Biochemical Pharmacology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Commentary

Targeting *GH-1* splicing as a novel pharmacological strategy for growth hormone deficiency type II

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ARTICLE INFO

Article history:
Received 2 June 2016
Accepted 21 July 2016
Available online xxxx

Keywords:
Growth hormone
GH-1 gene
Short stature
Growth hormone deficiency type II
Splicing modulation

ABSTRACT

Isolated growth hormone deficiency type II (IGHD II) is a rare genetic splicing disorder characterized by reduced growth hormone (GH) secretion and short stature. It is mainly caused by autosomal dominant-negative mutations within the growth hormone gene (*GH-1*) which results in missplicing at the mRNA level and the subsequent loss of exon 3, producing the 17.5-kDa GH isoform: a mutant and inactive GH protein that reduces the stability and the secretion of the 22-kDa GH isoform, the main biologically active GH form.

At present, patients suffering from IGHD II are treated with daily injections of recombinant human GH (rhGH) in order to reach normal height. However, this type of replacement therapy, although effective in terms of growth, does not prevent the toxic effects of the 17.5-kDa mutant on the pituitary gland, which may eventually lead to other hormonal deficiencies. As the severity of the disease inversely correlates with the 17.5-kDa/22-kDa ratio, increasing the inclusion of exon 3 is expected to ameliorate disease symptoms.

This review focuses on the recent advances in experimental and therapeutic strategies applicable to treat IGHD II in clinical and preclinical contexts. Several avenues for alternative IGHD II therapy will be discussed including the use of small interfering RNA (siRNA) and short hairpin RNA (shRNA) constructs that specifically target the exon 3-deleted transcripts as well as the application of histone deacetylase inhibitors (HDACi) and antisense oligonucleotides (AONs) to enhance full-length *GH-1* transcription, correct *GH-1* exon 3 splicing and manipulate GH pathway.

Contents

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http://dx.doi.org/10.1016/j.bcp.2016.07.016
0006-2952/

1. Introduction

Growth hormone (*GH*) is a member of the somatotropin/prolactin family of hormones and is considered of main importance for linear growth in childhood. Severe shortage or absence of GH causes a condition called isolated growth hormone deficiency (IGHD).

IGHD is a rare disorder (the incidence of congenital IGHD varies between 1:4000 and 10,000 live births) and although it is most commonly sporadic, depending on the cohort screened, between 3% and 30% of cases are familial implicating genetic factors [1].

Newborns with a genetic etiology of IGHD present a poor linear growth, which becomes progressively retarded with age, and generally share many clinical features such as truncal obesity, facial appearance younger than expected for their chronological age, delayed secondary dentition and a high-pitched voice [2]. At the genetic level, familial IGHD has been usually classified in four types depending on the inheritance pattern, as autosomal recessive (Type IA, IB), autosomal dominant (Type II) or X-linked inherited (Type III) [2].

The most common genes implicated in the genetic etiology of IGHD are those encoding GH (*GH-1*) and the receptor for GH releasing hormone (*GHRHR*), while IGHD may be the only or first presentation of mutations in genes encoding early (*OTX2*, *HESX1*, *SOX2*, *SOX3)* or late (*PROP1*, *POU1F1)* transcription factors. Depending on the cohort studied and the criteria used for defining IGHD, mutations in known genes are identified in almost 11% of IGHD patients, with a higher prevalence in familial compared to sporadic IGHD (34% *vs* 4% respectively) [3].

In this review, we focus on IGHD II that is, in the majority of the cases, due to mutations affecting splicing of the *GH-1* gene [4–6]. These mutations result in skipping of exon 3 during splicing and, therefore, production of a shorter GH variant: the 17.5-kDa GH isoform. At the cellular level, the 17.5-kDa GH isoform lacks the protein linker domain between the first two helices of GH and is therefore retained in the endoplasmic reticulum (ER). Basically, it acts as a dominant negative isoform by blocking the secretion of the full-length 22-kDa GH isoform (*wt*-GH) [7,8] (Graphical abstract).

For this reason, compared with healthy subjects, patients affected with IGHD II usually present with decreased GH concentration although variations are observed depending on the specific mutation affecting *GH-1* splicing. In fact, clinical severity varies, and it has been shown to correlate with the level of 17.5-kDa GH isoform [9]. Several mechanisms are believed to cause this dominant disorder, such as accumulation of toxic aggregates of mutant proteins, decreased stability of *wt*-GH due to cellular responses induced by unfolded proteins or impaired maturation of secretory vesicles (SVs). Nevertheless, a proper functional explanation for causing this disorder remains unknown.

At present, children suffering from IGHD II are treated with costly daily injections of recombinant human GH (rhGH) in order to reach normal height.

However, this type of approach, which is usually well tolerated and effective in terms of growth and metabolism, cannot replicate the normal, pulsatile pattern of GH secretion [10] and may not totally prevent toxic effects of the 17.5-kDa isoform on the pituitary gland, which can eventually lead to other pituitary-derived hormonal deficiencies or to anterior pituitary hypoplasia [11]. Moreover, children diagnosed with IGHD may require rhGH treatment for few years to lifelong and the surveillance of the long term safety of rhGH treatment is still controversial [12].

Therefore, therapies that specifically target the damaging 17.5-kDa GH isoform may be useful in patients with *GH-1* splicing defects.

This review summarizes the recent advances in experimental and therapeutic strategies applicable to treat IGHD II and their possible future development as treatment.

2. The *GH-1* gene cluster

GH is a single-chain peptide of 191 amino acids produced and secreted mainly by the somatotrophs of the anterior pituitary gland. It regulates many important processes such as somatic growth, bone mineralization, sugar and lipid metabolism, protein synthesis and stimulation of the immune system. GH production is, in turn, influenced by several factors including sex, age, adiposity, sleep, stress, diet and exercise [13–17].

The gene encoding growth hormone (*GH-1*) is located on the long arm of chromosome 17 (17q22-24) within a cluster of five homologous genes, including *CSH1* (chorionic somatomammotropin hormone 1), *CSH2*, *CSHL1* (CSH-like 1) and *GH2* (a GH variant expressed in the placenta) [2]. Expression of *GH-1* is controlled by the highly polymorphic proximal promoter and by a locus control region placed 15–32 kb upstream of the gene that confers the pituitary-specific, high-level expression of GH [18,19].

GH-1 consists of five exons and four introns (or intervening sequences; IVS), enabling transcription of five known variants through alternative splicing [1,20,21] (Fig. 1A). When *GH-1 is* correctly spliced, it produces the 22-kDa isoform, which includes all five exons and represents the major biologically active form of GH present in the circulation. However, even under normal condition, a small percentage of alternative splicing products is generated. These splicing variants include:

(a) The 20-kDa isoform (representing 5–10% of GH transcripts), as a result of the activation of an in-frame cryptic splice site within exon 3 which causes deletion of amino acids 32–46;
(b) The 17.5-kDa isoform, (representing 1–5% of GH transcripts) resulting from complete skipping of exon 3 with no biological activity;
(c) The severely truncated 11.3-kDa and 7.4-kDa isoforms, present in trace amount in normal human pituitary and encoded by transcripts that skip exons 3–4 or exons 2–4, respectively, yielding inactive protein products [2].

GH is produced as a 217-amino acid precursor that is transported into the lumen of the endoplasmic reticulum (ER) via a mechanism that involves the recognition of the signal peptide (amino acids 1–26). Following the cleavage of the signal peptide, GH is secreted as a mature 191 amino acid protein. X-ray crystallographic studies have shown that it comprises a core of two pairs of parallel α-helices arranged in an up-up-down-down fashion and stabilized by two intramolecular disulfide linkages (Cys53–Cys165, Cys182–Cys189) (Fig. 2) [22].

3. The GHRH-GH-IGF-1 secretory axis

GH is secreted into the bloodstream in a pulsatile manner, with a major surge at the onset of slow-wave sleep and less conspicuous secretory episodes a few hours after meals [23]. Two hypothalamic hormones regulate GH secretion: GH-releasing hormone (GHRH), which stimulates GH secretion, and somatotropin release-inhibiting factor (SRIF; somatostatin), which inhibits GH secretion [24,25].

Following secretion, GH circulates mainly bound to a GH-binding protein [26,27] and it reaches the liver and other target organs where it binds to the GH receptor (GHR) and mediates the release of insulin-like growth factor-1 (IGF-1), the primary

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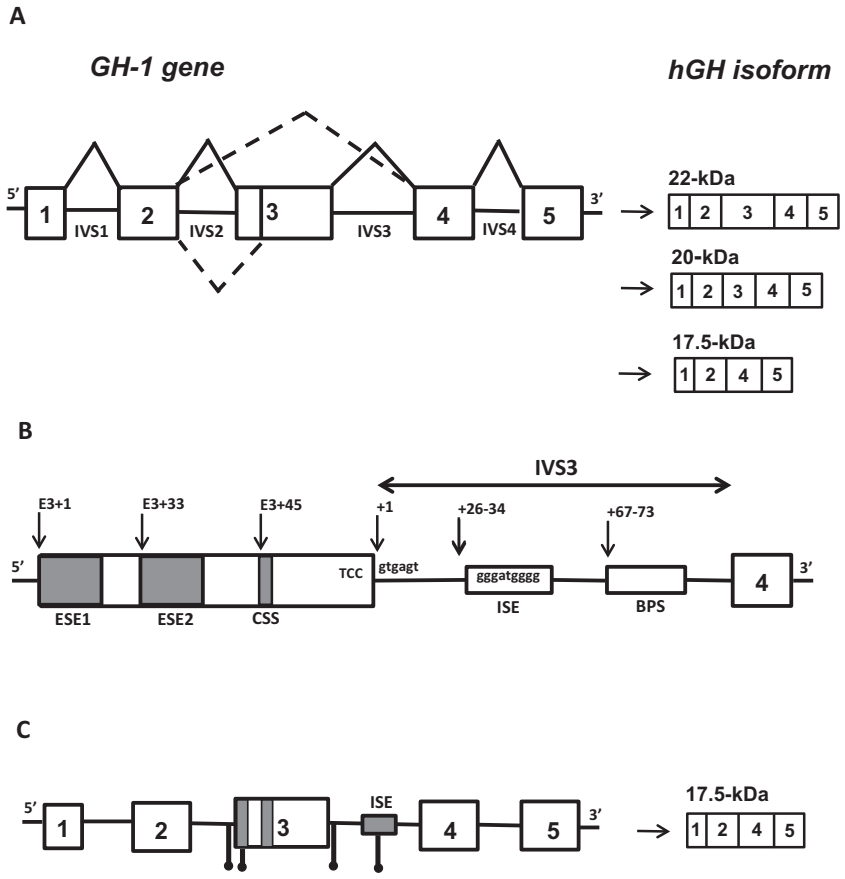

Fig. 1. Structure and splicing of *GH-1*. A) Growth hormone gene (*GH-1*) is constituted of five exons and four intronic intervening sequences (IVS1-4). When the *GH-1* is correctly spliced, three mRNA transcripts are produced. The major isoform present in the blood circulation of healthy humans is the 22-kDa isoform (*wt*-GH), translated from transcripts bearing all five exons. The most abundant minor isoform, representing 5–10% of all the transcripts, is produced as a consequence of a cryptic 3′ splice site in exon 3 (vertical black line in exon 3), resulting in a 20-kDa GH bioactive peptide once translated. Complete skipping of exon 3 leads to the production of the 17.5-kDa isoform. B) Elements crucial for the correct splicing of GH-1 and the inclusion of exon 3 are: the exonic splice enhancers (ESE1 and ESE2) and the cryptic splice site (CSS) within exon 3; the first six nucleotides of intron 3; the intronic splice enhancer (ISE) and the branching point site (BPS) within intron 3. The nucleotide position of these elements is indicated by arrows. C) Complete skipping of exon 3 is a very rare event in healthy individuals but patients harboring mutations within the following regions: intron 2–3′ splice site, exon 3–5′ splice site (or ESE1), intron 3–5′ splice site and ISE have a higher proportion of mutant protein produced and are affected by IGHD II (solid circles from left to right respectively).

mediator for the growth-mediating actions of GH. Although systemic IGF-1 is synthesized primarily in the liver, where its synthesis is partially GH dependent [28], it is also synthesized in multiple extrahepatic tissues, where it acts as a local growth factor under the control of different hormones. Consequently, IGF-1 circulates mostly as part of a 150-kDa ternary complex formed by one molecule each of IGF-1, IGF-1 binding protein (IGFBP), mainly IGFBP-3, but also IGFBP-5, and the labile acid subunit (ALS) [29–32]. The 150-kDa ternary complex stabilizes IGF-1, prolonging its circulating half-life and regulating its availability to the target tissues [29].

IGF-1 has, in fact, growth stimulating effects on a wide variety of tissues but it has principally stimulatory effects on osteoblast and chondrocyte activity to promote growth for development during childhood and puberty. Further, GH normally regulates its own expression and production by both direct and indirect feedback (via IGF-1): IGF-1 leads to decreased secretion of GH by suppressing the somatotrophs and by stimulating release of somatostatin from the hypothalamus. In parallel, GH inhibits GHRH secretion and probably has a direct autocrine inhibitory effect on secretion from the somatotrophs [24].

4. IGHD II

IGHD II is the autosomal dominant form of IGHD and it is caused by heterozygous *GH-1* mutations. In contrast to nonsense, missense and deletion mutations that are characteristic for the autosomal-recessive form of IGHD (IGHD I), the majority of IGHD II mutations described so far affect the splicing of *GH-1* by different mechanisms [5,6].

Splicing is the complex process by which exons are joined together to generate mature mRNA after removal of introns. It requires a complex interplay between *cis*- and *trans*-acting factors. In constitutive splicing, the *cis*-acting elements are the 5′ splice site, branch point, polypyrimidine tract and the 3′ splice site, each of which are defined by short, degenerate consensus sequences [33]. These elements are recognized by the spliceosome, a large macromolecular complex which assembles onto them and catalyzes the splicing reaction in two transesterification steps [34]. However, often splice sites are weak and need additional sequences to be properly recognized. These elements are called splicing enhancers and usually are purine-rich or A/C-rich sequences [35]. They are termed exonic splicing enhancers (ESEs) if located in exons or intronic splicing enhancers (ISEs) when they occur in introns.

Since *GH-1* has several weak splice sites and exhibits small amounts of aberrant splicing even in healthy individuals, multiple mechanisms have evolved to avoid production of skipped *GH-1* transcripts, especially those encoding the 17.5-kDa isoform [36] (Fig. 1B).

Multiple enhancers are essential to maintain full-length GH splicing: they contribute to exon 3 definition and to the enhancer-dependent activation of the intron 2 3′ splice site at

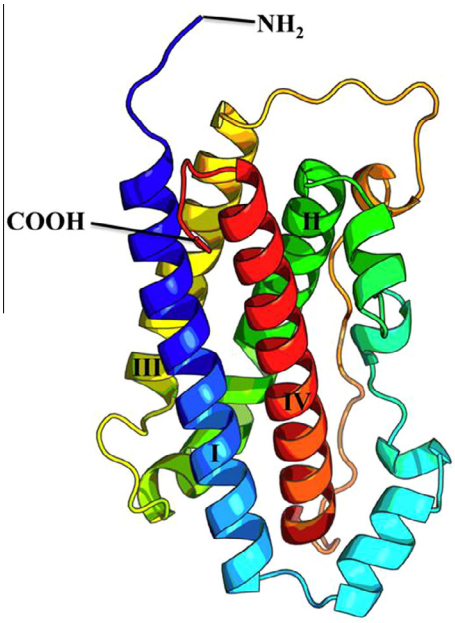

Fig. 2. Structure of human GH. The model proposed is based on human GH structures from PDB IDs 1HGU and 3HHR. The graphical view was generated as a ribbons diagram using Pymol (www.pymol.org) and represented as ray-traced image with POVRAY (www.povray.org). The four α-helices (I to IV) are orientated in an up-up-down-down conformation (Picture kindly provided by PD. Dr. Amit Pandey).

the expense of the stronger, nearby cryptic splice site [37]. Three *GH-1* splicing enhancers are well characterized: ESE1, exon splice enhancer motif 1, which encompasses the first seven bases of exon 3; ESE2, a second exon splicing enhancer, located 12 nt upstream of the cryptic splice site in exon 3; and ISE, intron splicing enhancer contained in intron 3. ESE1 is crucial for correct *GH-1* splicing because it strengthens the use of the weak 3′-splice site upstream of exon 3 suppressing at the same time, a downstream cryptic splice site.

Consequently, mutations in any of the bases of ESE1 lead to either complete or partial exon 3 skipping and the generation of the 17.5-kDa and 20-kDa isoforms at various concentrations (35–68% and 20–37%, respectively) [38,39] (Fig. 1C). More importantly, mutations within ESE1 have been demonstrated to result in abnormal splicing even if there is no amino acid substitution, which underlines the observation that even translationally silent mutations can affect splicing and lead to phenotypic manifestations [37,38].

Exon 3 skipping and generation of the 17.5-kDa isoform can also result from mutations that affect the conserved consensus splice site bordering exon 3. These mutations cause almost complete skipping of exon 3 by impairing U1 small nuclear ribonucleoproteins (snRNP) recognition and spliceosome assembly and are considered the most severe. A heterozygous missense mutation (Lys41Arg) in ESE2 leads to exon skipping in about 20% of transcripts with pleiotropic effects on the phenotype that ranges from normal to short stature [36,40].

Further, exon 3 skipping can result from disruption of sequences in IVS3 downstream of the consensus splice sites that affect ISEs [41] or the branching point site [42]. Besides enhancers, several findings support the notion that even the size of intron 3 is important for the integrity of the splicing mechanism [36,42]. Intron 3 in *GH-1* is 92 nt in length, well above the approximately 50 nucleotide minimum intron length required for spliceosome assembly. Decreasing the size of intron 3, rather than deleting the specific sequences within intron 3, by as little as 12 nt, prevents its accurate identification by the spliceosome causing increased exon 3 skipping [36,42].

In addition to splice-site mutations, GH missense mutation (P89L, R183H, V110F, R178H, L76P) have also been reported to cause IGHD II [43–45]. In these cases the mechanisms underlying the reduced GH secretion are even more complex and mainly involve profound and early disturbances in the secretory pathway.

4.1. IGHD II at the cellular level

Independent of the mechanism underlying its expression, both in cell cultures and in transgenic murine models, the 17.5-kDa isoform exhibits a dominant-negative effect on the secretion of 22-kDa isoform [38,46]. At the structural level, the 17.5-kDa isoform lacks the protein linker domain between the first two helices of GH and a cysteine residue (C53) that is involved in the creation of a disulfide bond between helix one and helix two and is retained in the ER. This triggers a misfolded protein response and disrupts the secretory pathway and the trafficking of GH [7,8]. After being produced, the 17.5-kDa isoform is detected by ER quality control mechanism and degraded via the proteasomal pathway. However, when the production rate of 17.5-kDa isoform exceeds the proteasome degradation capacity, it accumulates in the cytoplasm leading to reduced cell proliferation and apoptosis of GC cells [47]. The amount of the 17.5-kDa isoform has to reach a critical threshold to exert its dosage-dependent effect, with increasing amounts leading to reduced cell proliferation and apoptosis of somatotrophs [36].

Although it is clear that these mutant GH forms have an effect on the accumulation and secretion of GH, the exact cause of IGHD II is still unknown. Disrupting effects may occur at any stage during the secretory process.

4.2. IGHD II animal models

Additional insights into this disorder come from IGHD II animal models. Transgenic animals were generated by micro-nuclear injection of a fragment containing the locus control region of *GH-1* and the genomic sequence of the IVS3 +1 GH mutation [7]. Three lines expressing *hGH-1* transcript missing exon 3 in their pituitary cells with different relative copy numbers were established, giving phenotypes ranging from strongly to barely affected animals. The most severely affected line gave birth to mice that became progressively growth retarded compared to wild type littermates during early postnatal growth. They presented anterior pituitary hypoplasia, barely detectable levels of GH and multiple hormonal deficiencies and their pituitary was smaller than that of control mice due to the loss of somatotrophs. Their hypothalamic GHRH expression was upregulated and SRIF expression reduced consistent with their profound GHD. Few SVs were detected in the residual pituitary and somatotrophs were in turn invaded by macrophages. On the other hand, low copy number transgenic animals showed a mild effect with their body weight not significantly lighter than control animals, despite a significant decrease in their GH content.

4.3. IGHD II phenotype in patients and clinical implications

Patients with IGHD II show substantial variation in their clinical phenotype. They present with low but detectable serum GH levels, variable height deficit and can show anterior pituitary hypoplasia on MRI (38%, 50%) [4,48,49].

Reports on pedigrees with the Arg183His or E3+1G>A mutations highlight the fact that patients with the same mutation can vary considerably in height (⩽−4 SDS to normal) and even attain normal adult height without treatment [9,49].

Patients with the IVS3+1 or IVS3+2 splice site or the Pro89Leu mutations can develop additional pituitary hormone deficiencies,

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including ACTH, prolactin, TSH or gonadotropin deficiency [11,50,51]. The mechanism by which other endocrine axes are affected is not obvious, but might be through bystander damage from activated macrophages clearing dying somatotrophs, as observed in the transgene mouse study [7,11].

Generally splice-site mutations, which exclusively produce the 17.5-kDa isoform, cause, on average, an earlier age of onset and greater clinical severity [5], while ESE mutations cause a slightly less severe impact on patients height [38]. The quantitative and qualitative differences in the clinical phenotype are attributable to differences in the isoform ratio rather than the mutant transcript alone. Patients with the highest 17.5-kDa/22-kDa ratio also have the lowest height standard deviation scores (SDS) and GH peak following pharmacological stimulation, while, on the contrary, those with the lowest 17.5-kDa/22-kDa ratio can attain normal height despite never having been treated with rhGH [9].

In these patients, the GH-negative feedback on GHRH is reduced and it results in a chronic upregulation of GHRH and reduction of somatostatin expression. The constant upregulation of GHRH might increase the stimulatory drive on somatotrophs and the production of GH, both from the normal and mutant allele, creating a vicious circle of increased levels of the 17.5-kDa isoform that further accelerate damage to the anterior pituitary [52,53]. Interestingly, even in patients with a genetic cause (for example, Glu32Ala), IGHD seems to reverse when they are retested at the end of growth, in the transition period before transfer from pediatric to adult services, possibly because the treatment with rhGH removes the endogenous drive by GHRH and allows the recovery of somatotrophs. However, this effect is temporary, observed in patients tested at the time of transition, who should not be discharged from follow-up [39]. This evolving phenotype dictates the need for lifelong follow-up and possibly rhGH treatment in affected individuals [11]. Supplementation of rhGH therapy with somatostatin could be a valid option: it could increase the efficacy of GH therapy by inhibiting the production of endogenous GH and thereby preventing the production of the 17.5-kDa isoform to progressively damage the remaining somatotrophs.

However, because IGHD II arises as a direct consequence of excessive production of a specific dominant-negative isoform, strategies designed to decrease the levels of the 17.5-kDa isoform without affecting the normal 22-kDa product may be useful to treat IGHD II patients with splicing mutations while avoiding the side-effects of long-term post-pubertal rhGH therapy.

5. Rescue of IGHD II *in vitro* and *in vivo* by RNA interference (RNAi): a simple concept but a complex reality

Over the last decade, double-stranded short interfering RNAs (siRNAs) have become a molecular tool, widely used for silencing gene expression. They are usually 19–22 bp oligonucleotide duplexes. This short length prevents from a strong interferon response provoked by duplexes greater than 30 bp in length, when introduced inside cells. When a duplex RNA enters the cell, it is targeted by the RNA-induced silencing complex (RISC), one strand is lost and the remaining strand cooperates with RISC to bind complementary RNA through Watson-Crick base pairing. SiRNAs may be designed to target dominant, aberrantly spliced isoforms or other dominant alleles for degradation [54,55].

In 2004, Ryther et al. [36] proposed to use siRNAs specifically targeting the 17.5-kDa isoform mRNA. They targeted the exon 3 skipped transcripts for degradation by designing siRNA for the exon 2-exon 4 junction, because it represents the only sequence within *GH-1* gene that is unique to transcripts encoding the 17.5-kDa isoform. They showed that in cultured GH3 cells, plasmids expressing this siRNA efficiently and specifically degraded the exon 3-skipped GH transcripts by almost 90%. Following this initial report, Shariat et al. [56] were able to rescue pituitary function in IGHD II mice progeny by mating them with transgenic mice expressing a short hairpin RNAs (shRNAs) targeting the 17.5 kDa isoform. The rescued mice presented macroscopically and microscopically normal somatotroph populations, which promoted complete functional recovery without necessarily achieving complete suppression of the aberrant allele in every cell.

Both studies showed the efficacy of siRNA or shRNA against the17.5-kDa isoform and provided a promising approach to treat IGHD II in humans.

Clearly the methodology presents some limits: siRNA and shRNA may induce cytotoxicity *in vitro* and *in vivo* [57,58] and successful exogenous delivery of siRNAs is still missing. Clinical use of siRNAs will require an effective and safe delivery system because although the pituitary is not protected by the blood-brain barrier; it is relatively inaccessible to direct targeting due to its location.

6. Viral-based therapy for IGHD II: background and progress in using viral vectors

Gene therapy has been used successfully to increase protein level *in vivo* in several neurological disease models, including ALS, epilepsy and Parkinson's disease [59]. In the contest of IGHD II Lochmatter et al. tested a strategy involving lentiviral vector (LVs) expressing a human micro-RNA-30-based shRNA (shRNAmir) [60,61], targeting the anomalous junction of exon 2 and exon 4 (shRNAmir-Δ3) of the *GH-1* characterizing the misspliced 17.5-kDa isoform.

Using rat pituitary tumor GC cells expressing both human *wt*-GH and e17.5-kDa transcripts, they demonstrated that shRNAmir efficiently reduces the expression of mutant protein, leading to an increase in *wt*-GH secretion, without affecting the viability of the transduced cells. Furthermore, cells expressing 17.5-kDa mRNA upon doxycycline (DOX) induction transduced with shRNAmir-Δ3 significantly reduced the 17.5-kD isoform at protein level and improved human *wt*-GH secretion in comparison to an shRNAmir targeting a scrambled sequence. Importantly, no toxicity due to shRNAmir expression could be observed in cell proliferation assays. Furthermore, confocal microscopy data strongly suggested that shRNAmir-Δ3 (exon 3 missing) enabled the recovery of GH granule storage and secretory capacity. Taken together, these viral vectors have shown their ability to stably integrate, express shRNAmir and rescue IGHD type II phenotype in rat pituitary cells. This methodology, although effective, raises specific safety and ethical issues. LVs are widely used as promising vehicles for their ability to provide long-term and stable gene expression and to infect non-dividing cells, such as neurons. Despite constant efforts are being made to improve safety and specificity, many concerns remain to be addressed including the possible generation of replication competent lentiviruses during vector production, mobilization of the vector by endogenous retroviruses in the genomes of patients, insertional mutagenesis leading to cancer, germline alteration resulting in trans-generational effects and dissemination of new viruses from gene therapy patients. Moreover the risk for interaction of LVs with other retroviral elements in human subjects, such as Human Immunodeficiency Virus should be taken in account before these types of viral vectors can proceed to the clinic [62].

7. Rescue of IGHD II by modulation of *GH-1* splicing

IGHD II can be considered primarily as a splicing disorder in which the severity of disease correlates with the level of correctly spliced RNA and with the ratio of alternatively spliced isoforms

[9]. Because splicing regulation has a role as a genetic modifier of disease severity, therapies that specifically target aberrant splicing may be useful in patients with *GH-1* splicing defects. In contrast to gene-replacement and stem-cell therapies, targeting the RNA splicing to correct the effects of a mutation bypasses the need to correct or replace mutated DNA or diseased cells [63]. Emerging therapies targeting RNA splicing indicate that this could be a powerful approach, because it can correct a wide range of mutations and it is easy to deliver. In the following section, we aim to describe some of the basic tools that have been developed to manipulate splicing.

7.1. Small molecule compounds

The possibility that alter the regulation of alternative splicing might restore normal phenotype in inherited diseases caused by aberrant splicing, led to the development of molecular approaches aiming to increase the level of correctly spliced transcripts. Several small molecules, mainly classified as histone deacytalase inhibitors (HDACi), such as sodium butyrate (NaB), valproic acid (VPA), 4-phenylbutyrate, SAHA, M34491-96 have been recently identified and reported to fulfill this aim [64,65].

HDACi are a relatively new group of epigenetic agents that have multiple substrates including histone and non-histone proteins, suggesting their involvement in multiple cellular processes. They alter the acetylation status of chromatin and other non-histone proteins, resulting in changes in gene expression. In normal cells (non-cancer cells), the response to HDACi depends on the cell type, on the structure and concentration of HDACi as well as the exposure time to HDAC inhibitors [64]. They are able to modify splicing by directly modifying the expression of specific splicing factors belonging to the serine/arginine (SR) rich protein.

SR proteins modulate selection of alternative splicing sites in a concentration-dependent and tissue-specific manner. They are parts of a growing family of structurally related and highly conserved splicing factors characterized by the presence of 1–2 RNA recognition motifs (RRM) and by a different carboxyterminal domain that is highly enriched in Arg/Ser dipeptides (the RS domain). The RRMs mediate sequence-specific binding to RNA, determining in that way substrate specificity, whereas the RS domain seems to be involved mainly in protein–protein interactions. SR proteins that are bound to ESEs can promote exon definition by directly recruiting the splicing machinery through their RS domain and/or by antagonizing the action of nearby silencer elements. These two models of splicing enhancement are not necessarily mutually exclusive, as they might reflect different requirements in the context of different exons. Overexpression of splicing factors regulate the splicing pattern of alternatively spliced exons and differences in the levels of alternatively spliced transcripts were suggested to arise from differences in the relative levels of splicing factors (the SR and hnRNP families) [33].

This molecular approach was successfully tested for treatment of splicing disorders like cystic fibrosis (CF) and spinal muscular atrophy (SMA) [66–68].

SMA is an autosomal recessive neuromuscular disease characterized by degeneration of alpha motor neurons in the spinal cord. It is caused by homozygous mutations of the survival motor neuron 1 (SMN1) gene. Normally, both gene copies (*SMN1* and *SMN2*) are expressed, but they differ in the expression of full-length protein. *SMN2* gene preferentially gives rise to a truncated and less stable version of the SMN protein and thus cannot compensate for SMN1 loss or mutations unless it is not present in multiple copies. The differences between these highly homologous genes are in their RNA expression patterns. Most *SMN2* transcripts lack exons 3, 5, or most frequently, 7, with only a small amount of full-length mRNA generated. On the other hand, the *SMN1* gene expresses mostly a full-length mRNA, and only a small fraction of its transcripts are spliced to remove exons 3, 5, or 7. The amount of exon 7-containing SMN protein has been shown to be an inverse indicator of disease severity in SMA patients and mice. Therefore, increasing the expression of intact SMN protein may have clinically therapeutic effects on SMA patients. Chang et al. [66] showed that butyrate treatment of human SMA lymphoid cell lines increased the expression of exon 7-containing SMN protein from the *SMN2* gene. After butyrate treatment *in vitro* and *in vivo*, the transcription pattern of *SMN2* changed to an *SMN1*-like transcription pattern, which was almost identical to the *SMN* pattern in healthy individuals. Moreover, the clinical symptoms of the SMA-like mice improved after treatment with butyrate.

Following this initial report, Nissim-Rafinia et al. [67] restored the function of CFTR (cystic fibrosis transmembrane conductance regulator) channel: the ABC-transporter class ion-channel that transports chloride and thiocyanate across epithelial cell membranes. Mutations of the CFTR gene affect functioning of the chloride ion channels in epithelial cell membranes, leading to cystic fibrosis. The CFTR gene comprises 27 constitutively spliced exons, however several exons (3, 4, 9, 12, 14a, 16, 17b and 22) undergo in some individuals, partial aberrant splicing leading to a decrease in the level of full-length CFTR transcripts generating nonfunctional CFTR proteins. Butyrate treatment of cystic-fibrosis-derived epithelial cells carrying the 3849+10 kb C → T mutation increased the level of normal and full-length CFTR transcripts, restore the CFTR function which was sufficient to achieve channel activation. In both studies, butyrate restores the correct splicing of pre-mRNA by overexpression of SR protein and the transcription of both genes is modified through the alternative splicing rather than directly through inhibition of histone deacetylation.

Considering these successful results, we applied such a strategy to correct the aberrant splicing of different GH-mutants (GH-IVS3 +2 [69], GH-IVS3+6 [46], GH-ISE+28 [41,70]) known to cause IGHD II [71]. The splicing of exon 3 in *GH-1* is regulated by two members of the SR protein superfamily: ASF/SF2 and SC35 which act antagonistically. ASF/SF2 activates exon 3 inclusion by binding to ESE2, a while SC35, by acting through a region just downstream of ESE2, can block this activation [72].

Since patients' pituitary cells are not available we used an established *in vitro* model for IGHD II [53]. This model relies on rat pituitary GC cells stably expressing the receptor for GHRH and thus transiently transfected with *wt*-hGH and/or with different GH-splice mutants under the control of a hGH promoter. The transfected cells were further treated with a high concentration of butyrate (5 mM), which might be the proper concentration to use *in vivo*. In fact, while *in vitro* butyrate exerts its effects on gene expression and cell proliferation in a wide range of concentrations (from 50 μM to 10 mM) [67], *in vivo* the molecule is rapidly metabolized requiring a higher dose to be effective [73].

Upon butyrate treatment, GC-GHRHR cells coexpressing *wt*-hGH and each of the mutants displayed increased GH transcript level, intracellular GH content and GH secretion when compared with the corresponding untreated condition. The effect of butyrate was most likely mediated by the splicing factor ASF/SF2. Overexpression of ASF/SF2 in the same experimental setting promoted the amount of full-length transcripts and increased synthesis and secretion of the 22-kDa isoform. Thus, it mirrors the effects of butyrate at the level of mRNA, protein and secretion.

Our results propose that, in the presence of splicing mutations, the *wt*-GH secretion can be rescued by increasing the expression of the 22-kDa isoform. This might represent an alternative option to the down-regulation of the 17.5-kDa isoform as previously shown [56]. The knockdown of the 17.5-kDa transcripts may reduce autosomal dominant effects and probably improve IGHD II condition. However, in an *in vivo* context, the reduced *wt*-GH transcript levels from a severely mutated allele cannot be totally restored by RNAi, because shRNAmir acts by a posttranscriptional mechanism and

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does not reverse the mutant mRNAs into *wt*-molecules. Translation of the 17.5-kDa mRNA is blocked, avoiding dominant-negative effect, but *wt*-GH expression and then secretion are overall diminished, because the mutant GH allele only synthesizes a small amount of functional *wt*-mRNAs.

Overall, these results provided direct evidence that changing the 17.5-kDa/22-kDa ratio by increasing the level of 22-kDa transcripts can restore normal GH secretion and could represent a potential new and therapeutically beneficial approach to IGHD II.

7.2. Benefits and drawbacks of small molecule as therapeutics

Currently there are numerous classes of small molecules under investigation for their ability to modulate splicing. They are identified by high-throughput screening assay using cells that report alterations in a particular splicing event [74]. The major benefit of some small molecules is that many of them are already approved and in use in clinical practice to treat diseases apart from splicing diseases [75] and thus their use in humans is considered safe. Moreover the safe pharmacological profile strongly accelerates their development as a treatment for a disease.

Specifically butyrate has been used clinically for several years to treat patients with sickle cell anemia and thalassemia [76,77]. It has low toxicity and it is well tolerated in both humans and animals [78,79] as demonstrated by pharmacokinetics and toxicity studies.

A triglyceride analog of butyrate (tributyrin), which has 95% similarity in structure, has already been approved as a food additive in the US and it was well tolerated even by young children after oral administration [80]. Nevertheless frequently splicing modulator molecules function in an indirect way on splicing and this can lead to off-target effects: the pattern of exons in the same or other genes could be affected by the molecule used. Moreover the exact mechanisms of action as well as long-and short-term consequences on the molecular profile of patients, and the use of different doses and routes of administration in combination treatments are not completely known.

8. Future directions: antisense oligonucleotides (AONs) and trans-splicing

Unlike the use of HDACi, AONs can be specifically designed to base pair to specific nucleotide sequences. As a result AONs offer increased specificity and reduced target effects.

AONs are short oligonucleotides, generally 15–25 bases in length, which are the reverse complement sequences of a specific RNA transcript region. AONs and siRNA share their basic principle: an oligonucleotide binds a target RNA through Watson-Crick base pairing but in contrast to siRNAs, AONs survive and function inside the cell as a single strand [55].

In addition to their specificity, AONs are relatively non-invasive (they do not alter the genome directly) and very stable. They are efficiently and spontaneously internalized by cells *in vivo*, present high substrate specificity and low toxicity, and are not degraded by endogenous RNase H. The half-life of naked AONs in mouse, human plasma and many mouse tissues is approximately 10–15 days [81]. Treatment of mice with a single injection of AONs early in life has been shown to correct splicing and disease-associated phenotypes for up to a year [82,83]. The basis of this longevity is unclear but may be attributable to the stability of AONs in post-mitotic cells where they continue to influence splicing long after administration. Such phenomenon might be related to the ability of mammalian cells to accumulate systemically and locally administered single-stranded oligonucleotides and lipophilic-conjugated single- or double-stranded oligonucleotides inside the same subcellular compartment as the target RNA. The mechanisms for accumulation within cells are complex, but appear to involve novel endosomal transport mechanisms [81,84].

The basic AONs technology underwent over the years to different variations in order to provide additional functions. A number of different bifunctional AONs have been designed. They are characterized by an antisense base pairing of a sequence complementary to a consensus binding site sequence for a specific splicing factor. In this way, the AONs can be directed to bind to the target RNA and at the same time to recruit a protein. This target protein, depending on its function and the location where the AONs binds, can either enhance [85] or silence splicing [86,87].

Because rescuing IGHD II using AONs has not been tested so far but has been successfully tested to promote exon inclusion in SMA [86], we believe that a good strategy for the future would be using, in combination with HDACi, a chimeric antisense oligonucleotide comprising two parts: one which is complementary to the aberrantly spliced exon and provides exon specificity, and the other containing binding motifs for the recruitment of splicing factor (specifically ASF/SF2) to the mutation site [85,88].

The clinical development of oligonucleotide as drugs has been so far slow. AONs and siRNAs are not small molecules (<500–700 molecular weight) and much effort is required to define the pharmacological profile, to understand their properties and optimize them. Currently, while many AONs are in the process of clinical development, one AONs has been approved by the FDA and other 22 are in Phase II or III clinical trials [89,90].

Finally we would like to discuss a promising molecular strategy, which is however still far away to be considered a contender for therapy called trans-splicing or Spliceosomal-Mediated RNA trans splicing (SMaRT) [91]. Trans-splicing is particularly effective to correct gene expression when the splice site mutation is in the first or last nucleotides of the intron. This type of mutation, often causing the most severe phenotype in IGHD II patients, cannot be easily corrected using other molecular strategies because the base specificity at the border of the intron is crucial for the catalytic steps of the spliceosome reaction.

Trans-splicing approach targets RNA at the pre-mRNA level and is designed to replace the entire coding sequence 5′ or 3′ of a target splice site. It requires in parallel three distinct components: the spliceosome, the target pre-mRNA transcripts, and pre-mRNA *trans*-splicing molecules (PTMs). While the spliceosome and target pre-mRNA are already inside the cells, the PTMs are artificially engineered RNA molecules that are able to bind specifically to target pre-mRNAs in the nucleus. The PTM consists in a plasmid expressing three components: an AON that targets the endogenous intron of the mutated gene, a synthetic splice site that directs splicing from the mutated RNA to PTM, and a copy RNA sequence that will be spliced to the endogenous RNA rather than to the mutated, inactive endogenous splice site [63,92].

Since trans-splicing technology requires the delivery of DNA expression vectors to cells, the disadvantage of this approach are similar to those explained before. On the other hand, compared to gene replacement one benefit of trans-splicing is that the expression of pre-mRNA of the target gene remains under native control. The PTM replaces a small portion of the gene and thus the endogenous promoter controls the transcription of the pre-mRNA as well as tissue, temporal and quantity specific expression [91]. This technology might be particularly useful to treat severe form of IGHD II where even a small increase in full-length transcript over the exon 3 transcript would give a great clinical beneficial to the patients.

As a final conclusion, we are clearly some years away from contemplating clinical trials, but the possible future identification of compounds that possess specificity for a splicing event or for a

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certain step in the splicing process remain an attractive goal. Clear progress in research targeting alternative splicing has also been rapid and hopefully the field will quickly move closer to clinical application.

Disclosure statement

The authors have nothing to disclose.

Acknowledgements

This study was supported by a grant of Swiss National Science Foundation 320000-121998 to PEM.

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Please cite this article in press as: M.C. Miletta et al., Targeting *GH-1* splicing as a novel pharmacological strategy for growth hormone deficiency type II, Biochem. Pharmacol. (2016), http://dx.doi.org/10.1016/j.bcp.2016.07.016