Glycans to improve efficacy and solubility of protein aggregation inhibitors

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Misfolding and subsequent aberrant self-assembly of certain proteins into toxic amyloid deposits are hallmarks of various diseases, most notably neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (Chiti and Dobson, 2017). Aromatic residues in amyloidogenic proteins have been shown to be key factors in protein oligomerization and fibrilization, mostly driven by π-π interactions. Together with aromaticity, post-translational modifications can greatly affect a protein's solubility and conformation and, as a consequence, its propensity to aggregate. Among post-translational modifications, this perspective focuses on protein glycosylation, the decoration of a protein with carbohydrate motifs, its effect on amyloid formation and its employment in the inhibition of protein aggregation.

Glycosylation can strongly alter the physicochemical properties of a protein by restraining, limiting or increasing the number of possible conformations it can adopt. An emblematic example of the effect of glycosylation on protein aggregation is provided by the protein tau, found aggregated in neurofibrillary tangles in AD and other tauopathies. Altered glycosylation patterns of tau were reported in post-mortem brain samples of AD patients (Frenkel-Pinter et al., 2017), and it has been demonstrated that different types (Frenkel-Pinter et al., 2017; Losev et al., 2019) and positions (Losev et al., 2020) of glycosylation can either exacerbate or reduce the propensity of tau to form tangles. Along the same line, mannosylation and galactosylation of the prion protein were reported to reduce its propensity for amyloid formation (Lin et al., 2014). Similarly, addition of advance glycation end-products to the backbone of islet amyloid polypeptide, associated with type II diabetes, caused the formation of distinct, less toxic aggregates (Milordini et al., 2020).

While the detailed mechanisms underlying the effects of glycans are not fully understood, it has been proposed that the decoration of the backbone of a protein with carbohydrates can lead to alternative, more stable conformations, reducing the number of aggregation-prone species that can be generated upon misfolding. Furthermore, due to their hydrogen:oxygen ratio, carbohydrates can expand the number of interactions with the solvent, increasing the solubility of the protein. It has also been postulated that the pyranose ring of glycans could disturb aromatic interactions by conferring steric hindrance and preventing the early events required for the self-assembly of the monomers into pathogenic amyloid species.

These beneficial effects of glycans on protein stability and solubility have been exploited *in vitro* to mitigate the aggregation of amyloidogenic proteins. One approach is exemplified by PHF6, a highly aggregative synthetic hexapeptide derived from full-length tau that recapitulates the aggregating behavior of the wild type protein. A glycosylated derivative of PHF6 was found to exhibit enhanced solubility and attenuated aggregation propensity *in vitro*. In addition, co-incubation of the glycan-modified PHF6 with non-modified PHF6 resulted in inhibition of the aggregation of the latter (Frenkel-Pinter et al., 2016). The underlining causes of these effects may include the steric hindrance brought by the glycan, which could limit the conversion of PHF6 into β-sheet rich conformations, and the increment in the number of hydrogen bonding with polar amino acids. The glycosylated peptide may also have adopted energetically favorable conformations that allowed a higher number of CH–π stacking interactions with aligned aromatic side chain of nearby residues.

Despite these reported beneficial effects of glycosylation on aggregation-prone proteins, glycosylated variants of peptides have not yet been employed as therapeutics to reduce aggregation of the cognate amyloidogenic proteins. We postulate that the reason could lie in the complexity of understanding the molecular consequences and causes of different types of glycosylation and of their positions along the protein sequence, together with the limited drugability of peptides. To overcome these limitations, an alternative, simpler approach could be to combine the advantages offered by carbohydrates and peptides, i.e. increased polarity and aromaticity, into the design of a new class of hybrid small-molecule inhibitors of protein aggregation. Conjugating carbohydrates to small molecule inhibitors could be therapeutically beneficial, since glycans can improve their solubility, interfere with aromatic interactions of amyloids, as well as improve the penetration of the drug through the blood brain barrier, increase its metabolic stability and protect it from denaturation and oxidation.

Small aromatic molecules have proved to be a promising avenue for developing anti-aggregation inhibitors. Arenes are believed to interact with the aromatic amino acids in the target protein by intercalating into the π-π stacking and interfering with the self-assembly process of amyloid formation. Polyphenols are among the most common aromatic molecules reported to target successfully amyloids species, including those formed by Aβ, tau and α-synuclein (Joshi et al., 2016). The most common terminal structure found in phenol-based inhibitors of protein aggregation is catechol, a phenol with an additional hydroxyl group, underlining the importance of both the phenyl and the hydroxyl groups in targeting aggregation-prone proteins. One of our own attempts along these lines exemplifies the importance of both aromatic and hydroxyl elements for mitigating amyloid aggregation. In that study, we conjugated dopamine (DA), a catecholamine acting as neuromodulator in the central nervous system, with naphthoquinone (NQ), an organic aromatic compound previously shown to be able to interfere with the aggregation of amyloidogenic proteins (Paul et al., 2020b). DA was employed as a source of hydroxyl groups and NQ as a source of phenyl group. This combinatorial chemistry resulted in a hybrid molecule, naphthoquinone-dopamine (NQDA) that was capable not only of preventing the aggregation of α-synuclein, but also of disrupting its pre-formed aggregates into smaller species more efficiently than its parent molecules DA and NQ (Paul et al., 2020b). Intriguingly, NQDA was also found to cross a human blood brain barrier model (Paul et al., 2020b).

Despite these successes, this molecule presented a few limitations: it exhibited certain toxicity toward mammalian cell lines when employed at higher concentrations and it displayed limited solubility in aqueous media. To overcome these issues, we aimed at combining simple carbohydrates with small molecules to generate a new class of aggregation inhibitors. We explored the use of the amino acid tryptophan in place of the commonly used phenols as an aromatic core because its side chain consists of a 6-member benzene ring and a 5-member pyrrole ring, hence it can establish the highest number of π-π interactions among all amino acids. We conjugated tryptophan with one-unit glycans, which not only represent an abundant

source of new potential hydrogen bonds but, as described above, are also natural elements that could have profound impact on the conformational rearrangements of proteins. In a first work, we demonstrated that a tryptophan-glucosamine conjugate could slow down the aggregation of the tau-derived hexapeptide PHF6 into amyloids even at very low molar ratios (Paul et al., 2019a). The tryptophan-glucosamine hybrid was also able to disaggregate pre-formed PHF6 fibrils. Molecular dynamics simulations were employed to gain insight into the mechanism of the aggregation inhibition and revealed that, while the tryptophan side chain interacted mainly with the aromatic residues of the target peptides, these interactions were assisted by the formation of a net of hydrogen bonds involving the glucosamine. In a follow-up work, to evaluate the effect of glycans with different chemical modifications, we explored the conjugation of three galactosylamine variants to tryptophan as novel hybrid agents to target Aβ and islet amyloid polypeptide aggregation (Paul et al., 2020a). All tested tryptophan-glycan hybrid molecules mitigated aggregation of the target amyloids, leading to the formation of fewer, smaller, amorphous and non-toxic oligomers. The most effective hybrid molecule contained tryptophan conjugated to N-acetyl galactosylamine, underlining the importance of fine-tuning each position of the hexose ring of the glycan for improving efficacy. Notably, the two separate components, tryptophan and galactosylamine, displayed hardly any amyloid inhibitory effect, highlighting the power of synergistic effect of the conjugates.

Combining the advantages of the above described hybrid molecules, in yet another study we explored the conjugation of naphthoquinone-tryptophan (NQTrp) as a targeting moiety to the carbohydrate mannitol, previously shown to display inhibitory effect towards α-synuclein *in vitro* and in animal models, resulting in reduction of Parkinson's disease-related phenotypes (Paul et al., 2019b). The most powerful among the tested NQTrp-mannitol hybrids was a mannitol conjugated to NQTrp via a linker of three PEG units, which also exhibited higher inhibitory effect towards aggregation and cytotoxicity of α-synuclein compared to the two individual parent molecules.

Collectively, these findings indicate that the immense number of variants offered by glycan chemistry presents a rich source for designing novel powerful small molecules for targeting amyloid aggregation in various diseases. We believe that single-unit glycans, in combination with aromatic amino acids, represent a promising future generation of protein aggregation inhibitors. To expand this new class of inhibitors, other aromatic amino acids different from tryptophan, such as phenylalanine, tyrosine and their non-proteinogenic derivatives, could be explored. To further improve these novel aromatic amino acid-based inhibitors, conjugation of glycans displaying specific functionalities, such as antioxidant power or ability to binding to specific receptors, could be further exploited to create molecules that function both as protein aggregation inhibitors as well as tackle other therapeutic aspects of the same targeted disease.

To the best of our knowledge, no such hybrid has been tested yet in an animal model. However, we are confident that, with further optimization by medicinal and combinatorial chemistry techniques, these small molecules could attain improved bioavailability, blood-brain-barrier penetration and metabolic stability, compared to currently-used small molecule inhibitors of protein aggregation.

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