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REVIEW

Putative Biological Mechanisms of Efficiency of Substrate Reduction Therapies for Mucopolysaccharidoses

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Abstract Mucopolysaccharidoses (MPS) are inherited metabolic diseases caused by mutations in genes coding for lysosomal enzymes involved in the degradation of glycosaminoglycans (GAGs). Dysfunction of any of these enzymes results in the accumulation of GAGs, which leads to severe clinical symptoms and significantly shortened life span. Several kinds of therapies have been proposed to treat MPS, including bone marrow or stem cell transplantation, enzyme replacement therapy, and gene therapy. Another option is substrate reduction therapy (SRT), in which synthesis of GAGs is inhibited. Recent studies employing in vitro and animal models suggested that this therapy may be efficient in decreasing levels of GAGs in MPS cells, including those bearing two null alleles of the affected gene. Results of behavioral tests in animals as well as some preliminary clinical observations with pediatric patients corroborated the suggestions about possible efficacy of SRT in MPS treatment, including brain functions. Efficient reduction of GAG levels in MPS cells homozygous for null mutations may be intriguing in the commonly accepted scheme of SRT mode of action. In this paper, we propose an explanation of this phenomenon, based on already known facts. Thus, we suggest that SRT may lead to reduction of GAG levels in MPS cells due to inhibition of efficiency of GAG synthesis combined with (a) any read-through of the stop codon, (b) dilution of already accumulated GAGs due to cell growth followed by cell divisions, and (c) action of endoglycosidases degrading GAGs, e.g., heparanase, in combination with functional GAG-specific hydrolases.

Keywords Lysosomal storage diseases · Mucopolysaccharidoses · Substrate reduction therapy · Gene expression-targeted isoflavone therapy · Genistein · Glycosaminoglycans

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Introduction: Overview on Mucopolysaccharidoses

Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases (LSD), inherited metabolic disorders caused by dysfunctions of enzymes involved in the degradation of certain compounds or their transport through lysosomal membranes (Schultz et al. 2011). MPS are characterized by accumulation of glycosaminoglycans (GAGs) (formerly called mucopolysaccharides) caused by mutations in genes coding for enzymes involved in the degradation of GAGs (for a review, see Neufeld and Muenzer 2001). Impaired hydrolysis of these compounds [particularly dermatan sulfate (DS), heparan sulfate (HS), or keratan sulfate (KS)] results in their storage in virtually all cells of the affected organism (Dorfman 1964; Van Gemund et al. 1971; Benson et al. 1972; Cain et al. 1977; for a review, see Neufeld and Muenzer 2001). This causes

a progressive damage of tissues, leading to severe dysfunctions of various organs, including the heart, respiratory system, bones, joints, and central nervous system (CNS). In most cases, MPS are fatal diseases, with severe symptoms and significantly shortened life span, though patients with milder forms have been described (summarized by Neufeld and Muenzer 2001).

Depending on the nature of lacking or defective enzyme and the kind(s) of stored GAG(s), 11 types and subtypes of MPS have been distinguished. Interestingly, despite the fact that a defect in particular enzyme is responsible for particular MPS type, an enormously large variability in the spectrum and severity of symptoms occurs in each MPS type (Neufeld and Muenzer 2001). This contributes significantly to serious problems with prediction of progress and severity of the disease in newly diagnosed patients, as well as with establishing adequate tests for monitoring efficacy of treatments with newly developed therapies for MPS (Wegrzyn et al. 2004).

Current Therapeutic Options for MPS

Despite extensive studies on the development of novel therapies for MPS are being conducted, only a couple of therapeutic options is currently available to patients as approved procedures. These are bone marrow (BMT) or hematopoietic stem cells' (HSCT) transplantations and enzyme replacement therapy (ERT). Since placebo-controlled clinical trials with BMT or HSCT for MPS were not performed, our knowledge on this type of treatment comes mostly from anecdotal descriptions. Nevertheless, it appears that stabilization of the disease or slowing down of the disease progress can be obtained after transplantation in some MPS types, especially MPS I (de Ru et al. 2011). However, it has been suggested that some other MPS types, like MPS III, are generally significantly less responsive to BMT and HSCT (Lau et al. 2010 and references therein). Moreover, positive results in MPS I can be expected only if transplantation was performed in relatively young patients, i.e., below 2.5 years of age (summarized by de Ru et al. 2011).

ERT is currently used in a clinical practice for only three MPS types: I, II and VI (Clarke 2008; Wraith 2008; Prasad and Kurtzberg 2010). Unfortunately, despite an unquestioned success of ERT in treating of some MPS symptoms and halting the progress of deterioration of some somatic organs (though a high variability of efficacy occurs in different patients), this therapy also has important restrictions, like a highly limited ability of the intravenously administered recombinant enzyme to cross the blood–brain-barrier and thus, a lack of efficacy in the treatment of neurological symptoms (Wraith 2008).

Intrathecal ERT has been tested in some patients with quite encouraging results, especially in the treatment of spinal cord compression (Munoz-Rojas et al. 2008, 2010); however, these experimental treatments were performed with either adult or non-neuronopathic pediatric patients. In this light, it is important that there are questions about safety of this procedure in children, especially those expressing hyperactivity and being on a high risk during anesthetic procedures, like most pediatric MPS patients. One should also consider immunological problems of ERT, i.e., inactivation of the therapeutic enzyme by antibodies produced by a patient, especially if two null alleles of the defective gene occur in his/her genome (Ponder 2008).

Although gene therapy remains a hope for MPS patients, it is still a treatment under development (Cotrim and Baum 2008; Beck 2010; Anson et al. 2011). Therefore, there is a continuous need for alternative therapies, which could be helpful for patients suffering from various MPS types.

Substrate Reduction Therapies (SRPs) for MPS

Since MPS are caused by the accumulation of GAGs due to their inefficient degradation, it was assumed that a decrease in efficiency of synthesis of these compounds might lead to slowing down the storage process and improvement of cell functions (Wegrzyn et al. 2004). If working ideally, this kind of treatment could restore the balance between GAG synthesis and degradation, already lost in the MPS cells. This therapeutic concept is known as SRT. In fact, SRT has been introduced as a therapeutic option for treatment of other LSD, such as Gaucher disease (Weinreb et al. 2005) or Niemann-Pick C disease (Patterson et al. 2007). The product called miglustat (*N*-butyldeoxynojirimycin), an inhibitor of glucosylceramide synthase, has been approved as a drug for the above-mentioned diseases. Since it slows down the production of glycosphingolipids, it should be effective in reducing storage of these compounds. Although positive effects of such treatment in clinical trials were reported (Elstein et al. 2004; Pastores et al. 2005), its efficacy in Gaucher disease has recently been questioned (Tylki-Szymanska et al. 2011; Machaczka et al. 2012).

There are various possible means of reducing the efficiency of GAG synthesis. The most straight-forward method is the use of specific chemical inhibitors of GAG synthesis. However, finding of non-toxic inhibitors of enzymes involved in GAG production is extremely difficult, and was not successful to date. Therefore, a search for compounds inhibiting GAG synthesis indirectly has been performed (for review, see Jakobkiewicz-Banecka et al. 2007).

Rhodamine B ([9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene]-diethylammonium chloride), a compound

used as a staining fluorescent dye, has been demonstrated to reduce GAG synthesis rates indirectly in cultures of MPS IIIA and MPS VI cells (Roberts et al. 2006). Genistein (5, 7-dihydroxy-3-[4-hydroxyphenyl]-4H-1-benzopyran-4-one), a natural isoflavone occurring in many plants, significantly inhibited GAG synthesis when added to cultures of fibroblasts of MPS I, MPS II, MPS IIIA and MPS IIIB patients (Piotrowska et al. 2006). In contrary to rhodamine B, whose detailed mechanism of action remains unknown, it was possible to learn about the way by which genistein slows the GAG synthesis down. Epidermal growth factor (EGF) is a ligand, whose binding by its specific receptor (EGF receptor, EGFR) triggers a signal transduction pathway resulting in the activation of expression of certain genes, including those coding for enzymes required for GAG synthesis. Genistein was found to interfere with this pathway by inhibiting phosphorylation of EGFR (Jakobkiewicz-Banecka et al. 2009). Therefore, the potential therapy based on the use of genistein as an inhibitor of the EGF-mediated signal transduction pathway has been named 'gene expression-targeted isoflavone therapy' (GET IT) (Jakobkiewicz-Banecka et al. 2009; Malinowska et al. 2009; Piotrowska et al. 2011).

Specific silencing of expression of genes coding for GAG synthetases is another mean of reducing the efficiency of production of these compounds. In fact, two strategies based on such an idea have been developed, both employing the RNA interference (RNAi) mechanism. First, short hairpin RNA (shRNA) molecules, specific to *EXTL2* and *EXTL3* genes, were used (Kaidonis et al. 2010). Second, short interfering RNA (siRNA) oligonucleotides were employed to reduce mRNA levels of four genes (*XYLT1*, *XYLT2*, *GALTI*, and *GALTII*) whose products are required for GAG synthesis (Dziedzic et al. 2010).

Efficacy of SRT in In Vitro Studies, Experiments with Animal Models, and During Preliminary Clinical Trials

Irrespective of which method was used to inhibit GAG synthesis (treatment of cells with rhodamine B, genistein, shRNA or siRNA), the results of experiments performed in vitro with cell cultures or in vivo with animal models were quite surprising. Namely, not only a reduced rate of GAGs production and inhibition of their further accumulation was observed but also a significant decrease in the storage was evident. These studies are summarized briefly below.

Addition of rhodamine B into cultures of MPS IIIA and MPS VI fibroblasts resulted in a significant decrease in lysosomal storage of GAGs (Roberts et al. 2006). Rhodamine B was administered to MPS IIIA mice at the dose of 1 mg/ml, and GAG levels were reduced relative to untreated animals. Particularly, liver size, total GAG content, and lysosomal GAG were reduced as was urinary GAG excretion. Lysosomal GAG content in the brain was also significantly reduced by this treatment (Roberts et al. 2006). MPS IIIA mice treated with rhodamine B, in contrary to control MPS IIIA mice, improved performance toward normal animals (Roberts et al. 2007).

Genistein at concentrations between 10 and 30 μM inhibited GAG synthesis and reduced lysosomal GAG storage (Piotrowska et al. 2006). Some other flavonoids were also able to decrease GAG storage in MPS IIIA, IIIB, and VII cells (Arfi et al. 2010; Piotrowska et al. 2010; Kloska et al. 2011). Similar to rhodamine B, genistein caused a marked improvement in treated MPS IIIB mice, particularly in decreasing GAG storage in various organs (Malinowska et al. 2009) and in correction of otherwise severely changed behavior (Malinowska et al. 2010). The same tendency of improvement was observed in MPS II mice (Friso et al. 2010).

Lysosomal GAG levels were reduced in MPS I and MPS IIIA fibroblasts treated with *EXTL2*- and *EXTL3*-specific shRNAs (Kaidonis et al. 2010). Positive effects were also observed in MPS IIIA cells in experiment with siRNA-mediated silencing of expression of *XYLT1*, *XYLT2*, *GALTI*, and *GALTII* genes (Dziedzic et al. 2010).

Efficacy in pre-clinical studies and a lack of toxicity of genistein in experiments summarized above encouraged researchers and clinicians to perform pilot clinical studies on the use of GET IT in the treatment of patients suffering from MPS. In the first open-label study, 10 patients diagnosed as MPS IIIA or IIIB were treated for 12 months with a genistein-rich soy isoflavone extract at the dose corresponding to 5 mg genistein/kg of body weight daily (Piotrowska et al. 2008). This treatment, in which no adverse effects were noted, resulted in a statistically significant improvement in all tested parameters (Piotrowska et al. 2008). Particularly, urinary GAG levels were reduced, hair morphology (which may be a useful parameter in monitoring efficacy of treatment of patients suffering from various MPS types; Kloska et al. 2005; Wegrzyn et al. 2007; Malinowska et al. 2008) improved, and patients got higher scores in the psychological test by which cognitive functions could be assessed (Piotrowska et al. 2008). Nevertheless, one must note that the positive changes were not dramatic, occurred in only a fraction of patients, and the measured parameters did not reach values estimated for healthy children. The 2-year follow-up study generally confirmed a potential usefulness of GET IT in the treatment of MPS III patients; however, it also confirmed a limited efficacy of the treatment at least with the use of low genistein doses (Piotrowska et al. 2011). Two other studies with MPS III patients indicated that some disease-linked parameters and symptoms (like hair morphology

and urinary GAG level) could be improved during GET IT (Malinova et al. 2012), while changes in other parameters (like a disability score) were not observed (Delgadillo et al. 2011). Nevertheless, results of very recent double-blinded placebo-controlled clinical trial, which evaluated efficacy of GET IT for MPS III, indicated that despite the fact that a statistically significant decrease in GAG levels in urine and plasma was found when genistein was administered at the dose of 10 mg/kg/day, no statistically significant clinical improvement could be observed during 6-month treatment (de Ruijter et al. 2012). Therefore, it was suspected that higher doses of genistein might be considerably more efficacious (Wegrzyn 2012), especially since the most positive effects were reported in mice at genistein dose of 160 mg/kg/day (Malinowska et al. 2010), which is more than 10 times higher than the highest dose used to date in published human studies (Malinova et al. 2012). On the other hand, recent clinical report indicated that not only MPS III but also MPS II patients may potentially benefit from GET IT as a special kind of SRT (Marucha et al. 2011). An important finding is a lack of adverse effects in all the studies on SRT for MPS reported to date and cited above.

Putative Mechanisms of SRT-Mediated Decreasing of GAG Levels in MPS Cells

Results summarized in the preceding section, especially those obtained in experiments with cell cultures and animal models, were quite unexpected. Namely, based on the general principle of SRT, one should expect rather inhibition or slowing down of further accumulation of GAGs and thus, slowing down the disease progress, rather than a decrease in GAG storage and improvement of both MPS cells and animals. Therefore, the question appeared how could SRT lead to GAG storage decrease?, which was especially unexpected when two null alleles were present in genomes of affected individuals. In fact, the studies described in the previous section contained many such cases.

To answer this question, it is necessary to look at the mechanism of GAG degradation, which is schematically depicted in Fig. 1, showing HS degradation pathway as an example. In the generally accepted model of breakdown of this complex polysaccharide, there are sequential reactions of either chemical modifications of the external sugar unit or cutting off the whole external sugar unit. Therefore, if one of the enzymes involved in the degradation process is deficient, the whole pathway is blocked and undegraded metabolites accumulate (for recent overviews and discussion, see Wegrzyn et al. 2010; Jakobkiewicz-Banecka et al. 2011).

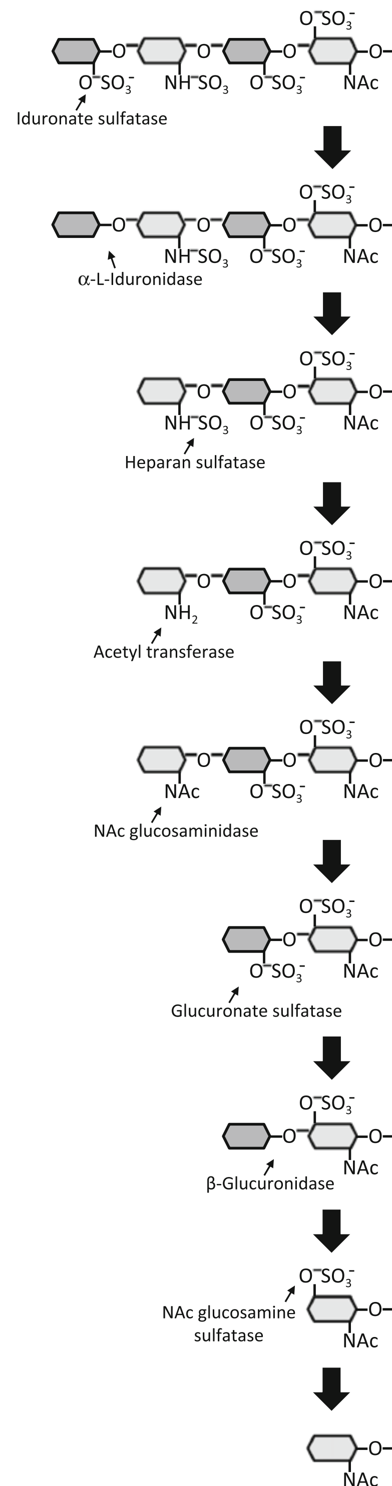

Fig. 1 A scheme for degradation of heparan sulfate, with indicated enzymes catalyzing particular reactions. Note that the picture is schematic, and chemical moieties are not necessary presented with all details. This scheme is based on figures published by Neufeld and Muenzer (2001) and Wegrzyn et al. (2010)

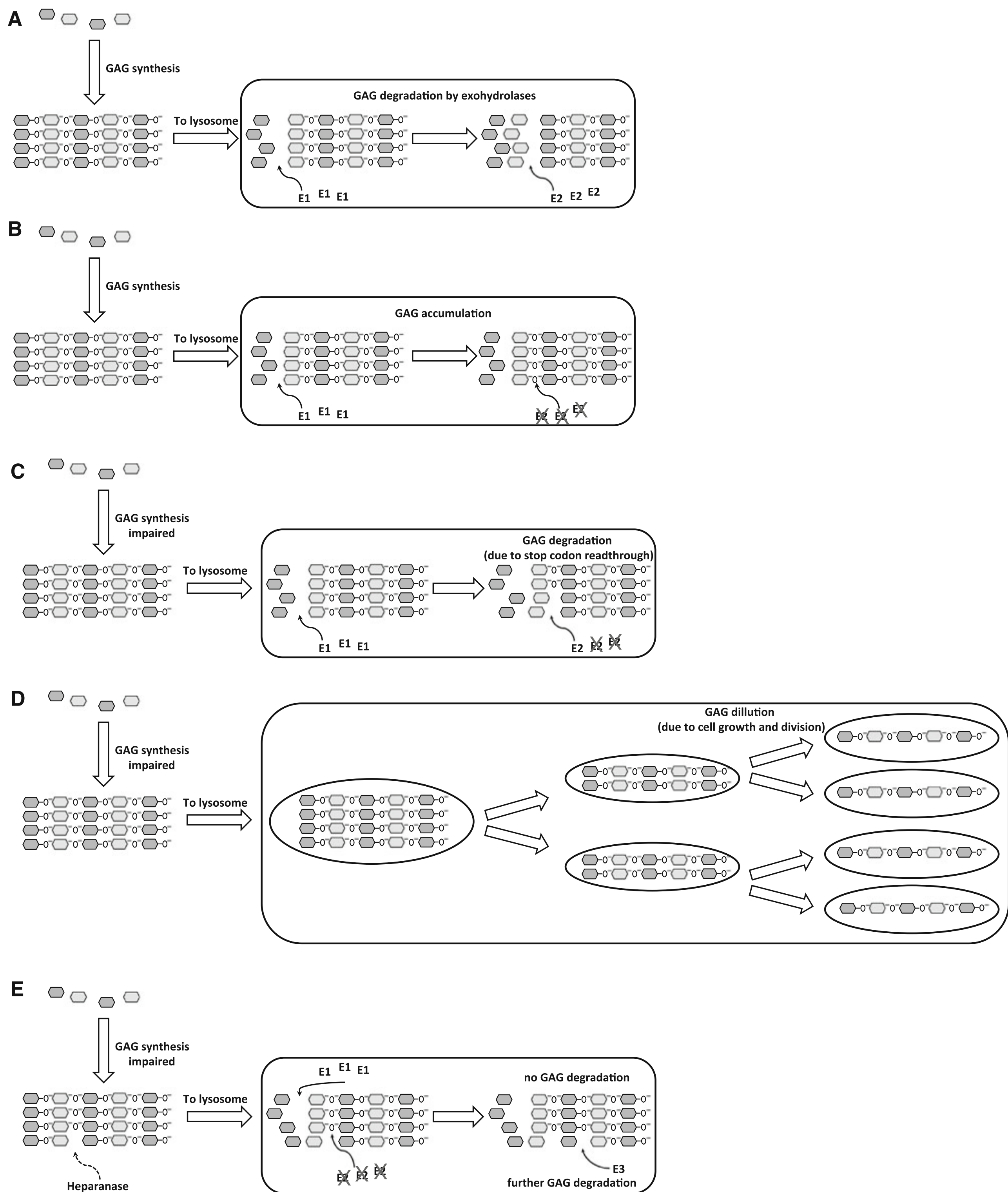

Fig. 2 The putative mechanisms of efficacy of SRT in the treatment of MPS III. **a** A normal situation in a healthy cell, where intensive GAG synthesis is in balance with effective GAG degradation (by enzymes marked as E1, E2 and others; see Fig. 1 for the complete list of these enzymes). **b** An MPS III cell, in which GAG accumulation occurs due to dysfunction of enzyme E2. **c–e** Different putative mechanisms of efficacy of SRT; namely, decrease of GAG storage may results from: stop codon readthrough and appearance of small amounts of the active enzyme E2 (**c**), cell growth and division which causes GAG dilution (**d**), and/or activity of an unspecific GAG endohydrolase (e.g., heparanase) and further degradation of some GAG molecules despite enzyme E2 dysfunction (**e**). Note that these mechanisms may be effective only when GAG synthesis is impaired due to SRT. For details of this hypothesis, see text

If GAG synthesis is inhibited, one may expect slowed accumulation of the substrate that cannot be efficiently degraded. However, if the defect of one of the enzymes involved in GAG degradation is not complete and some residual activity of such a protein is still present, it is likely that the efficiency of GAG degradation will be high enough to have additive effects towards its slow production. Then, a decrease in total GAG level is not a surprise. However, how to explain a GAG storage clearance if no residual activity of a GAG-degrading enzyme is present?

We provide a hypothesis presenting three possible scenarios that may lead to a decrease in the amount of accumulated GAGs in cells bearing two null alleles of a gene coding for one of the enzymes involved in GAG degradation. First, there is always a possibility of the leakiness of a null mutation, especially if it is a non-sense mutation. In such a case, the stop codon may be readthrough from time to time (generally with a very low frequency), leading to appearance of low amounts of a functional enzyme, able to degrade already accumulated GAGs (Fig. 2c). However, this mechanism, although possible, would be rather of low efficiency, thus it cannot serve as a sole explanation of the effects described in the preceding section. Second, it should be considered that cells used in experimental cultures are constantly growing and dividing. This is true also for various tissues of young animals used in studies as well as for children taking part in clinical trials. Therefore, if GAG synthesis is inhibited, the growing total cell volume would result in decreasing total GAG concentration, the phenomenon actually observed (Fig. 2d). This, however, is unlikely to operate in some cells and tissues, especially in neurons forming the CNS. Nevertheless, there is a third possibility. Namely, it is often forgotten that apart from exoglycosidases, another class of enzymes, endoglycosidases, are involved in GAG degradation. An example of such enzymes is heparanase that cleaves HS inside the chains, with the only, though not strong, preference to low sulfation sites (for a review, see Fux et al. 2009). Heparanase is located in lysosomes and at cell surface (Fux et al. 2009). One should take into consideration that GAGs play their physiological roles being linked to proteins, and thus, forming proteoglycans. They are located at cell surface, and only those molecules which are no longer required (including damaged ones) and are devoted to degradation can be directed to lysosomes for their hydrolysis. Hence, heparanase can potentially cleave the GAG chain before and after it is transported into lysosome. It is likely that at certain frequency this enzyme can cut the HS chain at the position just near the sugar monomer which cannot be excised or modified (for example, by removing the sulfate moiety) by the deficient hydrolase. In such a case, other and still active hydrolases have open access to their substrates, and previously blocked degradation of GAG can proceed (Fig. 2e).

We believe that each of the three putative mechanisms described above, and especially their combination, may lead to effects of a substantial decrease in GAG storage in various cell types during SRT, observed in experiments. One should note that efficiency of each of these mechanisms is too low to overcome accumulation of GAGs in the absence of one of specific exohydrolases when effective synthesis of these compounds proceeds. Therefore, without inhibiting the synthesis of GAGs, their storage is observed in MPS cells. However, when GAG production is impaired due to SRT, one can observe not only inhibition of further accumulation of the stored material but also a decrease in the storage. This phenomenon may be encouraging to perform further studies on the use of SRT for MPS, as a potential therapy, possibly able to help patients suffering from these severe diseases.

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Conflict of interest The authors declare that they have no competing interests.

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