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The importance of the glycosylation of antimicrobial peptides: natural and synthetic approaches

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Glycosylation is one of the most prevalent post-translational modifications of a protein, with a defining impact on its structure and function. Many of the proteins involved in the innate or adaptive immune response, including cytokines, chemokines, and antimicrobial peptides (AMPs), are glycosylated, contributing to their myriad activities. The current availability of synthetic coupling and glycoengineering technology makes it possible to customise the most beneficial glycan modifications for improved AMP stability, microbicidal potency, pathogen specificity, tissue or cell targeting, and immunomodulation.

Introduction

AMPs are ubiquitous, ancient, and highly effective host defense compounds that are a prominent aspect of the early innate immune response to infection. They vary in sequence and length, but are generally less than 30 amino acids, with a tendency to have a cationic charge that attracts them to bacterial membranes. Their mode of action is also diverse, ranging from direct integration and permeabilisation of the cell wall, binding with nucleic and enzyme targets, to indirect activity, such as immunomodulation of the host. AMPs are often synthesised in an inactive form and then post-translationally cleaved into an active state.

Glycosylation increases the protein and/or peptide diversity and extends their range of functionality. Four distinct types of glycosylation are currently known and being profoundly studied: *N*-, *O*-, *C*-, and *S*-glycosylation. The type of the glycosylation depends on the nature of the sugar–peptide bond and can be further diversified on the base of glycosidic linkage, glycan composition, structure, and length. *N*-type glycosylation, in which glycan is attached to the amino group of asparagine, is well studied. Oligosaccharides attach covalently upon recognition of the Asn-X-Ser/Thr (where X is any amino acid) via a nitrogen atom. Many bacterial glycosyltransferases are already in use for *in vitro* controlled glycosylation, resulting in the field of protein glycoengineering [1,2].

O-linked glycosylation is a dynamically explored field because of its potent role in mammalian pathophysiological processes. Defects in glycosylation in humans have broadly studied links to different diseases and malfunctions [3]. *O*-linked glycosylation is characterised by the covalent attachment of glycan through an oxygen atom. However, the *O*-linked consensus, unlike the *N*-linked one, is not as easily predictable [4]. It is initiated by the attachment of GalNac to Ser/Thr, but can also comprise *O*-linked β-*N*-acetylglucosamine; thus, classification of *O*-glycans is based on their initiating monosaccharide [5]. The glycan polymer can vary in heterogeneity, which makes the prediction of glycan building blocks variable *in vivo*. Additionally, the further branching of the *O*-glycans involves multiple glycosyltransferases, and our understanding of their function and structure is still in a discovery stage.

Protein *C*-linked glycosylation differs fundamentally from *N*- and *O*-glycosylation and defines a type of glycosylation in which a carbohydrate is linked to a protein via a carbon atom. In eukaryotes, *C*-glycosylation has been detected in multiple cell lines (e.g., human RNase 2 and IL-12 and rat liver microsomes) [6]. The biological role of *C*-glycosylation is still being elucidated, although the interest of the pharmaceutical industry in the *C*-glycosylation of proteins is related to its unique resistance to metabolic hydrolysis [7]. For *C*-mannosylation, the acceptor sequence comprises W-x-x-W, where the first Trp becomes *C*-mannosylated [8].

S-linked glycosylation is the most recent discovery, described for the first time as a post-translational modification of the

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sublancin glycopeptide [9], later also found in other bacteriocins [10]. In *S*-linked glycosylation, the hexose residue is linked to the thiol group of cysteine. An *in vitro* study of the glycotransferase SunS showed that the enzyme can transfer glucose as well as any hexose to multiple cysteine residues [9]. Therefore, the relaxed substrate specificity of *S*-glycotransferase is similar to that of *N*-glycosylation, and might have wide application in protein and/or peptide engineering.

By 1998, glycosylation had been described in five naturally occurring insect-derived AMPs, all *O*-linked, and proline rich: diptericin, drosocin, formaecin, lebocin, and phyrrorricin [11]. Whereas *O*-linked glycosylation appears to favour proline-rich regions, modifications are sometimes also observed in glycine-rich regions [12]. A curated database of AMPs describes many hundreds of different AMPs from humans, fishes, insects, reptiles, amphibians, molluscs, and crustaceans (http://aps.unmc.edu/AP/main.php). Although most are post-translationally modified in some manner, such as *C*-terminal amidation, cyclisation, or the formation of disulfide bridge(s), only a small fraction, around a dozen or so, are listed as carrying glycosylation motifs (most of these so-called 'gAMPs' are insect derived). Therefore, gAMPs represent a small subclass of AMPs, with broad-spectrum effects [11]. It is becoming clear that the glycosylation of both natural and synthetic AMPs can influence their antimicrobial activity, and their ability to affect host immunity, target specificity, and biological stability [13,14] (Fig. 1).

Glycosylation and antimicrobial activity

O-linked glycosylation of proline-rich AMPs has an important, and often essential, role in their antimicrobial activity. By far the most abundant type of *O*-glycosylation of prokaryotic and eukaryotic peptides is the *N*-acetylgalactosamine (GalNAc) attachment to serine (Ser) or threonine (Thr) by a glycosidic linkage, as occurs in *O*-mannosylation, *O*-fucosylation, or *O*-glucosylation [15–17].

The importance of glycosylation has been well studied among the insect AMPs, such as diptericin and formaecin and the bacteriocin-family member, enterocin F4-9 [18,19]. In all the above-mentioned peptides, the absence of glycosylation abolishes their antimicrobial activity. Treatment of enterocin with the deglycosylating enzyme, *N*-acetylglucosaminidase, cleaved the *O*-linked GlcNac linkage from Ser and Thr residues and resulted in a loss of activity [20]. In fish, the glycosylation and glycation of skin gelatin peptides showed enhanced activity against multiple species of bacteria, as well as antioxidant activity [21]. Finally, plants also produce gAMPs to defend against fungal pathogens. One recently identified gAMP, datucin, contains a terminal GlcNac-asparagine, which shows activity against both planktonic and biofilm *Candida albicans*, and even against multidrug-resistant clinical strains [22].

Evidence of glycosylation affecting the function of human AMP is scarcer, but has been documented [17]. Eosinophil cationic protein (ECP) is exceptionally large compared with typical AMP, but shares many of the same properties with the smaller peptides, such as membrane-lysis activity and affinity towards lipopolysaccharide (LPS) [15]. It is one of many proteins that are presynthesised and stored in granules in readiness to be released upon infection by parasitic invaders. Given that this is a relatively nonspecific process, in the sense that it can be triggered by many different species, diversity among the sequences and the post-translational modifications of AMP is advantageous [16,17]. Indeed, the level of glycosylation can account for as much as 3 kDa of the total mass of the ECP. Concurrently, damage to the host is a harmful consequence of such broad activity. The extent to which the native form ECP is glycosylated with *N*-linked oligosaccharides is influential. It is currently thought that heavily glycosylation of ECP limits cytotoxicity, and only upon degranulation is the protein deglycosylated to its low-molecular-weight form, which shows greatest cytotoxicity and enhanced lysis of model liposomes [16]. Further evidence that glycosylation of this AMP protects the host from harm comes from the identification of a single nucleotide polymorphism that introduces an additional locus for glycosylation, which reduces cytotoxicity without affecting catalytic activity [17,23].

Glycosylation of AMP does not necessarily result in generation of an efficacious peptide and can sometimes lead to a loss of activity or functionality. As an example, the prokaryotic AMP lysostaphin becomes glycosylated when expressed in mammalian cells, and loses potency against its target, *Staphylococcus aureus* [24]. Huang *et al.* identified the precise amino acid glycosylation site on lysostaphin that resulted in the peptide being able to bind, but no longer lyse, *S. aureus*. A single amino acid substitution prevented glycosylation and restored lysostaphin lytic activity [24]. Therefore, it is important to take into consideration the differential expression of peptides among hosts to avoid undesired types of glycosylation [25].

Finally, there are some remarkable examples of AMPs that are not themselves glycosylated but whose function depends upon a larger glycoprotein. Jellein is one such AMP, located within the larger amino acid sequence of major royal jelly protein 1 (MRJP1). Mannosylation of MRJP1-derived peptides facilitates the agglutination of bacteria through interaction with lectin receptors, whereupon jelleins are able to lyse the bacteria in an example of synergy between glycosylated proteins and nonglycosylated peptides [26].

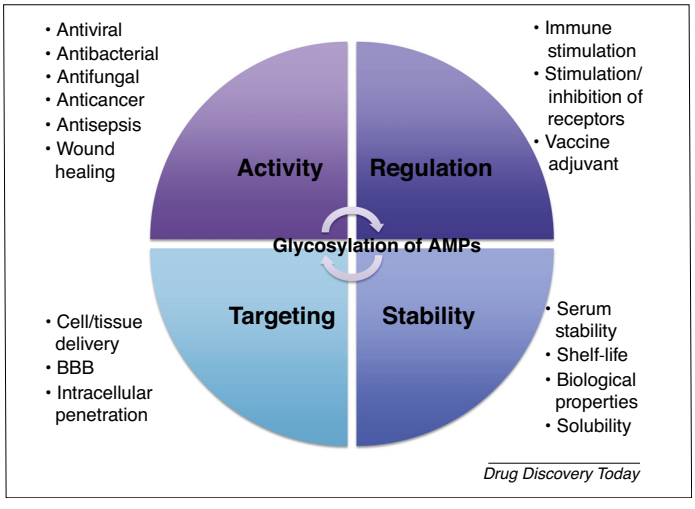

FIGURE 1

The broad-spectrum effects of glycosylation and its possible utilisation for antimicrobial peptide (AMP) optimisation. Abbreviation: BBB, blood–brain barrier.