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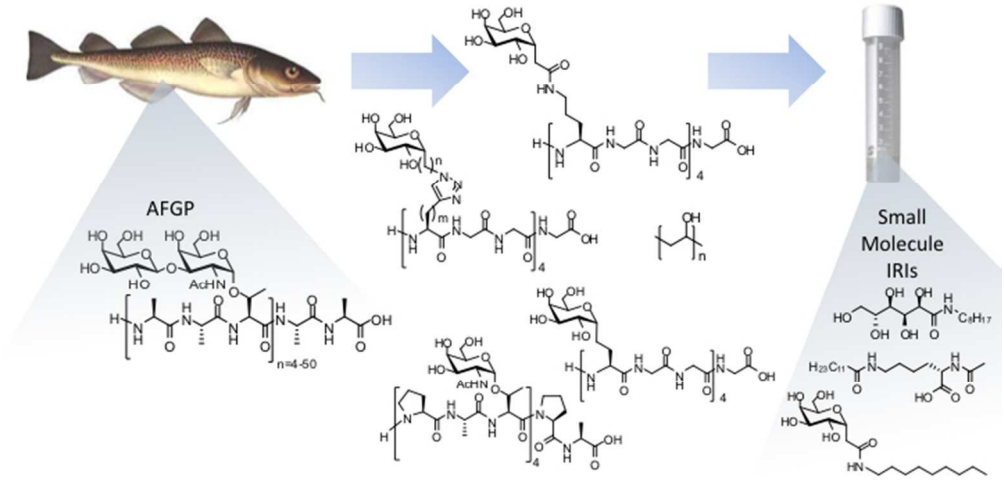
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ARTICLE

Designing Ice Recrystallization Inhibitors: From Antifreeze (Glyco)Proteins to Small Molecules

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Ice recrystallization occurs during cryopreservation and is correlated with reduced cell viability after thawing. Therefore, ice recrystallization inhibition (IRI) activity is a very desirable property for an effective cryoprotectant. Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) were the first compounds discovered with this property, however they are poor cryoprotectants due to their unique ability to bind to ice and alter habits of ice crystals. Consequently, AFGP analogues with "custom-tailored" antifreeze activity have been developed which exhibit potent IRI activity but do not bind to ice. Subsequent to this, it was reported that simple mono- and disaccharides exhibit moderate IRI activity and this has ultimately facilitated the discovery of several small carbohydrate-based ice recrystallization inhibitors with IRI activity similar to that of native AFGP-8. This represents a major advancement in the field of ice recrystallization inhibitors (IRIs). The recent developments of IRIs will be reviewed, focusing on novel small molecules that have great potential for use as cryoprotectants.

1. Introduction

Cryopreservation involves the use of very low sub-zero temperatures (>-180 °C) to store cells, tissues and other biological materials. At temperatures close to -196 °C all biochemical processes are effectively stopped, permitting storage for long periods of time. Ice recrystallization is a process that occurs predominately during the storage and thawing cycles of cryopreservation, and this process correlates with reduced post-thaw cell viability in a variety of cells.[1-5] The detrimental effects of ice recrystallization are exacerbated during the thawing phase of cryopreservation, and rapid warming rates are often employed to decrease the rate of recrystallization.[5,6]

Recently, the cryopreservation of certain progenitor cells such as hematopoietic stem cells[7] has become increasingly important with the recent advances in regenerative medicine therapies.[7,8] To date, treatment of spinal cord injury,[9] coronary artery disease,[9] heart attacks,[10] stroke,[11] various cancers, genetic diseases, immune deficiencies and blood disorders[12] have benefited from regenerative therapies. However, the outcome of a regenerative therapy is closely correlated to the quality and number of cells recovered post-thaw.[13] Reduced post-thaw cell viabilities as a result of cryo injury caused by the freezing process and/or ice recrystallization is still a major problem. Consequently, the development of small-molecule ice recrystallization inhibitors with the ability to protect cells against cryo injury during cryopreservation is a worthwhile goal that has not yet been realized.

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2. The Phenomenon of Ice Recrystallization

The phenomenon of recrystallization has been extensively studied in the metallurgical and geological sciences and many comprehensive reviews have been written on the subject.[14-18] This phenomenon also occurs in ice and can adversely affect biological materials when cryopreserved.[1,19-22] While ice recrystallization can occur during the freezing, storage and thawing cycles of cryopreservation, the rates are fastest when the frozen sample is being thawed. Ice recrystallization is a thermodynamically driven process whereby ice crystals grow larger at the expense of smaller ice crystals (Ostwald ripening), resulting in an overall reduction in free energy.[6,23-25]

The most common form of ice at atmospheric pressure is the hexagonal ice (I_h) lattice unit. This consists of a regular crystalline structure with an ordered arrangement of intermolecular hydrogen bonds between water molecules, in which a single oxygen atom is hydrogen-bonded to two hydrogen atoms.[26-28] In an ice crystal, water molecules at the bulk-water/ice crystal interface are higher in free energy than water molecules inside the ice crystal because they are not able to form the optimum number of hydrogen bonds. Therefore, smaller ice crystals having a higher surface area to volume ratio are thermodynamically less stable than large ice crystals having a lower surface area to volume ratio. As the system attempts to lower its free energy, water molecules migrate from an ice

crystal through a semi-ordered quasi-liquid layer (QLL). This layer is found between an ice crystal and bulk-water.[29-33] The water molecules then migrate to bulk-water and are subsequently transferred to a growing ice crystal, resulting in a net overall decrease in total surface area to volume ratio, ultimately reducing the free energy of the system.[24] During this process, the total amount of ice and liquid water within the sample remains constant while the number of ice crystals decrease and the average size of the ice crystals increase.

3. Biological Antifreezes

Nature has evolved "antifreezes" that protect organisms inhabiting sub-zero environments against cryo injury. These compounds are peptide-based and can be found in various plants, insects, and fish.[28,34-39] Scholander and co-workers first reported an abnormally low freezing temperature of blood serum in Arctic fish that was not explained by colligative effects of dissolved solutes in the blood.[40-42] Analysis of blood plasma revealed that salts only accounted for super-cooling to -0.8 °C.[43] It was subsequently discovered that antifreezes present in the blood plasma were responsible for the additional freezing point depression to -1.9 °C.[44,45] There are two classes of "antifreezes" that have the ability to depress the freezing point of blood serum or aqueous solutions: antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs).

AFPs found in fish are classified into four different categories: type I,[35-37,46-52] type II,[53,54] type III,[54-63] and type IV.[64-66] AFPs are also present in many insects such as the spruce budworm moth (*Choristoneura fumiferana)*,[67,68] yellow mealworm beetle (*Tenebrio molitor)*,[69,70] fire-coloured beetle (*Dendroides Canadensis)*,[71] and the snow flea.[72] In addition, AFPs are also found in plants,[73-80] fungi, and bacteria.[81-86] These proteins range in size from approximately 3 to 24 kDa and can have α-helix, β-sheet, globular or random coil secondary structure in solution. For instance, type I fish AFPs adopt α-helices in solution and this secondary structure is responsible for antifreeze activity (TH and IRI).[28,35,37,38,87] Recently, a newly discovered AFP from Antarctic yeast (*Glaciozyma antarctica*) displays four α-helices that were hypothesized to be responsible for TH and IRI activity.[88] To investigate this, several peptide fragments derived from the residues of the α-helical regions of the parent AFP were synthesized and analysed for both TH and IRI activity. While these peptide fragments possessed both TH and IRI activity, they were much less active than the wild type protein. Analysis of secondary structure by NMR and molecular dynamics simulation suggested a correlation between the degree of helicity and TH and IRI activity.[88]

AFGPs make up 3-4% of the blood serum of Antarctic notothenoids and Arctic cod and are classified by molecular mass.[89] AFGP-1 has a molecular mass of 33.7 kDa and AFGP-8 has a molecular mass of 2.6 kDa.[89] The typical structure of an AFGP consists of an alanine-alanine-threonine (Ala-Ala-Thr)$_n$ tripeptide repeat in which the hydroxyl group of the threonine is glycosylated with β-D-galactosyl-(1-3)-α-*N*-acetyl-D-galactosamine (Fig. 1).[90] Amino acid variations are present in some lower molecular weight AFGPs where the first alanine residue is replaced with a proline, or the glycosylated threonine is replaced with an arginine.[43,91-95] While the solution structures of AFPs have typically been well defined, the exact secondary structure adopted by AFGPs has been debated. Studies suggested that AFGPs adopt an ordered helix similar to a polyproline type II (PPII) structure[96-99] or an amphiphilic helix with a hydrophobic face from exposed methyl groups and a hydrophilic face from hydroxyl groups on the disaccharide.[52] Other studies suggest that the solution conformation of AFGPs is consistent with a random coil structure possessing short segments of localized order. [96,97,100]

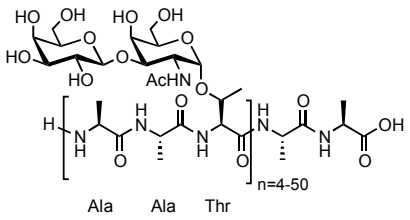

Fig. 1 Structure of native AFGPs.

AF(G)Ps exhibit two distinct types of antifreeze activity: thermal hysteresis (TH) and ice recrystallization inhibition (IRI) activity. Thermal hysteresis is defined as a localized freezing point depression relative to the melting point. The difference between these temperatures is the TH gap, in which the growth of ice is arrested.[101-103] This localized freezing point depression is the result of the irreversible binding of the AF(G)P to the surface of ice, that ultimately restructures the ice crystal giving rise to the characteristic single ice crystal habits (or shapes) observed with various AF(G)Ps.[101,103-105] Within the TH gap, a single ice crystal does not increase in size as the temperature of the solution is lowered below that of the melting point. However, at temperatures below the freezing point outside of the TH gap, the ice crystal will grow uncontrollably, often with extreme speed, into long spicules.[106] This rapid "burst" growth and change in ice crystal habit is hypothesized to be the reason why AF(G)Ps are poor cryoprotectants and often result in increased cellular damage and decreased cell viability post-thaw.[107-110] This property has been exploited for the cryo-ablation of various tumours.[111-113]

The second "antifreeze" property that AF(G)Ps exhibit is the ability to inhibit ice recrystallization. This property was discussed in section 2. Fig. 2 shows two images of ice crystals in frozen wafers generated in a splat-cooling assay after 30 minutes of annealing at -6.4 °C.[114-116] In panel A, large ice crystals are generated in the presence of phosphate buffered saline (PBS). This solution serves as a positive control for ice recrystallization. Other controls utilized in IRI assays include sodium chloride (NaCl), calcium chloride ($CaCl_2$) or 30-45% (w/v) sucrose solutions.[20,25,114,117,118] Panel B depicts the ice crystals in the presence of an ice recrystallization inhibitor and thus, much smaller ice crystals are observed after the 30 minute annealing period. The ability to maintain small ice crystal sizes

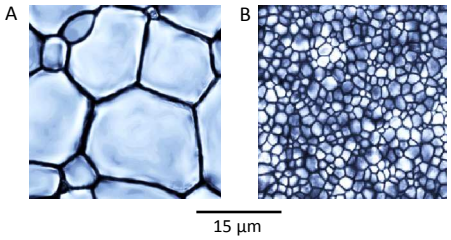

Fig. 2 Photographs of ice crystals from a splat-cooling assay depicting: A) no inhibition of ice recrystallization and B) inhibition of ice recrystallization.

is a very desirable property for many medical and commercial applications in addition to cryopreservation. For example, AF(G)Ps have been used to prevent ice recrystallization in frozen foods such as ice cream and frozen meats.[119-125] However, the difficulties associated with producing large quantities of these proteins have limited their use in industrial settings which has prompted the synthesis of AFGP analogues.

4. Antifreeze Analogues with "Customized" Activity

AF(G)Ps are potent inhibitors of ice recrystallization, however, the TH activity associated with these compounds prevents their use as cryoprotectants. The temperatures associated with cryopreservation of cells and tissues are well below the TH gap. This results in increased cellular damage because of the ability of AF(G)Ps to bind to the surface of ice and modify the shape of a growing ice crystal.[107,108] Consequently, there has been increasing efforts to design compounds that have the ability to inhibit ice recrystallization, but do not exhibit TH activity. This work has been difficult as the specific structural features necessary for IRI activity have not been clearly identified. To date, a number of structure-function studies have generated peptide and glycopeptide analogues of AFGPs. Some of these analogues are very effective inhibitors of ice recrystallization while others are not. The results of these studies are outlined in the following sections.

4. 1 *C*-linked Antifreeze Glycoprotein (*C*-AFGP) Analogues

The first AFGP analogues reported to inhibit ice recrystallization but exhibit very little TH activity were *C*-linked AFGP analogues **1-4** (Fig. 3).[126] Native AFGP-8 contains a disaccharide moiety covalently linked to a threonine residue *via* a carbon-oxygen bond, but these analogues possess a carbon-linked (*C*-linked) α-D-galactosyl unit conjugated to the ε-amine of lysine in a $(Lys\text{-}Gly\text{-}Gly)_n$ tripeptide repeat. The carbon linkage at C1 of the pyranose residue (*C*-linkage) is more resistant to hydrolysis under acidic, basic or enzymatic conditions. Although monomer **1** and analogue **2** with three tripeptide repeating units did not exhibit IRI activity, analogues **3** and **4** containing six and nine tripeptide repeats exhibited moderate IRI activity. However, **3** and **4** also exhibited very small TH gaps of 0.06 °C and formed hexagonal shaped ice crystals, indicating these compounds interacted with the ice lattice.[126]

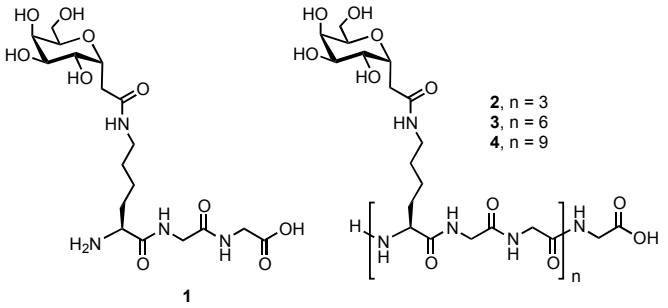

Fig. 3 Structures of first generation *C*-linked AFGP analogues **1**-**4**.

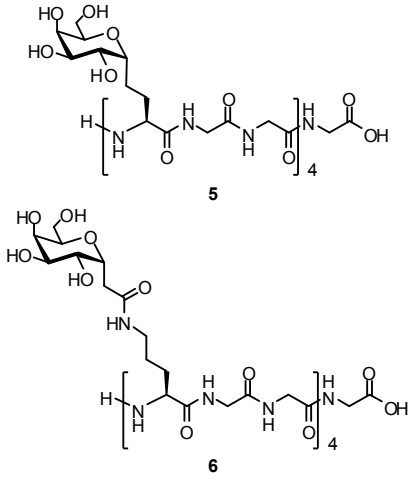

Fig 4. Structures of IRI active *C*-linked AFGP analogues **5** and **6**.

Second-generation AFGP analogues **5** and **6** (Fig. 4) were potent ice recrystallization inhibitors and showed activity comparable to that of AFGP-8 at equimolar concentration.[116,127] Analogue **5** is a *C*-linked serine-based derivative that contains four tripeptide repeating units with a *C*-linked galactosyl moiety. In analogue **6**, the *C*-linked galactosyl unit was coupled to the γ-amine of ornithine in the tripeptide repeat. At a very low concentration (5.5 μM), analogue **5** possessed IRI activity but not any measurable TH activity or dynamic ice-shaping capabilities.[127] Similarly, **6** possessed a very high degree of IRI activity without TH activity, at the same concentration as **5**, although it exhibited weak dynamic ice shaping.[116] These *C*-linked AFGP analogues were the first examples of compounds with "custom-tailored" antifreeze activity, meaning they had potent IRI activity without the undesirable property of TH activity.

Following this, additional studies by the Ben group have identified structural features that are important for the IRI activity of these compounds. Analogues of **6** in which the galactosyl moiety was modified were prepared to elucidate the importance of the carbohydrate residue. This included glucose derivative **7**, mannose derivative **8**, and talose derivative **9** (Fig. 5A). While galactose analogue **6** was highly IRI active, analogue **7** was only moderately active and mannose and talose derivatives **8** and **9** were inactive (Fig. 5B).[116] This was

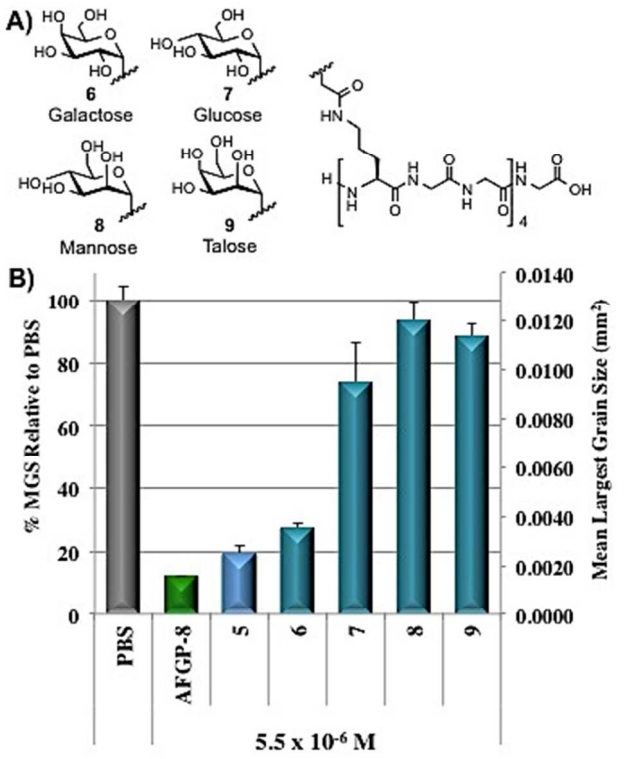

Fig 5. A) Structures of *C*-linked AFGP analogues **6-9** containing various monosaccharide moieties. **B)** IRI activity observed in a splat-cooling assay[116] of *C*-linked AFGP analogues **6-9** at 5.5 μM in PBS.[116,131] IRI activity is represented as % mean grain size (MGS) of ice crystals relative to a phosphate buffered saline (PBS) positive control for ice recrystallization.[115,116]

surprising as the only difference between these derivatives is the configuration of the C2 and/or C4 carbons of the pyranose residue, highlighting how sensitive IRI activity is to small structural changes.

Further investigation revealed that the relative orientation of hydroxyl groups on a pyranose ring (ie. axial *vs.* equatorial) affects the hydration of a carbohydrate in aqueous solution,[128-130] suggesting that carbohydrate hydration influenced IRI activity.[116] Hydration of a molecule is defined as the number of non-exchangeable water molecules associated with a solute to allow the solute to be compatible with, or to "fit" in, the three-dimensional hydrogen-bonded network of bulk-water. Carbohydrates that are more highly hydrated do not fit well into bulk water and tend to disorder this three-dimensional hydrogen-bond network. Galactose, with an axial hydroxyl group at C4 and an equatorial hydroxyl group at C2, is a highly hydrated carbohydrate and fits poorly into the three-dimensional bulk-water network. In contrast, talose, with axial hydroxyl groups at both the C2 and C4 positions, is less hydrated and has a better fit with the hydrogen-bond network of bulk water.

From TH experiments, *C*-AFGP analogues **6-9** did not interact with the ice lattice and did not exhibit any dynamic ice shaping activity.[116] It was therefore hypothesized that these compounds were inhibiting ice recrystallization by altering the structure of bulk-water and/or the QLL (Fig. 6). This ability was thought to be correlated to the hydration of the

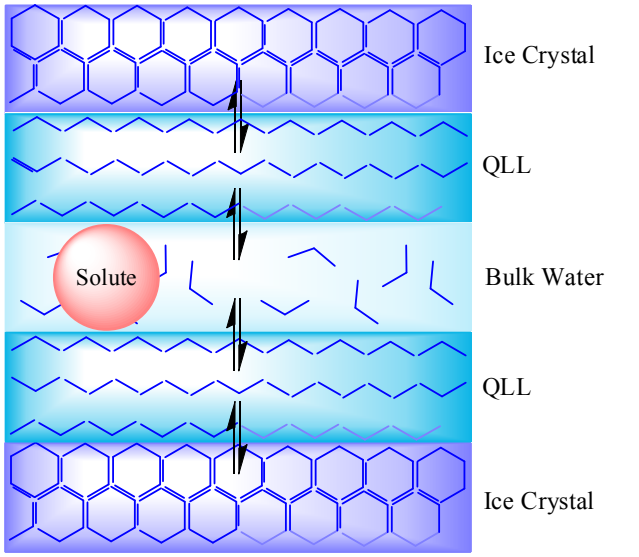

Fig 6. Illustration of the proposed mechanism by which carbohydrates and *C*-AFGP analgoues inhibit ice recrystallization. A solute will reside at the QLL–bulk water interface between two adjacent ice crystals. A greater disruption in the ordering of water molecules within these layers makes the transfer of water molecules to ordered ice crystals less favourable, resulting in smaller ice crystals.

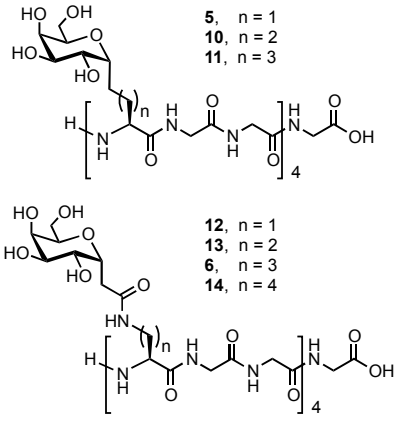

Fig 7. Structures of *C*-linked AFGP analogues containing different side-chain linker lengths between the monosaccharide residue and the polypeptide backbone.

carbohydrate residues.[116,131] Consequently glycopeptide **6**, with the more highly hydrated galactose residues, disrupts the ordering of water molecules in the QLL or bulk-water to a greater extent than talose-containing glycopeptide **9**. Due to this disorder in the presence of **6**, the addition of a water molecule from a more disordered QLL to an ordered ice crystal lattice is thus energetically unfavourable resulting in inhibition of ice recrystallization (Fig. 6). Conversely, if the QLL was more ordered, as in the case of talose derivative **9** which "fits" well within bulk water, the addition of water molecules to the ice crystal would be more favourable resulting in less inhibition and larger ice crystals.[131]

In addition to the carbohydrate residue, the Ben group has also examined how length of the linker between the monosaccharide moiety and the polypeptide backbone affects IRI activity of **5** and **6** (Fig. 7). The analogues of **5**, containing

three (**10**) and four (**11**) carbons in the linker, were both inactive.[127] Only analogue **5** with a two-carbon chain connecting the galactosyl residue to the polypeptide backbone, was highly IRI active. Similarly, analogues of **6** containing a total number of four (**12**), five (**13**), or seven (**14**) atoms in the side-chain conjugating the carbohydrate to the peptide backbone were inactive.[132] Molecular dynamic (MD) simulations suggested that **6** adopted a unique conformation in solution where the linker chain was "folded", creating a hydrophobic pocket between the carbohydrate and the polypeptide backbone. Only derivative **6** containing six atoms within the side-chain adopted this conformation, and **12-14** adopted a conformation where the carbohydrate was extended away from the peptide. This folded conformation with the hydrophobic pocket was proposed as the reason **6** was so active while **12-14** were inactive.[132]

In summary, two *C*-linked AFGP analogues, **5** and **6**, with dynamic structural modifications to the AFGP structure have been identified as novel potent IRIs.[116,127] These compounds are classified as "custom-tailored" antifreeze analogues as they exhibit IRI activity similar to AFGP-8 at equimolar concentration, however, they do not possess the undesirable activity for cryopreservation, TH or dynamic ice shaping. Structure-function studies have indicated that the potent activity of **6** is highly influenced by the carbohydrate moiety, and that a highly hydrated galactose residue is crucial for this activity.[116] These studies also highlighted the correlation of IRI to carbohydrate hydration for these glycopeptides. Finally, for both derivatives **5** and **6** there is an optimal number of atoms in the side chain connecting the carbohydrate residue to the polypeptide backbone that retains potent activity. For galactosyl *C*-serine derivative **5** a two-carbon linker is optimal for activity, whereas for galactosyl-ornithine derivative **6**, six atoms is optimal. An increase or decrease in these optimal linker lengths abrogates activity.[132]

4.2 Other AFGP Analogues

In addition to *C*-linked AFGP derivatives, several other AFGP analogues have been reported. The Sewald group prepared analogues of AFGPs in which the native disaccharide was replaced with an *N*-acetyl-D-galactosamine residue and the $(Ala\text{-}Ala\text{-}Thr)_n$ tripeptide was repeated three, four or five times (**15-17** respectively, Fig. 8).[133] Analogues containing proline residues (**18-21**) were also prepared as proline has a restricted angle of rotation and can cause a protein to adopt a distinct secondary structure in solution.[133] Furthermore, in native AFGPs alanine residues are occasionally substituted by proline.[43,91-95,134] Analogues **15-17** showed strong inhibition of ice recrystallization in a 45% (w/v) sucrose-based IRI assay and induced hexagonal ice crystal shaping in this solution indicating an interaction with the ice lattice. Proline-containing analogues **18-20** were only slightly IRI active at higher concentrations

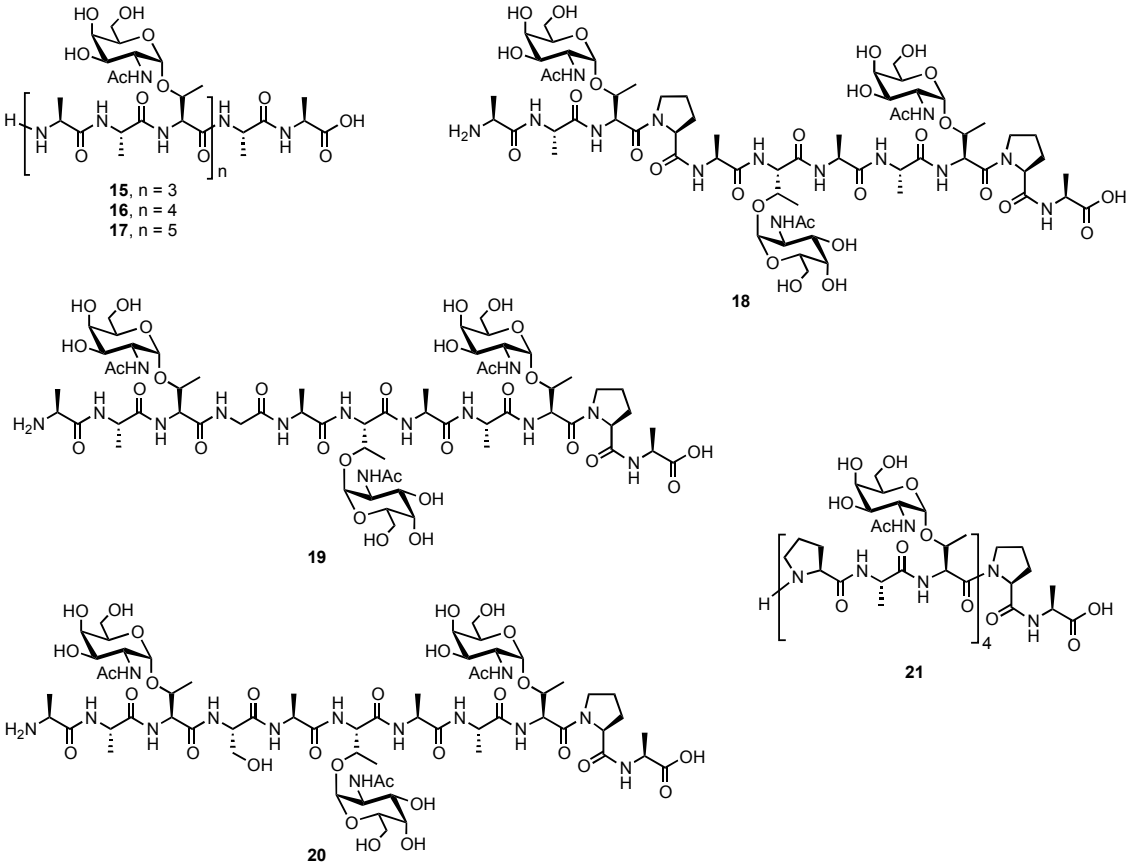

Fig. 8 Structures of AFGP analogues by Sewald group.[133]

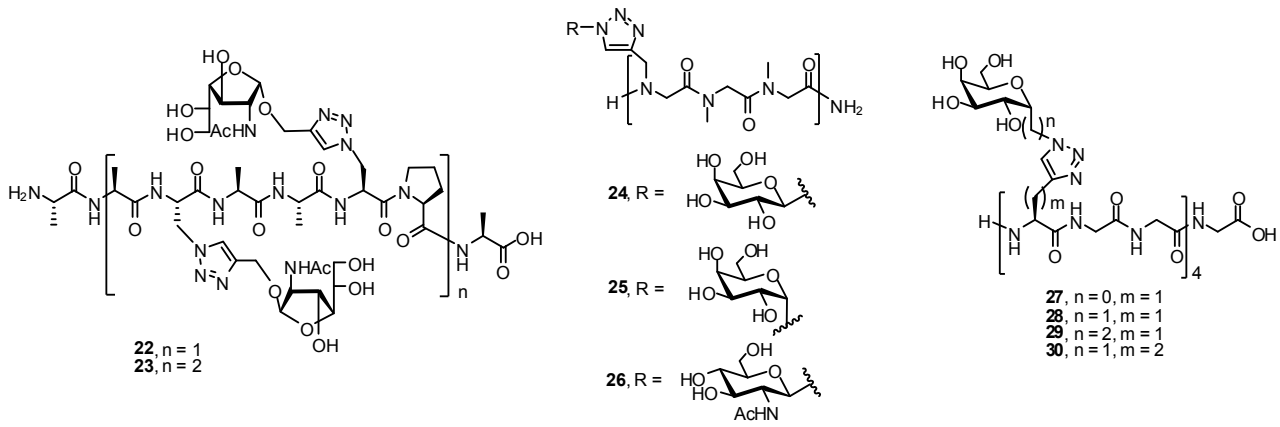

Fig 9 Structures of triazole-containing AFGP analogues by the Brimble,[135] Sewald,[136] and Ben[137] groups.

than analogues **15**-**17**, indicating that the flexibility of the peptide backbone is important for IRI activity. However **21** containing the tripeptide repeat (Pro-Ala-(GalNAc)Thr)$_n$ was only slightly less IRI active than corresponding Ala-containing analogue **16**, demonstrating that the regular incorporation of proline retains high IRI activity.[133]

Triazole-containing AFGP analogues have also been reported by the Brimble,[135] Sewald,[136] Ben[137] and Liskamp[138] groups. The triazole moiety was employed as a synthetic strategy to quickly and efficiently synthesize AFGP analogues utilizing "click" chemistry *via* the copper(I)-catalyzed Huisgen azide-alkyne cycloaddition (CuAAC reaction). The structures of some of these analogues are shown in Fig. 9. While analogues **22** and **23,** containing a triazole-linked furanose *N*-acetyl glucosamine residue conjugated to the peptide backbone were not assessed for IRI activity, they did not exhibit TH activity or dynamic ice shaping capabilities.[135] Similarly, triazole-containing peptoid analogues **24**-**26** did not exhibit IRI activity or ice shaping capabilities.[136] Analogues **27**-**30** which are based on the IRI active *C*-linked AFGP analogue **6** exhibited very weak inhibition of ice recrystallization, indicating that the replacement of the amide moiety in the side chain with a triazole is detrimental for IRI activity.[137] Furthermore, Liskamp and co-workers also demonstrated that replacing amide bonds within the polypeptide backbone of *C*-linked AFGP analogue **6** with triazole-linkages also abrogates IRI activity (structures not shown).[138] Collectively, these data indicate that although "click" chemistry is an efficient synthetic strategy for the preparation of AFGP derivatives, thus far triazole-containing derivatives have failed to exhibit high levels of IRI activity.

Overall, AFGP analogue structure-function studies have elucidated specific structural requirements necessary for IRI activity. These studies indicate that the polypeptide backbone and the number of tripeptide repeats is important, as well as the nature of the carbohydrate residues and side chain conjugating the carbohydrate to the polypeptide. While these analogues provided a very useful starting point for determining the structural features necessary for IRI activity, these compounds are not amenable to the large-scale synthesis necessary to produce sufficient quantities required for applications in cryopreservation. Consequently, significant interest has arose in synthetic polymers (section 4.3) and small molecule (section 5) IRIs.

4.3 Synthetic Polymers

The *O*- and *C*-linked glycoconjugates described in sections 4.1-4.2 involve complex, multi-step syntheses. Therefore, traditional synthetic polymers that are easily synthesized have been explored for their ability to inhibit ice recrystallization. The first observation that synthetic polymers could inhibit ice recrystallization was made by Knight *et al.* in 1995.[20] This study demonstrated that poly-L-histidine (**31**), poly-L-hydroxyproline (**32**), and polyvinyl alcohol (**33**, PVA) had IRI activity, while poly-L-aspartic acid (**34**), poly-L-asparagine (**35**), polyacrylic acid (**36**) and polyvinylpyrrolidone (**37**) did not (Fig. 10). However, in this study, many of these polymers were only assessed for IRI activity in water, with the exception of PVA, which retained activity in a NaCl solution. The presence of solutes (salts or sucrose) in the solution used to dissolve the analyte is required to negate false-positive ice recrystallization inhibition effects.[20]

Following this initial study, Inada showed that the IRI activity of PVA increased as the molar concentration and molecular weight of PVA was increased. The degree of activity exhibited by high molecular weight PVA was similar to a type I AFP at the same molar concentration. It is important to note however, that due to the larger difference in molecular masses, the mass quantity of PVA required for this activity was significantly higher than the AFP.[118] It has also been demonstrated that PVA exhibits very weak but measurable TH activity (0.037 °C at 50 mg/mL)[139] and dynamic ice shaping capabilities.[140] It is hypothesized that the spacing of the hydroxyl groups of PVA closely match those of the prism planes of the ice lattice, allowing adsorption of these planes.[140]

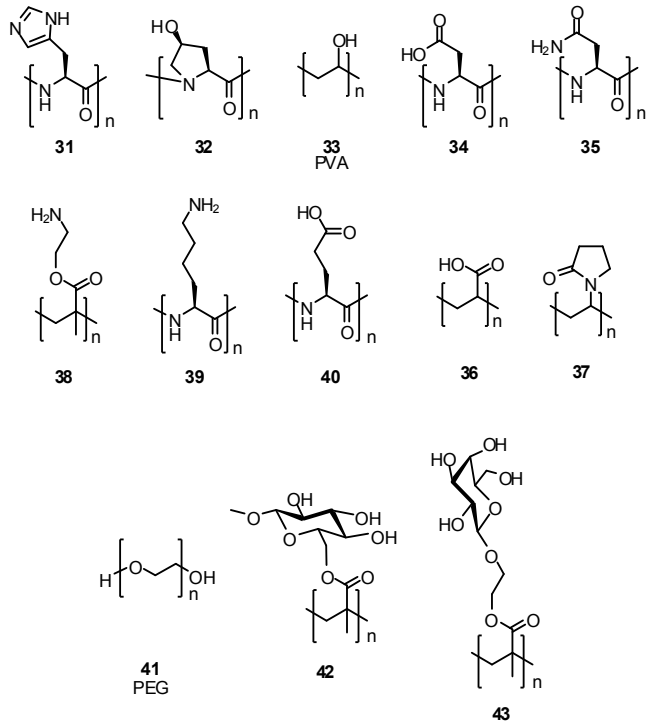

Fig. 10 Structures of synthetic polymers previously assessed for IRI.

The Gibson laboratory has also reported various other synthetic polymers with the ability to inhibit ice recrystallization.[141] Poly(2-aminoethyl methacrylate) (**38**), poly-L-lysine (**39**), poly-L-glutamic acid (**40**), and polyethylene glycol (**41**, PEG) were weakly IRI active[117] and polyacrylic acid (**36**) and poly-L-hydroxyproline (**32**) exhibited weak to moderate IRI activity which is in agreement with the previous study conducted by Knight *et al*.[20] The activity of poly-L-hydroxyproline (**32**) was determined to be concentration-dependent.[117] Poly-L-hydroxyproline (**32**) adopts a PPII helical structure[142] which is a proposed structure that AFGPs are suggested to adopt in solution (see section 3). However, it appears that this solution structure is not a requirement for IRI activity as PVA exhibits similar activity as poly-L-hydroxyproline, but PVA is unstructured in solution. Payne and co-workers further validated this hypothesis by demonstrating that many hydroxyproline-containing AFGP analogues that adopt PPII helical structures in solution fail to inhibit ice recrystallization.[143] The two structurally diverse glycopolymers **42** and **43** displayed only moderate IRI activity.[117] It was also shown that poly(tartar amides) significantly change the morphology of ice crystals, however, it is unknown to what extent ice recrystallization was inhibited.[144] Overall, of all polymers investigated, PVA exhibits the highest degree of IRI activity to date.

Recently, zirconium(IV) acetate was shown to be a strong inhibitor of ice recrystallization.[145] The conditions under which zirconium acetate presents ice recrystallization inhibition is thought to be in the structure of a polymer where the zirconium atoms are linked by the hydroxyl groups of the acetates (Fig.

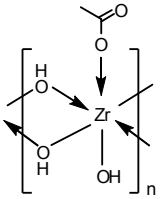

Fig. 11 Proposed structure of zirconium(IV) acetate polymer.

11). In one proposed mechanism of action, the acetate carbonyl oxygen and methyl group bind to the ice lattice. In this model, the distance between the hydroxyl groups matches the periodicity of an ice crystal. The side that does not interact with ice would contain hydroxyl groups to interact with bulk water.[146,147] This arrangement is similar to the binding faces of AFPs containing threonine.[148-150] Zirconium(IV) acetate is able to slow the growth of ice in a buffer solution but does not completely halt ice growth and therefore does not exhibit TH activity.

5. Small molecule ice recrystallization inhibitors

5.1 Simple Mono- and Disaccharide Derivatives

In 2008, the Ben laboratory demonstrated that the relative orientation of the hydroxyl groups of the carbohydrate moiety in *C*-linked AFGP analogue **6** influenced IRI activity.[116] This work led to the conclusion that carbohydrate hydration ultimately modulates IRI activity. The presence of a more hydrated galactose residue on the glycopeptide (derivative **6**) resulted in more potent IRI activity compared to less hydrated glucose, mannose or talose containing derivatives (**7-9**, Fig. 5). This was discussed in greater detail in section 4.1.

To further probe the correlation between carbohydrate hydration and IRI activity, the Ben group assessed the ability of simple mono- and disaccharides to inhibit ice recrystallization and correlated activity to hydration metrics.[131] Similar to the results with glycopeptides **6-9**, galactose was found to be the most IRI active monosaccharide, while talose was the least effective at inhibiting ice recrystallization. These results correlated well with the hydration parameters of these monosaccharides (Fig. 12).[128-130] A similar correlation was observed with the disaccharides where more hydrated disaccharides exhibited greater IRI activity. The most IRI active disaccharide was melibiose, followed by lactose, trehalose, maltose, and sucrose which exhibited the least activity (Fig. 12).

As previously discussed in section 4.1, the hydration of a carbohydrate is dependent upon the relative configuration of the hydroxyl groups on the pyranose ring.[128-130] Hydration is also dependent on the number of monosaccharide units present within the molecule as disaccharides (containing two monosaccharide units) typically have hydration numbers twice that of monosaccharides. However, in the study conducted by the Ben laboratory, disaccharides were not twice as IRI active as monosaccharides.[131] This result was attributed to the difference in total volume between mono- and disaccharides,

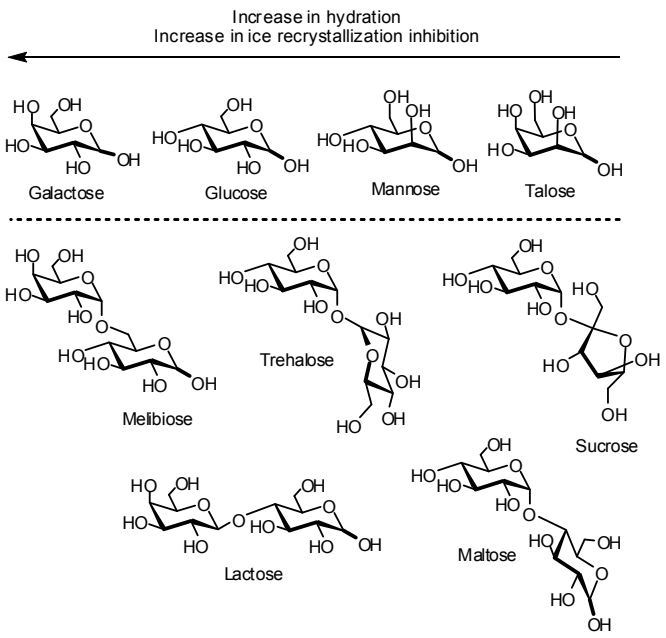

Fig. 12 Correlation between hydration of carbohydrates and IRI activity.

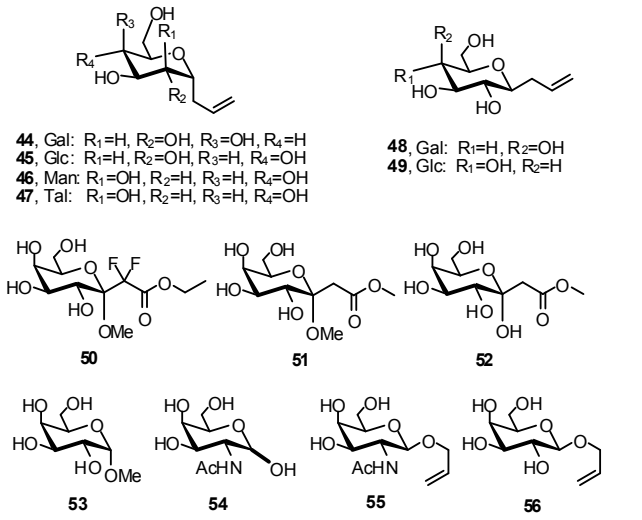

Fig. 13 Structures of monosaccharide derivatives assessed for IRI activity by the Ben group.

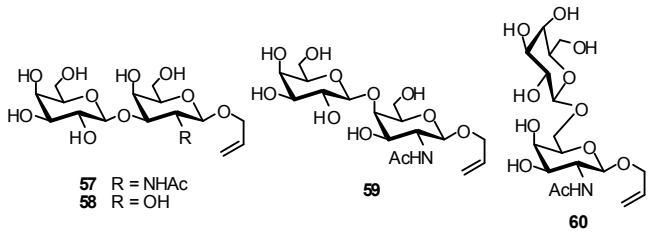

Fig 14. Structures of AFGP disaccharide.

where disaccharides with twice the hydration values also occupied twice the volume in aqueous solution compared to monosaccharides. This observation led to the correlation between IRI activity and the *hydration index* (HI) of the carbohydrate.[131] The HI is defined as the hydration number divided by the partial molar volume of the carbohydrate. It provided an indication of the degree of hydration of the substrate as a function of its size or volume. Using this metric, a linear correlation was observed between IRI activity and HI for all of the mono- and disaccharides assessed in this study. It should be noted that the activity of the mono- and disaccharides examined in this study was moderate despite the fact these compounds were assessed for IRI activity at a significantly higher concentration (22 mM) compared with glycopeptide derivatives **5** and **6** and AFGP-8.[116,127,131]

Following this work, a number of structurally different carbohydrate derivatives have been prepared and assessed for IRI activity (Fig. 13). Derivatives with a *C*-linked allyl group have been investigated containing both an α-linkage (analogues **44**-**47**) and a β-linkage (**48** and **49**) to various monosaccharide residues (Fig. 13).[131] Fluorinated derivatives, methoxy-containing derivatives, and derivatives with *C*-linked methyl esters conjugated to galactose have also been explored.[151] In addition, some C2 *N*-acetyl derivatives (this moiety is present in the native AFGP disaccharide) have been investigated (**54**-**56**) for IRI activity.[152] All of the analogues described above exhibited only moderate IRI activity.

Structure-function studies have demonstrated that the β-D-galactosyl-(1,3)-α-D-*N*-acetyl-galactosamine disaccharide in native AFGPs is an essential structural feature for TH activity.[153-155] However the importance of this moiety for inhibiting ice recrystallization has not been investigated. In fact, previously synthesized *C*-linked AFGP analogues that are potent inhibitors of ice recrystallization do not possess this disaccharide.[116] The Ben group explored the structural features of this disaccharide that were important for IRI activity.[152] Compounds **57**, **59**, and **60** were regioisomers of the native AFGP disaccharide containing a β-(1,3), β-(1,4), or β-(1,6) glycosidic linkage (Fig. 14). Disaccharide **58** was an analogue of **57** in which a hydroxyl group replaced the *N*-acetyl group. In native AFGPs, the oxygen of the threonine is glycosylated with the *N*-acetyl galactosamine. However, in these disaccharides, this was replaced with a β-linked *O*-allyl group.[131] While all disaccharides exhibited moderate IRI activity at 22 mM in PBS, **59** containing the β-(1,4)-glycosidic linkage was the most active. This was interesting as the native disaccharide in AFGPs possesses a β-(1,3)-linkage (similar to **57**), and this disaccharide was not the most IRI active. As these disaccharides were not conjugated to the $(Ala\text{-}Ala\text{-}Thr)_n$ polypeptide backbone, it is unknown whether a glycoconjugate containing the more IRI active disaccharide **59** would also be more effective at inhibiting ice recrystallization than the native AFGP.[152]

5.2 Carbohydrate-based surfactants and hydrogelators

In 2012, it was reported that small molecule carbohydrate-based surfactants and hydrogelators were capable of inhibiting

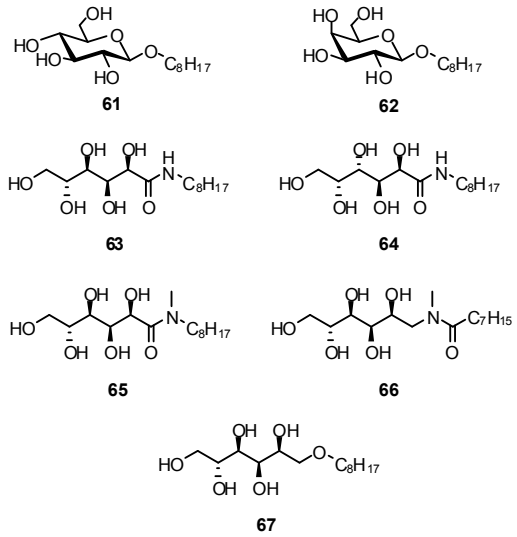

Fig. 15 Structures of carbohydrate-based surfactants and hydrogelators. β-Octyl-D-galactopyranoside **62** and *N*-octyl-D-gluconamide **63** are the first reported potent small molecule IRIs that were highly active at 22 mM and 0.5 mM, respectively.[156]

ice recrystallization. This was the first report where small molecules exhibited very potent IRI activity.[156] Many of these compounds were much more active than the previous reducing sugars. As described in section 5.1, highly hydrated carbohydrates tended to exhibit moderate IRI activity, and this activity was attributed to the ability of these compounds to disrupt the structure of bulk water.[116,131] It was therefore hypothesized that other compounds that have the ability to alter the structure of bulk water, such as surfactants, hydrogelators and organogelators, may also be effective IRIs.

The small-molecule surfactants and hydrogelators investigated in this study are shown in Fig. 15.[156] The glucose-derived non-ionic surfactant β-octyl-D-glucopyranoside **61** exhibited moderate IRI activity at 22 mM that was comparable to D-glucose alone while the galactose-based non-ionic surfactant β-octyl-D-galactopyranoside **62** was highly IRI active even at 11 mM. Both **61** and **62** are surfactants and form micelles in solution. However, it was subsequently determined that the ability to form micelles was not a prerequisite for potent activity.

In addition to surfactants, hydrogelators of the *N*-octyl-D-aldonamides, **63** and **64**, were also assessed for their ability to inhibit ice recrystallization. The glucose derivative, *N*-octyl-D-gluconamide **63**, was found to have unprecedented potent IRI activity at a low concentration of 0.5 mM (for comparison, all other compounds were tested at 22 mM).[156] To this date, this is the most active small molecule ice recrystallization inhibitor. Interestingly the galactose derivative, *N*-octyl-D-galactonamide **64** was weakly active at 0.5 mM. This was also the first instance where a glucose-based compound was more IRI active than a galactose-based compound. *N*-octyl-D-gluconamide **63** can form hydrogels at a concentration of 33 mM. However, the fact that **63** was highly active at 0.5 mM, suggested that activity was not related to the ability to form gels. *N*-Methylated

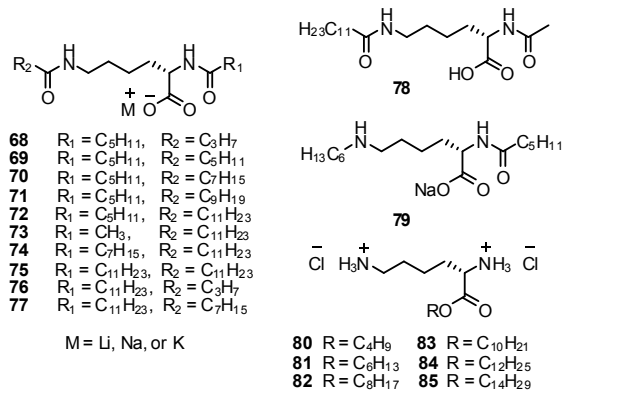

Fig. 16 Structures of lysine-based anionic and cationic surfactants.

analogues **65** and **66** had weak activity, indicating that the proton of the amide bond is important. In addition, replacement of the amide with an ether linkage (**67**) significantly decreased IRI activity, highlighting the importance of the amide bond. None of these compounds (**61-64**) were found to exhibit TH activity or dynamic ice shaping capabilities suggesting these compounds were not binding to ice, and solid-state NMR studies failed to indicate interactions with the ice lattice. This study concluded that the potent IRI activity of these compounds is not dependent upon micelle formation, gelation, or interaction with the ice lattice.[156] This work was the first example of small molecules exhibiting "custom-tailored" antifreeze activity that were as potent as AFGPs and AFGP analogues.

5.3 Lysine-based surfactants/gelators

Another class of surfactants possessing high levels of IRI activity are based on low-molecular weight organogelators.[157-161] In lysine analogues **68-77** (Fig. 16), the α- and ε-amino termini were acylated with alkyl chains of different lengths.[162] To increase solubility, the carboxylic acid was converted into a lithium, sodium, or potassium carboxylate salt, thereby creating an anionic lysine surfactant. When assessed as sodium salts for IRI activity, the most potent analogues had an alkyl chain of eleven carbons at R_2 (**72-75**) at 22 mM in a 0.5 mg/mL NaCl standard solution.[20] The alkyl chain length at R_1 did not significantly alter IRI activity. The presence of the carboxylate salt was important for IRI activity, as the IRI activity of carboxylic acid **78** was lower than the activity of the carboxylate salt direct analogue, **73**. This was the first study demonstrating that different positively charged counterions have various effects on IRI activity. When derivatives **70-73** were converted into lithium or potassium salts, the IRI activity was altered compared to the corresponding sodium salts. In carbohydrate-based IRIs, the amide moiety was found to be important for IRI activity.[156] Similarly, removing the carbonyl group from the amide bond in the lysine-based derivative (**79**) resulted in a loss of IRI activity when compared to the direct analogue containing the carbonyl group (**69**).

Cationic lysine derivatives were also investigated containing alkyl ethers of various lengths at the C-terminus

with both amines protonated as hydrochloride salts (**80-85**, Fig. 16). At 22 mM in a 0.5 mg/mL NaCl solution, the most active analogue contained a ten-carbon alkyl chain (**83**). Interestingly, increasing this chain to twelve or fourteen carbons, analogues **84** and **85** respectively, resulted in a decrease in IRI activity. The conclusions from these studies were that hydrophobic moieties are important for ice recrystallization inhibition and that the position and size of these hydrophobic domains also affects IRI activity. Therefore a delicate balance between hydrophobic and hydrophilic groups is important for IRI activity.[162]

5.4 Truncated *C*-Linked Glycopeptides

Recently, another structure-function study was reported with IRI active *C*-linked glycopeptide **6**. Given that IRI active carbohydrate-based and lysine-based surfactants and/or gelators contained hydrophobic moieties (Figs 15-16),[156,162] the *C*-linked glycopeptide was truncated and conjugated to hydrophobic alkyl chains to determine the smallest sub-unit that can possess potent IRI activity.[163] These *C*-linked substrates are illustrated in Fig. 17. Compounds **86-99** contain increasing alkyl lengths from zero to sixteen carbons. The effect of ionisable amine and carboxylic acid groups and alcohol groups were also explored using compounds **100-102**, respectively. Additionally, structurally diverse branched alkyl chains were investigated (**103-106**), as well as cyclic structures (**107** and **108**), and various alkenes and alkynes (**109-113**). Derivatives containing alkyl chains less than six carbons in length (**86-92**) were weak to moderately IRI active. The ionisable groups did not improve IRI activity as **100** and **101** were similar in activity to moderately active parent compound **90**. However, the presence of an alcohol in **102** did increase IRI activity. From the unsaturated alkyl chains (**109-113**), the analogues with the terminal alkene and alkyne (**109** and **110**) had improved activity while the remainder were moderately IRI active. Analogues with branched alkyl chains (**103-106**) were slightly less active than galactose at 22 mM and analogues containing rings (**107** and **108**) possessed IRI activity similar to galactose.

Interestingly, when the alkyl chain lengths were increased greater than six carbons in length (compounds **93** and **94**) a significant increase in IRI activity was observed. Both **93** and **94**, with seven and eight carbon atoms, respectively, exhibited very high IRI activity at 22 mM. A further increase in hydrocarbon chain length improved activity where analogues containing nine and ten carbon atoms (**95** and **96**) exhibited greater activity than **93** and **94** at 5.5 mM. Compounds with longer alkyl chains were tested at lower concentrations (5.5 mM and lower) due to the solubility of these compounds in aqueous solutions. At 5.5 μM, the longest alkyl chain analogues, **98** and **99**, were as active as galactose at 22 mM while **93-97** had substantially decreased IRI activity at this concentration. Unfortunately, none of these compounds exhibited activity as potent as parent glycoconjugate **6** at 5.5 μM. The most potent analogues are very similar to the non-ionic surfactants described in section 5.2 and conjugation of long alkyl chains to the *C*-linked galactosyl moiety **86** resulted

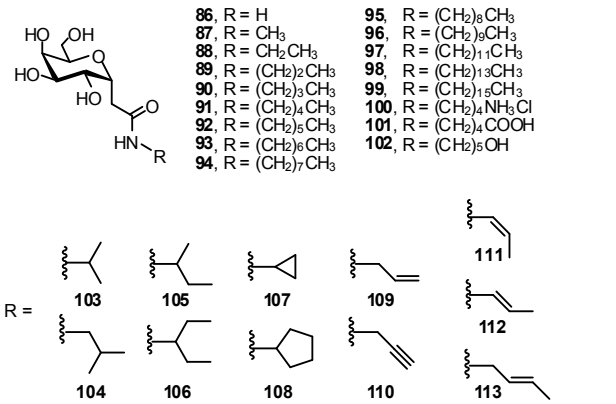

Fig. 17 Structures of truncated alkylated derivatives of *C*-AFGP analogue **6**.

in IRI active small molecules (**93-96**) at low concentrations. These results confirm that the addition of hydrophobic moieties is beneficial for IRI activity and are consistent with previous carbohydrate-based (section 5.2) and lysine-based (section 5.3) small molecules. The results from this study suggest that a balance between hydrophobic and hydrophilic moieties is important for potent IRI activity. Cumulatively, these studies described in section 5 have demonstrated that it is possible to design small molecules that are IRI active at low concentrations.

6. Cryopreservation of biological material with ice recrystallization inhibitors

Currently, the most widely used cryoprotectant is dimethyl sulfoxide (DMSO). However, cryopreservation of biological material with DMSO does not afford 100% recovery and the survival rate of some cell types is still very low. A major problem with DMSO is that it is cytotoxic.[164] Furthermore, with the increasing cases of transplantation of peripheral blood stem cells, there have been many examples that have correlated the amount of DMSO present in a transfused sample with the intensity and frequency of cytotoxic effects observed in patients.[165-168] AF(G)Ps are not typically used as cryoprotectants as temperatures in cryopreservation decrease below the TH gap where AF(G)Ps can exacerbate cellular damage.[110] Therefore, cryoprotectants that inhibit ice recrystallization and are not cytotoxic may be able to replace DMSO or at least reduce the amount of DMSO required for cryopreservation and hence reduce its toxic effects.

A recent study was performed where human embryonic liver cells were cryopreserved with the highly IRI active *C*-linked AFGP analogues **5** and **6** presented in Fig. 4 which do not exhibit TH activity.[169] These types of cells were chosen for the study because they can model stem cells.[170] Analogues **5** and **6** at 1 mg/mL were reported to double post-thaw cell viabilities relative to the negative control (cell medium only). Both **5** and **6** were also as effective as a 2.5% DMSO solution for cryopreserving this cell type. The increase in post-thaw cell viability was attributed to the IRI activity of the *C*-linked AFGP analogues, thus showing a correlation between IRI and

cryopreservation outcome. This was further validated when it was demonstrated that IRI active mono- and disaccharides exhibiting minimal cytotoxicity significantly increased post-thaw cell viabilities of $CD34^+$ hematopoietic progenitor cells and human embryonic liver cells compared to less IRI active carbohydrates.[171,172] In addition, carbohydrates exhibiting a greater degree of IRI activity reduce the induction of early apoptosis of the hematopoietic progenitor cells during thawing and increase the proliferation of functional progenitor cell colonies.[171] These validate the hypothesis that inhibiting ice recrystallization is very useful for the cryopreservation of cells and highlight the potential to replace DMSO with IRIs.

Work by Gibson and co-workers demonstrated that 9 kDa PVA has the potential to cryopreserve ovine and human erythrocytes.[173] PVA was shown to slow the rate of ice crystal growth during the thawing of red blood cells (RBCs). The addition of 0.1% (w/v) PVA (1 mg/mL) to erythrocytes resulted in post-thaw recoveries of 40% without the addition of other cryopreservatives. Furthermore, when the same concentration of PVA was utilized in combination with the non-penetrating cryoprotectant hydroxyethyl startch (HES), post-thaw recoveries could be increased to 60%. No significant haemolysis of RBCs was seen after six days of incubation with 15% (w/v) 9 kDa PVA.

In summary, these studies demonstrate that highly IRI active molecules have great potential for use as novel cryoprotectants. However, thus far the highly IRI active compounds that have been explored as cryoprotectants have been limited to AF(G)Ps, "custom-tailored" *C*-linked AFGP analogues and polymers. To date, the only small molecules that have been reported in cryopreservation assays are simple carbohydrates that are only moderately IRI active. Recent advancements in the field of ice recrystallization inhibitors has led to the discovery of small molecules that exhibit potent IRI activity. Thus, a novel class of compounds has been identified which have great potential to be beneficial cryoprotectants that may be able to significantly reduce or eliminate the need for conventional toxic cryoprotectants such as DMSO.

7. Conclusions

Currently, the most potent ice recrystallization inhibitors are the AF(G)Ps. However, these biological compounds have not been developed as cryoprotectants due to their TH activity and ice binding capabilities. *C*-linked AFGP analogues have been developed that exhibited "custom-tailored" antifreeze activity. These compounds were equipotent as AFGP-8 for IRI activity, but do not possess TH activity or dynamic ice shaping capabilities. Interest has since arisen into developing small molecule ice recrystallization inhibitors that are more amenable to large-scale preparation for the medical and commercial applications of IRIs. Simple mono- and disaccharides and their derivatives have been shown to exhibit moderate IRI activity. However, a major advancement in this field was the discovery of the first small molecules that were highly potent IRIs, the carbohydrate-based surfactants and hydrogelators. Subsequently, lysine-based derivatives and *C*-linked galactosyl derivatives have been identified as other highly IRI active small molecules and have uncovered the importance for hydrophobic residues and a balance between hydrophobic and hydrophilic groups for IRI activity. These novel small molecules exhibiting a high degree of IRI activity have great potential for use in cryopreservation applications, and further development of these molecules will ultimately lead to the identification of novel cryoprotectants that can significantly improve upon currently limited cryopreservation protocols.

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