and EXTL3 as a substrate deprivation therapy for heparan sulfate storing mucopolysaccharidoses. *Eur. J. Hum. Genet.* **18**, 194–199.

15. Smith, R. A., Miller, T. M., Yamanaka, K., Monia, B. P., Condon, T. P., et al. (2006) Antisense oligonucleotide therapy for neurodegenerative disease. *J. Clin. Invest.* **116**, 2290–2296.

16. Juliano, R., Alam, M. R., Dixit, V., and Kang H. (2008) Mechanism and strategies for effective delivery of antisense and siRNA oligonucleotides. *Nucleic Acids Res.* **36**, 4158–4171.

17. Węgrzyn, G., Węgrzyn, A., and Tylki-Szymańska, A. (2004) A general model for genetic regulation of turnover of glycosaminoglycans suggests a possible procedure for prediction of severity and clinical progress of mucopolysaccharidoses. *Med. Hypoth.* **62**, 986–992.

18. Piotrowska, E., Jakóbkiewicz-Banecka, J., Tylki-Szymańska, A., Czartoryska, B., Węgrzyn, A., et al. (2009) Correlation between severity of mucopolysaccharidoses and combination of the residual enzyme activity and efficiency of glycosaminoglycan synthesis. *Acta Paediatr.* **98**, 743–749.

19. Pisano, M. M. and Greene, R. M. (1987) Epidermal growth factor potentiates the induction of ornithine decarboxylase activity by prostaglandins in embryonic palate mesenchymal cells: effects on cell proliferation and glycosaminoglycan synthesis. *Dev. Biol.* **122**, 419–431.

20. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., et al. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J. Biol. Chem.* **262**, 5592–5595.

21. Tsai, T. H. (2005) Concurrent measurement of unbound genistein in the blood, brain and bile of anesthetized rats using microdialysis and its pharmacokinetic application. *J. Chromatogr. A* **1073**, 317–322.

22. Piotrowska, E., Jakóbkiewicz-Banecka, J., Barańska, S., Tylki-Szymańska, A., Czartoryska, B., et al. (2006) Genistein-mediated inhibition of glycosaminoglycan synthesis as a basis for gene expression-targeted isoflavone therapy for mucopolysaccharidoses. *Eur. J. Hum. Genet.* **14**, 846–852.

23. Jakóbkiewicz-Banecka, J., Piotrowska, E., Narajczyk, M., Barańska, S., and Węgrzyn, G. (2009) Genistein-mediated inhibition of glycosaminoglycan synthesis, which corrects storage in cells of patients suffering from mucopolysaccharidoses, acts by influencing an epidermal growth factor-dependent pathway. *J. Biomed. Sci.* **16**, 26.

24. Arfi, A., Richard, M., Gandolphe, C., and Scherman, D. (2010) Storage correction in cells of patients suffering from mucopolysaccharidoses types IIIA and VII after treatment with genistein and other isoflavones. *J. Inherit. Metab. Dis.* **33**, 61–67.

25. Kloska, A., Jakóbkiewicz-Banecka, J., Narajczyk, M., Banecka-Majkutewicz, Z., and Węgrzyn, G. (2011) Effects of flavonoids on glycosaminoglycan synthesis: implications for substrate reduction therapy in Sanfilippo disease and other mucopolysaccharidoses. *Metab. Brain Dis.* **26**, 1–8.

26. Friso, A., Tomanin, R., Salvalaio, M., and Scarpa, M. (2010) Genistein reduces glycosaminoglycan levels in a mouse model of mucopolysaccharidosis type II. *Br. J. Pharmacol.* **159**, 1082–1091.

27. Malinowska M., Wilkinson, F. L., Bennett, W., Langford-Smith, K. J., O'Leary, H. A., et al. (2009) Genistein reduces lysosomal storage in peripheral tissues of mucopolysaccharide IIIB mice. *Mol. Genet. Metab.* **98**, 235–242.

28. Malinowska, M., Wilkinson, F. L., Langford-Smith, K. J., Langford-Smith, A., Brown, J. R., et al. (2010) Genistein improves neuropathology and corrects behaviour in a mouse model of neurodegenerative metabolic disease. *PLoS One* **5**, e14192.

29. Jakóbkiewicz-Banecka. J., Węgrzyn, A., and Węgrzyn, G. (2007) Substrate deprivation therapy: a new hope for patients suffering from neuronopathic forms of inherited lysosomal storage diseases. *J. Appl. Genet.* **48**, 383–388.

30. Piotrowska, E., Jakóbkiewicz-Banecka, J., Tylki-Szymańska, A., Liberek, A., Maryniak, A., et al. (2008) The use of genistein-rich isoflavone extract in substrate reduction therapy for Sanfilippo disease: open-label, pilot study in 10 pediatric patients. *Curr. Ther. Res. Clin. Exp.* **69**, 166–179.

31. Kloska, A., Bohdanowicz, J., Konopa, G., Tylki-Szymańska, A., Jakóbkiewicz-Banecka, J., et al. (2005) Changes in hair morphology of mucopolysaccharidosis I patients treated with recombinant human α-L-iduronidase (laronidase, Aldurazyme). *Am. J. Med. Genet. A* **139**, 199–203.

32. Malinowska, M., Jakóbkiewicz-Banecka, J., Kloska, A., Tylki-Szymańska, A., and Czartoryska, B. (2007) Abnormalities in the hair morphology of patients of same but not all types of mucopolysaccharidoses. *Eur. J. Pediatr.* **167**, 203–209.

33. Piotrowska, E., Jakobkiewicz-Banecka, J., Maryniak, A., Tylki-Szymańska, A., Puk, E., et al. (2011) Two-year follow-up of Sanfilippo disease patients treated with a genistein-rich isoflavone extract: assessment of effects on cognitive functions and general status of patients. *Med. Sci. Monit.* **17**, CR196–CR202.

34. Delgadillo, V., del Mar O'Callaghan, M., Artuch, R., Montero, R., and Pineda, M. (2011) Genistein supplementation in patients affected by Sanfilippo disease. *J. Inherit. Metab. Dis.* **34**, 1039–1044.

35. Malinova, V., Węgrzyn, G., and Narajczyk, M. (2012) The use of elevated doses of genistein-rich soy extract in the gene expression-targeted isoflavone therapy (GET IT) for Sanfilippo disease patients. *JIMD Rep.*, in press (DOI: 10.1007/8904_2011_87).

36. de Ruijter, J., Valstar, M. J., Narajczyk, M., Węgrzyn, G., Kulik, W., et al.(2012) Genistein in Sanfilippo disease: a randomized controlled cross-over trial. *Ann. Neurol.* **71**, 110–120.

37. Marucha, J., Tylki-Szymańska, A., Jakóbkiewicz-Banecka, J., Piotrowska, E., Kloska, A., et al.(2011) Improvement in the range of joint motion in seven patients with mucopolysaccharidosis type II during experimental gene expression-targeted isoflavone therapy (GET IT). *Am. J. Med. Genet. A* **155**, 2257–2262.

38. Piotrowska, E., Jakóbkiewicz-Banecka, J., and Węgrzyn, G. (2010) Different amounts of isoflavones in various commercially available soy extracts in the light of gene expression-targeted isoflavone therapy. *Phytother. Res.* **24** (Suppl 1), S109–S113.

39. Szkudelska, K. and Nogowski, L. (2007) Genistein—a dietary compound inducing hormonal and metabolic changes. *J. Steroid Biochem. Mol. Biol.* **105**, 37–45.

40. Rusin, A., Krawczyk, Z., Grynkiewicz, G., Gogler, A., Zawisza-Puchałka, J., et al.(2010) Synthetic derivatives of genistein, their properties and possible applications. *Acta Biochim. Pol.* **57**, 23–34.

41. Węgrzyn, G., Jakóbkiewicz-Banecka, J., Gabig-Cimińska, M., Piotrowska, E., Narajczyk, M., et al.(2010) Genistein: a natural isoflavone with a potential for treatment of genetic diseases. *Biochem. Soc. Trans.* **38**, 695–701.

42. Hamano, K., Hayashi, M., Shioda, K., Fukatsu, R., and Mizutani, S. (2008) Mechanisms of neurodegeneration in mucopolysaccharidoses II and IIIB: analysis of human brain tissue. *Acta Neuropathol.* **115**, 547–559.

43. Villani, G. R., Gargiulo, N., Faraonio, R., Castaldo, S., Gonzalez, Y., et al.(2007) Cytokines, nuerotrophins, and oxidative stress in brain disease from mucopolysaccharidosis IIIB. *J. Neurosci. Res.* **85**, 612–622.

44. Villani, G. R., Di Domenico, C., Musella, A., Cecere, F., Di Napoli, D., et al.(2009) Mucopolysaccharisosis IIIB: oxidative damage and cytotoxic cell involvement in the neuronal pathogenesis. *Brain Res.* **1279**, 99–108.

45. Ohmi, K., Kudo, L. C., Ryazantsev, S., Zhao, H. Z., Karsten, S. L., et al.(2009) Sanfilippo syndrome type B, a lysosomal storage disease, is also a tauopathy. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 8332–8337.

46. Bang, O. H., Hong, H. S., Kim, D. H., Kim, H., Boo, J. H., et al.(2004) Neuroprotective effect of genistein against beta amyloid-induces neurotoxicity. *Neurobiol. Dis.* **16**, 21–28.

47. Liang, H. W., Qiu, S. F., Shen, J., Sun, L. N., and Wang, J. Y. (2008) Genistein attenuates oxidative stress and neuronal damage following transient global cerebral ischemia in rat hippocampus. *Neurosci. Lett.* **438**, 116–120.

48. Valles, S. L., Dolz-Gaiton, P., Gambini, J., Borras, C., Loret, A. L., et al.(2010) Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression cultured astrocytes. *Brain Res.* **1312**, 138–144.

49. Kajta, M., Domin, H., Grynkiewicz, G., and Lason, W. (2007) Genistein inhibits glutamate-induced apoptotic processes in primary neuronal cell cultures: an involvement of aryl hydrocarbon receptor and estrogen receptor/glycogen synthase kinase-3beta intracellular signaling pathway. *Neuroscience* **145**, 592–604.

50. Chindewa, R., Lapanantasin, S., Sanvarinda, Y., and Chongthammakun, S. (2007) Genistein effects on synaptophysin expression in rat hippocampal neurons. *Ann. Microsc.* **7**, 95–101.