Research Article
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Life Sciences, Medicine and Biomedicine

Heparin octadecasaccharides influence iduronate-2-sulphatase activity in a mutation-dependent manner in Mucopolysaccharidosis type II

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Received 24 October 2025; Revised 29 January 2026; Accepted 20 February 2026; Published online 18 March 2026

ABSTRACT

Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is a lysosomal storage disorder caused by deficiency of iduronate-2-sulphatase (IDS), resulting in glycosaminoglycans (GAG) accumulation and progressive multi-system pathology. Enzyme replacement therapy improves somatic manifestations but has limited neurological benefit. Therefore, pharmacological chaperone therapy has been proposed as a mutation-dependent strategy to stabilise misfolded IDS variants with residual activity. This study evaluated the effect of heparin octadecasaccharides (HO18) in patient-derived fibroblasts representing five genotypes and in HEK293T cells expressing IDS mutants. HO18 treatment (15 µM, 96 hours) produced mutation-specific effects across both experimental systems, with GAG reduction observed only in selected IDS variants. In fibroblasts carrying the p.Ile360Arg variant, IDS activity increased by approximately 1.8-fold along with a marked reduction in GAG levels (~71%). Other genotypes showed partial responses, including increased IDS activity without significant GAG reduction or GAG reduction in the absence of detectable IDS activity enhancement. In recombinant systems, HO18 increased IDS activity in p.Asn63Asp (~1.2-fold) and reduced GAG accumulation in p.Asn63Asp and p.Leu314Pro variants, while catalytically inactive mutants showed no response. Overall, HO18 exhibited pharmacological chaperone-like effects in selected IDS variants but also produced mutation-specific outcomes not fully explained by IDS activity enhancement alone. These findings support further *in vitro* investigation of HO18 as a mutation-dependent small-molecule approach for IDS-related pathology, while highlighting the need to better understand how HO18 acts and to confirm its effects in *in vivo* models.

Keywords: *Heparin Octadecasaccharides; in vitro assay; mucopolysaccharidosis Type II; pharmacological chaperone therapy and small molecule*

INTRODUCTION

Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is a rare X-linked lysosomal storage disorder caused by a deficiency of iduronate-2-sulphatase (IDS), an enzyme responsible for the degradation of glycosaminoglycans (GAG), particularly heparan sulphate and dermatan sulphate. The lack of functional IDS results in progressive accumulation of

https://doi.org/10.28916/lsmb.10.1.2026.239

GAG within lysosomes, leading to multisystem pathology that includes hepatosplenomegaly, skeletal dysplasia, cardiac disease and in many patients, progressive neurocognitive decline (Neufeld & Muenzer, 2019; Vollebregt et al., 2017). The clinical spectrum of MPS II ranges from severe, neuronopathic forms with early onset to attenuated phenotypes with longer survival, reflecting the underlying heterogeneity of IDS mutations (Wraith et al., 2008). To date, over 600 IDS mutations have been reported, many of which cause misfolding or instability of the enzyme rather than complete catalytic loss suggesting that therapeutic strategies targeting enzyme folding may be beneficial.

Enzyme replacement therapy (ERT) with recombinant IDS is currently the standard treatment for MPS II and has been shown to improve somatic manifestations such as hepatosplenomegaly and respiratory function (Muenzer et al., 2006; Okuyama et al., 2010). However, ERT has a limited effect on neurological symptoms because the infused enzyme does not efficiently cross the blood-brain barrier. Additionally, the treatment requires lifelong intravenous infusions, incurs a high financial burden and may induce immune responses or lead to reduced efficacy in some patients (Vollebregt et al., 2017). Substrate reduction therapy and gene therapy are emerging alternatives but their long-term effectiveness and safety remain under investigation (Leal et al., 2023). These challenges highlight the need for complementary strategies that aim to restore endogenous enzyme function in patients with MPS II.

Among the potential complementary strategies, pharmacological chaperone therapy offers a targeted approach to stabilise misfolded lysosomal enzymes, thereby promoting proper folding, trafficking and lysosomal localisation. The clinical utility of this strategy has been demonstrated in Fabry disease, where migalastat is approved for patients with amenable mutations (Germain et al., 2016). In the context of MPS II, several small molecules and glycosaminoglycan-derived fragments have been evaluated as potential chaperones (Hoshina et al., 2018; Keyzor et al., 2023; Valenzano et al., 2011). However, their efficacy is highly dependent on the underlying IDS mutations, as only variants retaining partial catalytic activity can be rescued to functional levels.

Heparin-derived oligosaccharides have been reported to influence the activity of several lysosomal enzymes, supporting their evaluation as mutation-dependent modulators of IDS function (Díaz et al., 2020; Hoshina et al., 2018). Most previous studies, however have focused on short heparin fragments such as dissaccharides or tetrasaccharides, leaving the functional effects of longer heparin oligosaccharides largely unexplored. Longer heparin chains are expected to provide increased interaction surface area and charge density, which may enhance protein-oligosaccharide binding stability compared with shorter fragments (Mulloy et al., 2015). On this basis, heparin octadecasaccharides (HO18) were selected for investigation in the present study as a representative longer heparin-derived oligosaccharide.

Despite the promising properties, HO18 present inherent physicochemical limitations, including high molecular weight, strong negative charge, limited cellular uptake and poor central nervous system permeability (Arnold et al., 2023; Mulloy et al., 2015; Xu et al., 2011). These factors may restrict their therapeutic applicability, particularly in neuronopathic MPS II (Markowicz-Piasecka et al., 2022; Pardridge, 2020).

The present study aimed to evaluate the pharmacological chaperone potential of a defined heparin octadecasaccharides (HO18) in MPS II. Using two complementary *in vitro* models - patient-derived fibroblasts with clinically relevant IDS mutations and recombinant IDS mutants expressed in HEK293T cells, we investigated whether HO18 enhances IDS activity and reduces GAG accumulation in a mutation-dependent manner. The IDS variants examined in this study represent clinically observed missense, splice-site and catalytically impaired mutations associated with a spectrum of MPS II phenotypes, enabling evaluation of mutation-dependent responses to HO18. This comparative approach was designed to evaluate the *in vitro*, mutation-specific effects of HO18 using patient-derived fibroblasts and recombinant IDS mutant models.

MATERIALS AND METHODS

Patient-derived fibroblasts

Primary skin fibroblasts were established from biopsy samples of genetically confirmed MPS II patients. The IDS genotypes included p.Leu259Pro (n=2), p.Ile360Arg (n=2), p.Arg110Lys (n=2), splice variants c.1007-1G>A (n=1) and complex rearrangements (n=1). All mutations had been verified by sequencing analysis. Although fibroblasts were obtained from eight patients, these samples represented five distinct IDS genotypes, with some genotypes shared by biological related individuals (including PC03 and PC07). Cells were cultured in Modified Eagle Medium (MEM, Sigma-Aldrich) supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin-streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 5 % CO_2. All fibroblast lines were processed under identical culture conditions and used between passages 3-8 at comparable confluency.

Recombinant mutant IDS expression in HEK293T cells

HEK293T cells were maintained under the same conditions as fibroblasts. DNA sequences of wildtype and mutant *IDS* gene were designed, sent for synthesis, and cloned into pCMV-script expression vector (Integrated DNA Technologies, USA). The IDS constructs tested in HEK293T cells comprised wild-type IDS and seven mutant variants: p.Asn63Asp, p.Leu67Pro, p.Ala85Thr, p.Arg88His, p.Tyr108Ser, p.Pro231Leu, and p.Leu314Pro. These variants were selected to represent IDS mutations with differing reported effects on enzyme activity, including variants with residual

activity and catalytically impaired forms.

The recombinant expression system provided a controlled experimental setting for assessing HO18 effects on individual IDS variants, complementing analyses performed in patient-derived fibroblasts. Wild-type IDS served as the

positive control for recombinant expression experiments. Plasmid constructs were confirmed by sequencing prior to transfection. Transient transfections were performed using Lipofectamine™ 3000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Transfected cells were allowed to express the recombinant IDS proteins for 24 hours before the start of treatment.

Heparin Octadecasaccharides (HO18) treatment

Heparin octadecasaccharides (HO18) were obtained from Iduron Ltd (UK). Stock solutions (385 µM) were prepared in sterile deionised water and diluted in culture medium immediately before use. HO18 purity (>95%) was certified by the manufacturer and used as supplied without further modification. For treatment experiments, fibroblasts and transfected HEK293T cells were seeded at approximately 70-80 % confluency in a 24-well plate and incubated with 15 µM HO18 for 96 hours under standard culture conditions. Non-treated controls received culture medium only, without HO18 supplementation. Each experiment was conducted in at least three independent biological replicates.

Protein quantification

Total cellular protein was quantified using the Bradford protein assay (Bio-Rad Laboratories, USA) with bovine serum albumin (BSA) as a standard. Absorbance was measured at 595 nm using a microplate reader (Spark™ 20M, Tecan, Mannedorf). All enzyme activity and GAG values were normalised to total protein content.

IDS enzyme activity assay

IDS activity was measured using 4-methylumbelliferyl-α-iduronide-2-sulphate substrate (TRC, Canada), following the method described by Voznyi et al. (Voznyi et al., 2001). Briefly, cell lysates were incubated in 0.05 M acetate buffer (pH 4.5) for 4 hours at 37 °C. The reactions were terminated by adding 0.5 M carbonate buffer (pH 10.4) and liberated 4-methylumbelliferone (4MU) was quantified fluorometrically (excitation 365 nm, emission 450 nm). IDS activity was normalised to total protein content and expressed as nmol 4MU released per 4 hours per mg protein.

For assay validation, fibroblasts from a healthy donor (normal IDS activity) and untreated MPS II fibroblasts (deficient or low IDS activity) were included as positive and negative controls, respectively. In HEK293T-based experiments, cells transfected with wild-type IDS plasmid served as normal control, while cells transfected with empty vector or IDS mutant constructs known to abolish activity were included as abnormal controls.

Glycosaminoglycan (GAG) quantification

Total cellular GAG content was quantified using the dimethylmethylene blue (DMB) assay as described by de Jong et al. (De Jong et al., 1992) . Briefly, 15 µL of cell lysate (treated and untreated) was diluted to 30 µL with deionised water in a 96-well plate, followed by the addition of 165 µL of freshly prepared DMB reagent. Chondroitin sulphate (CS) was used to generate a standard curve. Absorbance was immediately read at 520 nm using a Spark™ 20M spectrophotometer. GAG levels were normalised to total protein and expressed as ng GAG per mg total protein. Each GAG measurement was performed using a freshly prepared CS standard curve and reagent blank in each assay run. For each fibroblast line, lysates were analysed in technical triplicates and averaged and experiments were repeated independently on three separate occasions. The reported variability (SD) therefore reflects inter-experiment biological variability rather than within-assay technical impression.

Statistical analysis

All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Comparisons between treated and untreated groups were analysed using a two-tailed Student's *t*-test. Statistical significance was considered at $p < 0.05$. GraphPad Prism 9 software (GraphPad Software, USA) was used for analysis and data visualisation.

RESULTS

Effects of HO18 in patient-derived fibroblasts

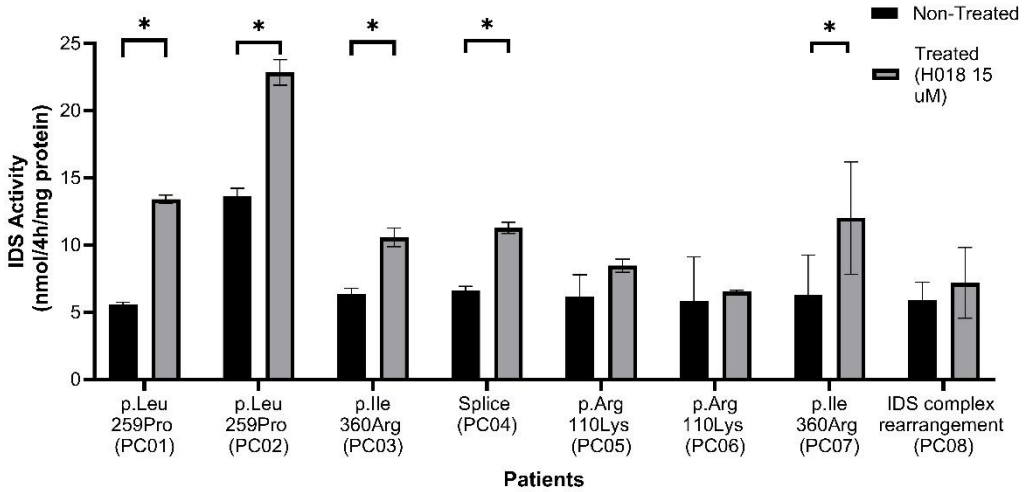
Treatment of patient fibroblasts with 15 µM HO18 for 96 hours resulted in increased IDS in a mutation-dependent manner (Figure 1, Table 1). Fibroblasts carrying p.Leu259Pro (n=2), p.Ile360Arg (n=2) and a splice variant exhibited

approximately 1.5 to 2.0-fold increase in IDS activity compared with untreated controls ($p < 0.05$). No significant change was observed in fibroblasts with complex IDS gene rearrangements ($p > 0.05$).

In parallel, total GAG quantification (Figure 2) revealed significant reductions in fibroblasts carrying p.Ile360Arg (-70.9 %, $p = 0.0083$) and p.Arg110Lys (-39.8%, $p = 0.0121$). However, fibroblasts harbouring p.Leu259Pro or splice variants, despite showing higher IDS activity, did not consistently display reductions in GAG levels (Table 1). This indicates that enzyme recovery does not always translate directly into substrate reduction, possibly due to limited catalytic turnover or the short treatment duration. For p.Leu259Pro, GAG measurements showed marked variability, with standard deviations similar to the mean values. Because of this variability, it is difficult to determine whether HO18 consistently increased or reduced GAG levels in this variant, since individual samples displayed different baseline levels and responses to treatment across experiments.

Figure 1

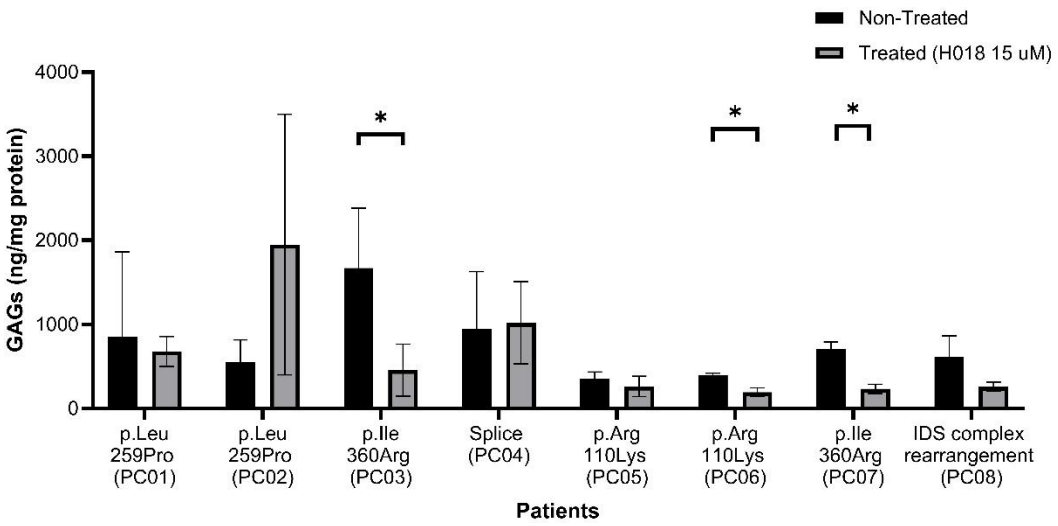
Effects of HO18 on IDS activity in patient-derived fibroblasts



Note: Fibroblasts from MPS II patients with different IDS mutations were incubated with HO18 (15 µM). Non-treated cells received culture medium only. IDS activity was determined fluorometrically and normalised to total protein. Data are presented as mean ± SD from three independent experiments (n = 3). p < 0.05 compared with untreated controls.

Figure 2

Effects of HO18 on glycosaminoglycans (GAG) levels in patient-derived fibroblasts



Note: Fibroblasts from MPS II patients with different IDS mutations were incubated with HO18 (15 µM). Non-treated cells received culture medium only. GAG content was quantified using the DMB assay. Significant reductions were observed in fibroblasts carrying p.Ile360Arg and p.Arg110Lys mutations. Data are presented as mean ± SD from three independent experiments (n = 3). p < 0.05 compared with untreated controls.

Effects of HO18 in recombinant IDS mutants expressed in HEK293T cells

A similar mutation-dependent pattern was observed in the recombinant system (Figure 3, 4; Table 2). Among the tested variants, p.Asn63Asp showed a significant increase in IDS activity (from 14.83 ± 1.16 to 17.94 ± 1.16 nmol 4MU/4 hours/mg protein; p = 0.0009), while p.Arg88His which has inactive catalytic sites showed no improvement. GAG quantification revealed notable reductions in p.Asn63Asp (-33.0 %, p = 0.0388) and p.Leu314Pro (-34.8 %, p = 0.0026) mutants following HO18 treatment. Several recombinant IDS variants, including p.Leu67Pro, p.Ala85Thr, p.Tyr108Ser and p.Pro231Leu, showed no significant changes in IDS activity or GAG levels following HO18 treatment.

Comparative summary

Overall, HO18 exhibited mutation-specific chaperone-like effects in patient-derived fibroblasts and recombinant IDS mutants expressed in HEK293T cells. Fibroblasts with p.Ile360Arg and p.Leu259Pro mutations showed the most consistent enzyme enhancement. In recombinant IDS mutants, HO18 increased IDS activity in p.Asn63Asp, whereas p.Leu314Pro exhibited a significant reduction in GAG levels without a detectable increase in IDS activity. The absence of effect in p.Arg88His which was a catalytically inactive variant (Balzano et al., 1997), supports the notion that HO18 acts by stabilising partially functional IDS proteins rather than restoring activity to inactive enzymes.

DISCUSSION

This study investigated the pharmacological chaperone potential of heparin octadecasaccharides (HO18) in MPS II using two complementary *in vitro* systems: patient-derived fibroblasts and recombinant IDS mutants expressed in HEK293T cells. The findings indicate that HO18 produces mutation-dependent effects on IDS activity and cellular GAG accumulation, which are not uniformly coupled across all variants. While some mutations showed increased IDS activity accompanied by reduced GAG levels, other variants exhibited inconsistent responses, including GAG reduction without detectable enhancement of IDS activity or increased IDS activity without corresponding substrate clearance. Importantly, the pattern of response differed between the two systems, reflecting the influence of genetic background and expression context on HO18 effects.

The concentration of 15 µM HO18 was chosen based on previous studies showing effective chaperone activity of similar glycosaminoglycan fragments on recombinant IDS with minimal cellular toxicity (Omar Affandi et al., 2023). MTT assays demonstrated no significant reduction in cell viability up to 18 µM, indicating that 15 µM lies within the non-toxic range for cell-based experiments. This concentration was therefore selected to minimise cytotoxic effects while allowing assessment of HO18's stabilising capacity. The consistent responses observed across both fibroblast and recombinant systems suggest that this concentration is suitable for assessing mutation-dependent chaperone effects *in vitro*. Although not intended for dose optimisation, the reproducible changes in IDS activity and GAG levels indicate that 15 µM provides a reliable experimental reference point for future comparative analyses.

In patient-derived fibroblasts, HO18 increased IDS activity in p.Leu259Pro, p.Ile360Arg and splice variants, whereas significant GAG reductions occurred in p.Ile360Arg and p.Arg110Lys. These findings indicate that HO18 can modulate IDS activity and substrate accumulation in a mutation-dependent manner, although the underlying mechanisms appear to differ across variants (Hoshina et al., 2018; Valenzano et al., 2011). For p.Leu259Pro, GAG measurements showed substantial variability across experiments, making it difficult to determine whether HO18 consistently increased or reduced GAG levels. This variability likely contributes to the apparent dissociation between increased IDS activity and the absence of a clear GAG reduction with the experimental timeframe.

However, the dissociation between increased enzyme activity and GAG clearance observed in some fibroblast lines, particularly p.Leu259Pro and splice variants, indicates that enzyme recovery does not always translate directly into substrate clearance within the experimental timeframe. Similar patterns have been reported in other lysosomal disorders, where rescued enzymes exhibit partial catalytic efficiency or insufficient lysosomal delivery, leading to delayed substrate degradation (Díaz et al., 2020; Leal et al., 2023). HO18 showed no detectable effect on complex IDS rearrangement variants, consistent with the requirement for residual enzyme function for responsiveness to small-molecule modulators.

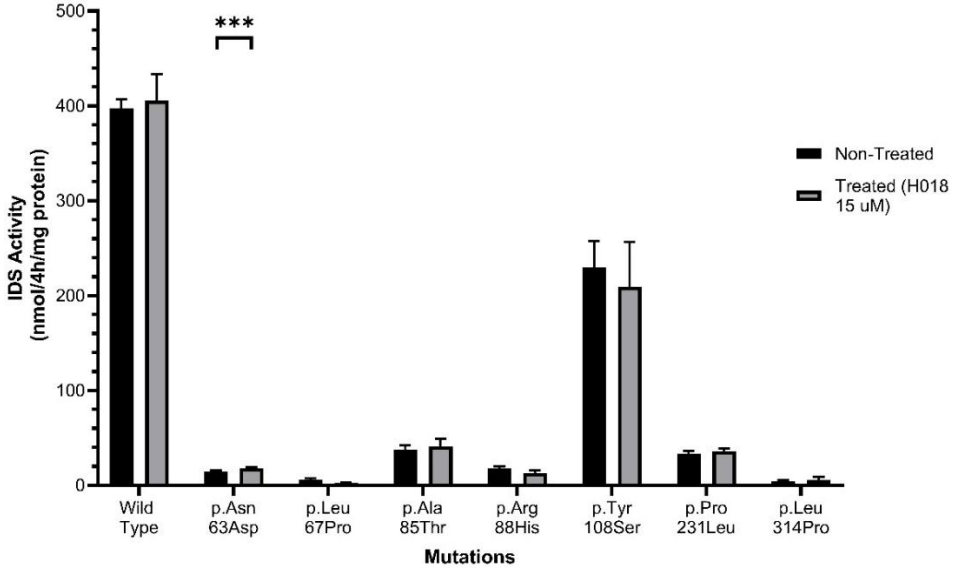
In recombinant IDS mutants, HO18 increased IDS activity in p.Asn63Asp, and reduced GAG levels in p.Asn63Asp and p.Leu314Pro, while catalytically inactive variants such as p.Arg88His remained unresponsive. These observations indicate that responsiveness to HO18 is restricted to IDS variants retaining some degree of functional integrity, although the specific pattern of response varies between activity enhancement and substrate reduction (Cubero et al., 2024). The differences between patient-derived and recombinant systems likely arise from intrinsic differences in protein folding, trafficking and post-translational processing between endogenously expressed IDS in fibroblasts and heterologous expressed protein in HEK293T cells. Although recombinant models are valuable for high-throughput evaluation, patients' fibroblasts remain a more physiologically relevant system for assessing the therapeutic potential of small molecule chaperones in MPS II (Hoshina et al., 2018; Keyzor et al., 2023).

The mutation-specific responses observed in this study align with the known biochemical impact of these variants.

The p.Ile360Arg and p.Leu259Pro mutations are located in structurally sensitive regions of the IDS protein that may affect local folding stability rather than the catalytic site, making them potentially responsive to chaperone binding. In contrast, p.Arg88His is situated near the catalytic pocket and disrupts substrate recognition, which likely explains the lack of response. The low but measurable basal activity observed in p.Arg88His is likely due to the highly efficient recombinant expression/trafficking machinery and highlights the limitation of using such systems to mimic the complex native cellular environment. These mutation-specific differences emphasise the importance of stratifying candidate molecules according to molecular phenotype rather than treating all genotypes uniformly.

Figure 3

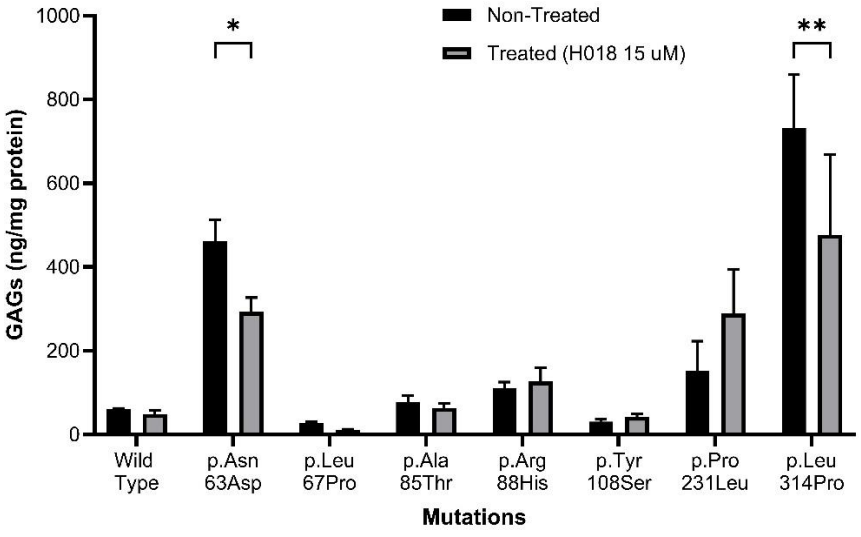
Effects of HO18 on IDS activity in recombinant IDS mutants expressed in HEK293T cells



Note: Cells were transfected with IDS constructs carrying selected mutations and were incubated with HO18 (15 µM). Non-treated cells received culture medium only. IDS activity was normalised to total protein. A significant increase was observed in p.Asn63Asp ($p < 0.05$). Data are shown as mean ± SD from three independent experiments ($n = 3$). $p < 0.05$ compared with untreated controls.

Figure 4

Effects of HO18 on glycosaminoglycans (GAG) levels in recombinant IDS mutants expressed in HEK293T cells.



Note: Cells were transfected with IDS constructs carrying selected mutations and were incubated with HO18 (15 µM). Non-treated cells received culture medium only. HO18 treatment led to reduced GAG in cells expressing p.Asn63Asp and p.Leu314Pro mutants ($p < 0.05$). Notably, for p.Leu314Pro, the reduction in GAG levels occurred in the absence of a statistically significant change in IDS activity. Data are expressed as mean ± SD from three independent experiments ($n = 3$). $p < 0.05$ compared with untreated controls.

Table 1

Summary of IDS activity and GAG level in patient-derived fibroblasts treated with HO18

Mutation	IDS activity (Non treated)[#]	IDS activity (HO18)[#]	Fold change	*p*-value	GAG levels (Non treated)^	GAG levels (HO18)^	Percentage change (%)	*p*-value
p.Leu259Pro (n=2)	9.61±4.44	18.13±5.21	1.89	<0.0001	701.5±681.1	1313±1208	+87.2	0.3944
p.Ile360Arg (n=2)	6.32±1.90	11.29±2.79	1.79	0.0007*	1187±696.9	345±233.6	-70.9	0.0083*
Splice variant	6.64±0.29	11.28±0.42	1.70	0.0003*	948.1±679.6	1019±488.4	+7.5	0.9261
p.Arg110Lys (n=2)	6.01±2.32	7.50±1.11	1.25 (n.s.)	0.1817	375.8±58.88	227.2±92.18	-39.5	0.0121*
Complex IDS rearrangement	5.87±1.35	7.19±2.64	1.23 (n.s.)	0.3168	611.5±253.1	259.2±56.16	-57.6	0.1085

*Note: Data represent mean ± SD values from three independent replicates (n=3); n.s. = not significant. [#]IDS activity, nmol 4MU/4 hours/mg protein. ^GAG level, ng/mg protein. *Statistically significant compared with untreated control (p < 0.05).*

Table 2

Summary of IDS activity and GAG levels in recombinant IDS mutants expressed in HEK293T cells

Mutation	IDS activity (Non treated)[#]	IDS activity (HO18)[#]	Fold change	*p*-value	GAG levels (Non treated)^	GAG levels (HO18)^	Percentage change (%)	*p*-value
Wild type (control)	397.4±9.44	405.5±28	n.s.	0.4946	61.67±2.23	49.04±19.17	n.s.	0.2421
p.Asn63Asp	14.83±1.16	17.94±1.16	1.21-fold	0.0009*	438.5±65.12	293.6±58.41	-33.0	0.0388*
p.Leu67Pro	5.75±2.00	2.31±0.82	n.s.	0.0673	27.56±6.50	11.63±2.45	n.s.	0.1065
p.Ala85Thr	37.24±4.80	41.28±7.57	n.s.	0.2882	77.26±31.74	64.17±21.06	n.s.	0.2187
p.Arg88His	17.92±2.37	13.26±2.82	n.s.	0.1121	110.4±30.47	127.0±66.23	n.s.	0.7720
p.Tyr108Ser	229.9±27.57	209.1±47.09	n.s.	0.5041	30.89±11.01	42.76±12.90	n.s.	0.3202
p.Pro231Leu	33.15±3.36	36.35±2.41	n.s.	0.0831	151.8±123.3	289.60±181.00	n.s.	0.0603
p.Leu314Pro	4.29±1.55	5.63±3.30	n.s.	0.6067	731.8±221.5	476.9±271.6	-34.8	0.0026*

*Note: Data represent mean ± SD of three independent experiments; n.s. = not significant. [#]IDS activity, nmol 4MU/4 hours/mg protein. ^GAG level, ng/mg protein. *Statistically significant compared with untreated control (p < 0.05).*

These results highlight both the promise and limitations of HO18. The positive responses in selected fibroblasts and recombinant variants indicate potential therapeutic benefit because these effects were observed in patient-derived fibroblasts, which are the most clinically relevant *in vitro* system. Nevertheless, the inconsistent reduction of GAG and limited coverage across diverse mutations make its potential unclear. The study included only a limited set of mutations and therefore does not represent the full mutational spectrum of MPS II. The treatment duration was short, which may have underestimate the true potential of HO18 to reduce GAG storage. Pharmacological chaperones are unlikely to be effective in patients carrying null or active-site mutations, as shown by the lack of effect in p.Arg88His.

Future investigations should therefore expand to a broader range of IDS mutations and include longer treatment durations to evaluate the kinetics of enzyme rescue and GAG clearance. *In vivo* studies in animal models of MPS II will be essential to determine the pharmacokinetic behaviour, biodistribution and safety of HO18. Since unmodified heparin fragments may not penetrate the brain effectively, approaches such as chemical modification, conjugation with transport carriers, or direct intrathecal administration could be considered. Potential strategies such as nanoparticle-based delivery systems, prodrug design or conjugation to transport carriers may improve cellular uptake and tissue targeting of HO18 derivatives. Combining pharmacological chaperones with enzyme replacement or substrate reduction therapy may also yield additive or synergistic effects by enhancing enzyme stability and intracellular activity (Fernández-Pereira et al., 2021; Grabowski & Hughes, 2022; Leal et al., 2023).

Overall, HO18 exhibited mutation-selective effects on IDS activity and cellular GAG accumulation across both patient-derived fibroblasts and recombinant IDS mutants. While some response patterns were compatible with chaperone-like behaviour, other outcomes were not fully explained by classical pharmacological chaperone mechanisms. These findings highlight the complexity of HO18-IDS interactions and underscore the importance of mutation-stratified evaluation. Further studies will be required to clarify the mechanisms underlying these effects and to assess the translational relevance of HO18 in appropriate *in vivo* models. Together, these findings provide a mutation-specific *in vitro* framework for understanding HO18 effects on IDS-related pathology, while highlighting the need for further mechanistic clarification and validation beyond cell-based models.

CONCLUSION

The present study demonstrates that heparin octadecasaccharides (HO18) show mutation-selective effects on IDS activity and cellular GAG accumulation in *in vitro* models of mucopolysaccharidosis type II (MPS II). HO18 enhanced IDS activity and/or reduced GAG levels in a subset of IDS variants, while for other variants it showed limited or no response. These patterns indicate that HO18 effects are dependent on the underlying molecular phenotype and are not uniformly explained by a classical pharmacological chaperone mechanism.

These findings indicate that small-molecule compounds such as HO18 may be beneficial only for selected IDS mutations, supporting treatment approaches tailored to individual mutation profiles in MPS II. Future studies should focus on HO18's pharmacokinetics and safety profile and evaluating its therapeutic effect in *in vivo* MPS II models. The integration of pharmacological chaperones with existing treatments such as enzyme replacement or substrate reduction therapy, may provide a more effective approach to improve clinical outcomes in MPS II. While HO18 shows promise for selected IDS mutations, further studies are required to assess its *in vivo* efficacy, safety and translational potential.

AUTHOR CONTRIBUTIONS

Affandi Omar conceptualised the study, performed data analyses, interpreted the results and drafted the manuscript. Affandi Omar, Fatimah Diana Amin Nordin, Nur Azian Aziz, and Siti Aishah Mohd Erham conducted the experiments and contributed to manuscript preparation. Salina Abdul Rahman, Balqis Kamarudin, Rosnani Mohamed and Mohd Khairul Nizam Mohd Khalid contributed to the experimental protocol design and data acquisition. Nur Jannaim Muhamad, Lock Hock Ngu, and Huey Yin Leong assisted with patients' recruitment, sample collection, and clinical data management. Julaina Abdul Jalil and Mohd Shihabuddin Ahmad Noorden supervised the study and provided critical revision of the manuscript. All authors have read and approved the final version of the manuscript.

ETHICS APPROVAL

Primary fibroblasts from patients with MPS II were obtained with written informed consent from parents or legal guardians, in accordance with the Declaration of Helsinki. The study protocol (NMRR-20-669-54509), including the collection and use of patient-derived fibroblasts, was reviewed and approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia (KKM/NIHSEC/P20-1999). All experimental procedures adhered to national and institutional guidelines for research involving human samples.

FUNDING

This study was supported by the Ministry of Health Research Grant (NMRR ID-20-669-54509). The funding body had no role in study design, data collection and analysis, decision to publish or manuscript preparation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest in this work.

ACKNOWLEDGEMENT

The authors would like to thank the Director General of Health Malaysia and the Director of the Institute for Medical Research (IMR) for their permission to publish this article. We thank Dr. Suraiami Mustar and Ts. Dr. Foo Phiaw Chong for critical reading of the manuscript and valuable comments. We also acknowledge the technical support from the staff of the Inborn Errors of Metabolism and Genetics Unit, Institute for Medical Research (IMR), and the assistance of clinicians from the Department of Genetics, Hospital Kuala Lumpur, for providing patient samples and clinical data.

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Citation:

Omar, A., Abdul Rahman, S., Amin Nordin, F. D., Aziz, N. A., Mohd Erham, S. A., Kamarudin, B., Mohamed, R., Mohd Khalid, M. K. N., Muhamad, N. J., Ngu, L. H., Leong, H. Y., Abdul Jalil, J., & Ahmad Noorden, M. S. (2026). Heparin octadecasaccharides influence iduronate-2-sulphatase activity in a mutation-dependent manner in Mucopolysaccharidosis type II. *Life Sciences, Medicine and Biomedicine*, *10*(1). https://doi.org/10.28916/lsmb.10.1.2026.239

Life Sciences, Medicine and Biomedicine
ISSN: 2600-7207