Bioorganic & Medicinal Chemistry Letters 20 (2010) 5251–5254

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Assessing the ability of a short fluorinated antifreeze glycopeptide and a fluorinated carbohydrate derivative to inhibit ice recrystallization

Jennifer L. Chaytor, Robert N. Ben *

Department of Chemistry, University of Ottawa, 10 Marie Curie, Ottawa, Ont., Canada K1N 6N5

ARTICLE INFO

Article history:
Received 25 May 2010
Revised 25 June 2010
Accepted 29 June 2010
Available online 23 July 2010

Keywords:
Antifreeze glycoprotein
AFGP 8
Ice recrystallization inhibition
Fluorine
Carbohydrate hydration

ABSTRACT

A short fluorinated antifreeze glycopeptide (**2**) was synthesized and evaluated for ice recrystallization inhibition (IRI) activity. The activity of **2** was compared to native biological antifreeze AFGP 8 and a rationally designed C-linked AFGP analogue (OGG-Gal, **1**). In addition, a simple fluorinated galactose derivative was prepared and its IRI activity was compared to non-fluorinated compounds. The results from this study suggest that the stereochemistry at the anomeric position in the carbohydrate plays a role in imparting ice recrystallization inhibition activity and that incorporation of hydrophobic groups such as fluorine atoms cause a decrease in IRI activity. These observations are consistent with the theory that fluorine atoms increase ordering of bulk water resulting in a decrease of IRI activity, supporting our previously proposed mechanism of ice recrystallization inhibition.

The design of novel carbohydrates that inhibit ice recrystallization has been an ongoing initiative in our laboratory. Ice recrystallization inhibition (IRI) activity is associated with biological antifreezes such as antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs). IRI activity has also been reported in carbohydrates and various polymers.[1–5] Recent work in our laboratory has suggested that carbohydrate hydration is important in IRI activity[4,5] and that IRI activity is correlated to cryopreservation ability. In other words, compounds able to inhibit the recrystallization of ice were also able to protect against cell damage and death during cryopreservation. With this knowledge, we have been studying how structural modifications to carbohydrates and carbohydrate derivatives modulate IRI activity. These structure–function studies will facilitate the rational design and synthesis of novel cryoprotectants required for various medical applications, including cryopreservation and cryosurgery.[6–10]

We have previously reported that the best inhibitors of ice recrystallization are highly hydrated carbohydrates known to fit poorly within the three dimensional hydrogen bonded network of water.[5,11] We hypothesize that these compounds disorder bulk water molecules, thus slowing the transfer of bulk water molecules to the quasi liquid layer (QLL) and subsequently to the growing ice crystal.[5] For example, galactose (one of the most potent monosaccharide inhibitors of ice recrystallization) is known to fit very poorly into the hydrogen bonded network of water resulting in a large disruption in the order of these bulk water molecules.[5] In contrast, talose, which is poorly hydrated has a very good fit into the hydrogen bonded network of water, and consequently exhibits very little IRI activity.[5] Based upon this precedent, we expect compounds that are poorly hydrated (those that cause bulk water molecules to become more ordered) to be poor inhibitors of ice recrystallization. To test this hypothesis, we have prepared galactose derivatives that contain poorly hydrated substituents and examined the effect on IRI activity.

It has been demonstrated that fluorine atoms mediate the biological and chemical properties of carbohydrates.[12–14] Consequently, glycosyl fluorides are often used as both substrates[15,16] and inhibitors[15–17] of carbohydrate enzymatic reactions. A fluorine atom has a similar size to that of a hydroxyl group, which makes the fluorine atom an appropriate replacement for the hydroxyl group in binding studies.[18,19] Furthermore, the difluoromethylene group is known to be isopolar and isosteric to oxygen.[17] The hydration of fluorine atoms has been studied and it has been observed that fluorine atoms behave like hydrocarbons in that they facilitate the ordering of water molecules.[13] For instance, Nishi et al., have examined the effects of 4-hydroxyproline and 4-fluoroproline on the stability of the collagen triple helix and confirmed that fluorine is more hydrophobic than oxygen ensuring that fluorinated proline residues are poorly hydrated relative to proline in the native triple helix.[20] The trend reported here suggests that fluorine atoms order

Abbreviations: AFGP, antifreeze glycoprotein; AFP, antifreeze protein; IRI, ice recrystallization inhibition; OGG, Ornithine–glycine–glycine; TH, thermal hysteresis; PBS, phosphate buffered saline.

* Corresponding author. Tel.: +1 613 562 5800x6321; fax: +1 613 562 5170.
E-mail address: rben@uottawa.ca (R.N. Ben).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.06.148

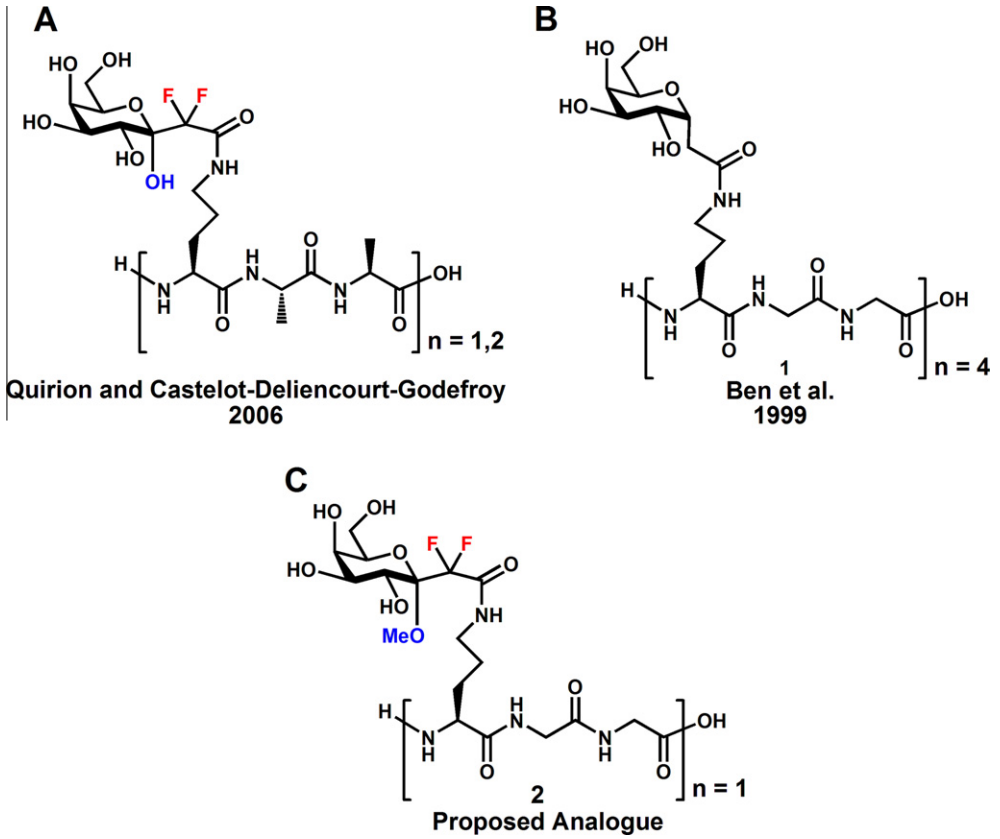

Figure 1. Image A: fluorinated AFGP analogues reported by Quirion and Castelot–Deliencourt–Godefroy. Image B: C-linked AFGP analogue OGG-Gal (**1**) reported by Ben et al. Image C: proposed analogue (**2**).

water molecules to the same degree as hydrocarbons. Based upon our proposed mechanism of IRI,[5] we would therefore expect fluorinated carbohydrates and those containing hydrocarbon chains to be equally poor inhibitors of ice recrystallization due to their enhanced ability to order bulk water molecules and facilitate the transfer of these water molecules to the ice lattice.

Introduction of fluorine is also a prudent choice as it has been previously shown that fluorinated AFGP analogues (Fig. 1, image A) were able to preserve human embryonic kidney cells, erythrocytes, blood platelets, and skin fibroblasts at physiological and sub-zero temperatures.[21] However, these compounds were not evaluated for antifreeze-specific activity such as IRI and thermal hysteresis (TH). Thermal hysteresis is defined as the selective depression of the freezing point of a solution below that its melting point. This property ultimately prevents the seeding of ice crystals in organisms inhabiting sub-zero temperatures and prevents subsequent cryo-injury and death.[22,23]

We have previously synthesized C-linked AFGP analogue **1** that exhibited potent IRI activity but had no TH activity (Fig. 1, image B). To properly investigate the effect of fluorine incorporation into this analogue, we have prepared **2** (Fig. 1, image C) and evaluated its IRI activity. Fluorinated tripeptide **2** was synthesized (Supplementary data) and assessed for IRI activity relative to native AFGP 8 and C-AFGP analogue OGG-Gal (**1**). IRI activity was assessed using a splat cooling assay developed by Horwath et al.[24] In this assay, ice crystals are measured after a 30 min annealing time at −6.4 °C and compared to a positive control for ice recrystallization to give a quantitative measure of ice grain size. In this case, the positive control is phosphate buffered saline (PBS). Since mechanical damage to cells as a result of large ice crystals formed by ice recrystallization has been proposed as a major cause of cell death during cryopreservation,[10,25–27] potent inhibitors of ice recrystallization have potential to be used as novel cryoprotectants. Additional details of this assay are described in the Supplementary data.

Figure 2 shows that **2** did not exhibit any IRI activity, as ice crystals had the same mean grain size as PBS (a positive control for ice recrystallization). These results indicate that this short fluorinated glycopeptide is not an effective inhibitor of ice recrystallization. The reported cryopreservation ability[21] of the structurally similar compound shown in Figure 1 (having a hydroxyl group instead of a methoxy group at the anomeric position) may therefore be due to some other mechanism that does not involve ice recrystallization inhibition.

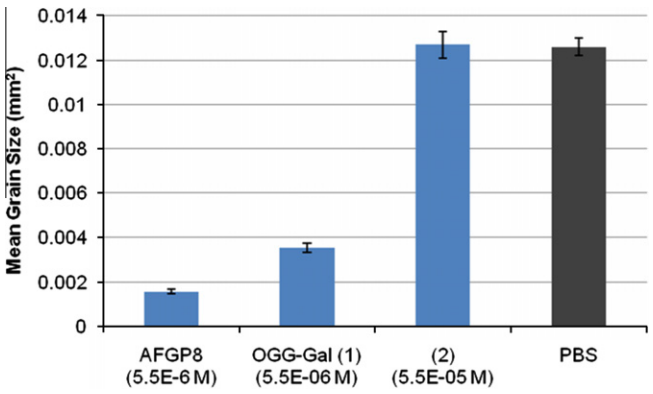

Figure 2. Ice recrystallization inhibition activity of native AFGP 8 (positive control), OGG-Gal (**1**), and fluorinated tripeptide **2**. The fluorinated tripeptide solution formed ice crystals that were identical in size to those in a phosphate buffered saline (PBS) solution (negative control).

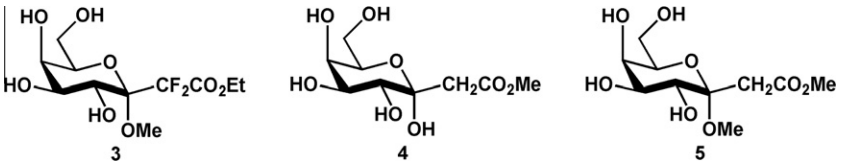

Figure 3. Fluorinated and non-fluorinated carbohydrate analogues **3**–**5**.

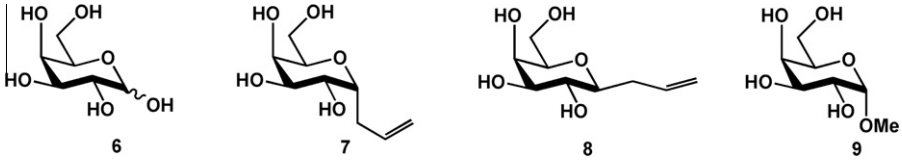

Figure 4. Galactose (**6**), α-*C*-allyl-galactose (**7**), β-*C*-allyl-galactose (**8**), and α-methyl galactopyranoside (**9**) were evaluated for IRI activity and compared to compounds **3**–**5**.

It is important to note that although the fluorinated tripeptide **2** was tested at a higher concentration than OGG-Gal (**1**), it still appeared to have no IRI activity. There are several plausible explanations for this observation. The lack of activity in this short glycopeptide may indicate that more than one tripeptide unit is required for successful inhibition of ice recrystallization. A similar effect has been observed for thermal hysteresis (TH) activity in AFGP analogues.[28] Alternatively, the lack of IRI activity may be due to the change in anomeric stereochemistry compared to OGG-Gal (**1**). Our laboratory has previously observed that small structural variations can result in changes in the presentation of the carbohydrate moiety of short glycopeptides and this can affect IRI activity.[29] Nevertheless, this was an interesting result that we felt was worthy of further study.

Earlier work in our laboratory has demonstrated that IRI activity of complex glycoconjugates can be assessed using only the carbohydrate component of the glycoconjugate.[5] While the magnitude activity of IRI active carbohydrates was generally lower than for the glycoconjugates, trends in IRI activity were the same. This is important as it allowed us to assess the potential for IRI activity of a proposed glycoconjugate before expending the effort to synthesize it. Utilizing this approach, we chose to further examine the effect of fluorine atoms on IRI activity by synthesizing a galactose derivative containing fluorine atoms that was structurally similar to the fluorinated carbohydrate moiety in the Quirion AFGP analogue.[21] The structure of this fluorinated compound and the corresponding non-fluorinated monosaccharides are illustrated in Figure 3.

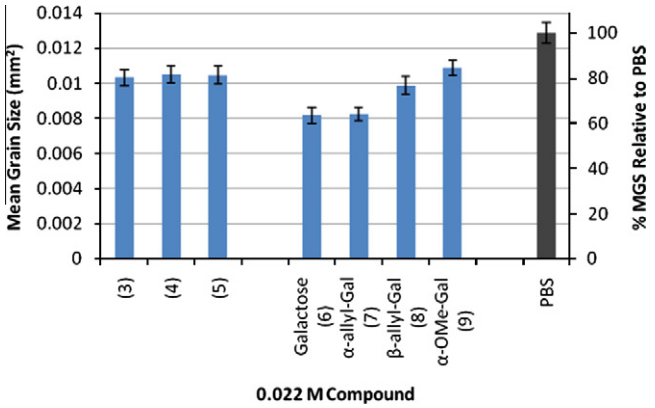

Figure 5. IRI activity of fluorinated monosaccharide **3**, non-fluorinated control monosaccharides **4** and **5**, galactose (**6**), α-*C*-allyl galactose (**7**), β-*C*-allyl galactose (**8**), and α-methyl galactopyranoside (**9**). All compounds are relative to the PBS negative control. MGS = mean grain size.

The IRI activity of these carbohydrates was compared to galactose (**6**) and the galactose derivatives (**7**–**9**) shown in Figure 4. Compounds **3**–**5** were prepared as reported in Supplementary data, and exhibited poor IRI activity as shown in Figure 5. In fact, these compounds were much less active than galactose (**6**), which is the most active monosaccharide tested to date. Interestingly, α-*C*-allyl galactose (**7**) is as active as galactose itself, yet we have previously observed a decrease in activity when the anomeric stereochemistry was changed from α- to the β-anomer (**8**).[5] This supports our theory that IRI activity is linked to the hydration of the carbohydrate, as β-linked carbohydrates have been reported to order bulk water to a greater degree than α-linked compounds.[30,31] Consequently, the observed decrease in activity of **3**–**5** relative to α-*C*-allyl galactose (**7**) was not surprising due to the change in anomeric stereochemistry.

The activity of non-fluorinated carbohydrate derivatives **4** and **5** was statistically identical[21] to the activity of fluorinated **3**, suggesting that fluorine atoms have no effect on the IRI activity of monosaccharides. Our observation that the non-fluorinated compounds had equally poor IRI activity to **3** was consistent with our hypothesis that carbohydrates containing hydrocarbon chains will be poor inhibitors of ice recrystallization. Incorporation of a methoxy group at the anomeric position of **3** and **5** did not result in a change in IRI activity relative to **4** which contains a free hydroxyl group even though the methoxy group is more hydrophobic. This increased hydrophobicity is supported by the fact that **9** was much less active than galactose and suggests that the overall hydration of compounds **3**–**5** and **9** is similar. Based upon this trend, it is plausible that the hydrophobic groups (methoxy groups, CF_2 groups, CH_2 groups and/or β-linked esters) in **3**–**5** result in poorer hydration relative to galactose itself. However, it should be noted that the hydration of these compounds has not been quantified in the literature and was not assessed in this study. Irrespective of this, these preliminary results indicate that fluorine atoms and methoxy groups do not improve the IRI activity of monosaccharides.

In conclusion, a short fluorinated glycopeptide displayed no IRI activity relative to a PBS negative control. Studies of fluorinated and non-fluorinated carbohydrates showed that these compounds were poor inhibitors of ice recrystallization. We have observed that increasing the hydrophobicity of carbohydrate derivatives causes a decrease in their IRI activity. These results support our proposed mechanism of IRI activity which is dependent on the hydration of the carbohydrate.

Acknowledgements

The authors acknowledge NSERC, CIHR, and CFI for financial support. J.L.C. thanks NSERC for a PGS-D award.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.148.

References and notes

1. Inada, T.; Modak, P. R. *Chem. Eng. Sci.* **2006**, *61*, 3149.
2. Inada, T.; Lu, S.-S. *Cryst. Growth Des.* **2003**, *3*, 747.
3. Gibson, M. I.; Barker, C. A.; Spain, S. G.; Albertin, L.; Cameron, N. R. *Biomacromolecules* **2009**, *10*, 328.
4. Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
5. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
6. Rubinsky, B.; Arav, A.; Hong, J.-S.; Lee, C. Y. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 732.
7. Koushafar, H.; Pham, L.; Lee, C. Y.; Rubinsky, B. *J. Surg. Oncol.* **1997**, *66*, 114.
8. Pham, L.; Dahiya, R.; Rubinsky, B. *Cryobiology* **1999**, *38*, 169.
9. Eroglu, A.; Russo, M. J.; Bieganski, R.; Fowler, A.; Cheley, S.; Bayley, H.; Toner, M. *Nat. Biotechnol.* **2000**, *18*, 163.
10. Baust, J. M. *Cell Preserv. Technol.* **2002**, *1*, 17.
11. Galema, S. A.; Hoiland, H. *J. Phys. Chem.* **1991**, *95*, 5321.
12. Brigaud, T.; Lefebvre, O.; Plantier-Royon, R.; Portella, C. *Tetrahedron Lett.* **1996**, *37*, 6115.
13. Biffinger, J. C.; Kim, H. W.; DiMagno, S. G. *ChemBioChem* **2004**, *5*, 622.
14. Giuffredi, G. T.; Purser, S.; Sawicki, M.; Thomspon, A. L.; Gouverneur, V. *Tetrahedron: Asymmetry* **2009**, *20*, 910.
15. Miethchen, R. *J. Fluorine Chem.* **2004**, *125*, 895.
16. Williams, S. J.; Withers, S. G. *Carbohydr. Res.* **2000**, *327*, 27.
17. Plantier-Royon, R.; Portella, C. *Carbohydr. Res.* **2000**, *327*, 119.
18. Street, I. P.; Armstrong, C. R.; Withers, S. G. *Biochemistry* **1986**, *25*, 6021.
19. Blackburn, G. M.; Kent, D. E.; Kolkmann, F. *J. Chem. Soc., Perkin Trans. 1* **1984**, *1984*, 1119.
20. Nishi, Y.; Uchiyama, S.; Doi, M.; Nishiuchi, Y.; Nakazawa, T.; Ohkubo, T.; Kobayashi, Y. *Biochemistry* **2005**, *44*, 6034.
21. Quirion, J.-C.; Castelot–Deliencourt–Godefroy, G. France Patent WO 2006/059227 A1, 2006.
22. DeVries, A. L.; Komatsu, S. K.; Feeney, R. E. *J. Biol. Chem.* **1970**, *245*, 2901.
23. Scholander, P. F.; Maggert, J. E. *Cryobiology* **1971**, *8*, 371.
24. Horwath, K. L.; Easton, C. M.; Poggioli, G. J., Jr.; Myers, K.; Schnorr, I. L. *Eur. J. Entomol.* **1996**, *93*, 419.
25. Gage, A. A.; Baust, J. *Cryobiology* **1998**, *37*, 171.
26. Mazur, P. In *Life in the Frozen State*; Fuller, B., Lane, N., Benson, E. E., Eds.; CRC Press, 2004; p 3.
27. Fowler, A.; Toner, M. *Ann. N.Y. Acad. Sci.* **2005**, *1066*, 119.
28. Tachibana, Y.; Fletcher, G. L.; Fujitani, N.; Tsuda, S.; Monde, K.; Nishimura, S.-I. *Angew. Chem., Int. Ed.* **2004**, *43*, 856.
29. Tam, R. Y.; Rowley, C. N.; Petrov, I.; Zhang, T.; Afagh, N. A.; Woo, T. K.; Ben, R. N. *J. Am. Chem. Soc.* **2009**, *131*, 15745.
30. Cheetham, N. W. H.; Lam, K. *Carbohydr. Res.* **1996**, *282*, 13.
31. Suggett, A. *J. Solution Chem.* **1976**, *5*, 33.