In Vitro Studies of Antifreeze Glycoprotein (AFGP) and a *C*-Linked AFGP Analogue

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Antifreeze glycoproteins (AFGPs) are a subclass of biological antifreezes found in deep sea Teleost fish. These compounds have the ability to depress the freezing point of the organism such that it can survive the subzero temperatures encountered in its environment. This physical property is very attractive for the cryopreservation of cells, tissues, and organs. Recently, our laboratory has designed and synthesized a functional carbon-linked (*C*-linked) AFGP analogue (**1**) that demonstrates tremendous promise as a novel cryoprotectant. Herein we describe the *in vitro* effects and interactions of *C*-linked AFGP analogue **1** and native AFGP 8. Our studies reveal that AFGP 8 is cytotoxic to human embryonic liver and human embryonic kidney cells at concentrations higher than 2 and 0.63 mg/mL, respectively, whereas lower concentrations are not toxic. The mechanism of this cytotoxicity is consistent with apoptosis because caspase-3/7 levels are significantly elevated in cell cultures treated with AFGP 8. In contrast, *C*-linked AFGP analogue **1** displayed no *in vitro* cytotoxicity even at high concentrations, and notably, caspase-3/7 activities were suppressed well below background levels in cell lines treated with **1**. Although the results from these studies limit the human applications of native AFGP, they illustrate the benefits of developing functional *C*-linked AFGP analogues for various medical, commercial and industrial applications.

Introduction

Antifreeze glycoproteins (AFGPs) are a subclass of naturally occurring biological antifreezes. AFGP 1 is the largest fraction (33.7 kDa), and AFGP 8 (2.6 kDa, Figure 1) is the lowest molecular weight fraction.[1] These compounds are found predominately in Teleost fish and protect against cryoinjury in subzero environments by preventing the uncontrolled growth of seeded ice crystals *in vivo*. The mechanism of this phenomenon is an adsorption−inhibition process that facilitates a localized freezing point depression at the surface of the ice crystal.[2] The difference between the freezing point and the melting point is referred to as thermal hysteresis (TH). AFGPs also inhibit the enthalpically driven re-organization of ice crystals in a frozen sample. This activity is referred to as recrystallization−inhibition (RI) activity.[3] Any substance possessing TH and/or the ability to inhibit recrystallization has many potential medical, commercial, and industrial applications. Medical applications are particularly attractive because most cellular damage during the cryopreservation of cells, tissues, and organs is due to recrystallization.[4]

Studies have shown that AFGPs have successfully protected pig oocytes at cryogenic and hypothermic storage temperatures[5,6] and also increased the viability of pig oocytes and mouse embryos after vitrification.[7] In addition, AFGPs enhance the viability of carp spermatozoa after hypothermic storage.[8] Other studies have suggested that AFGPs protect cell membranes during thermatropic phase transitions[9] and this has been exploited for the long-term storage of human platelet cells.[10] The preservation of tissues and organs using AFGPs has not been extensively explored, but preliminary studies have produced conflicting results. For instance, low concentrations of AFGPs have been successfully used to preserve mammalian livers at subzero temperatures.[11,12] However, high concentrations of AFGPs failed to protect an isolated rat heart during hypothermic storage and cryopreservation.[13] In the latter case, it was proposed that AFGPs actually exacerbated cellular damage because of the needle-like ice crystal morphology occurring as a result of dynamic ice shaping that transpires outside of the TH gap.[14] This effect has subsequently been exploited to enhance the effectiveness of experimental noninvasive cryosurgical procedures.[14d,15]

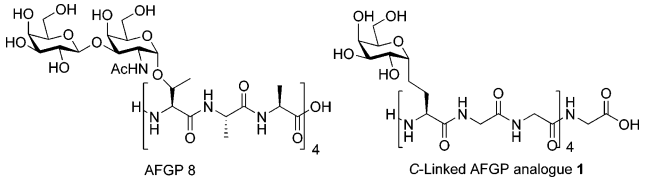

Figure 1. Structures of AFGP 8 and *C*-linked AFGP analogue **1**.

On the basis of these preliminary reports, it is likely that AFGPs will play an important role in the development of new preservation protocols for cells, tissues, and organs.[8] However, there are several issues that must be overcome before AFGPs can find widespread application. First, the natural abundance is low, and the isolation and purification of native AFGPs from fish are very labor-intensive and costly procedures.[16] Second, the anomeric carbon−oxygen bond in native AFGPs is prone to cleavage under acidic or basic conditions[17] as well as

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hydrolysis by glycosidases. Third, dynamic ice shaping (a result of TH activity) exacerbates cellular damage at temperatures below the TH gap. Finally, the potential for cytotoxicity must be investigated before these compounds can be used in human applications. Although preliminary studies of AFP types I and III suggest that there are no long-term health effects or immunogenicity associated with these compounds,[15b,18,19] detailed studies need to be performed. There has been only one report of AFGP cytotoxicity,[20] but it was not in a mammalian system.

In an effort to address these issues, our laboratory is exploring whether carbon-linked (*C*-linked) AFGP analogues could serve as structural and functional mimics of native AFGP. Over the last several years, we have reported significant progress toward this goal and described the synthesis and assessment of several functional *C*-linked AFGP analogues possessing the ability to inhibit the growth of ice.[21] Of these compounds, *C*-linked AFGP analogue **1** (Figure 1) is a potent inhibitor of recrystallization[22] that does not possess TH activity. Consequently, AFGP analogue **1** is an extremely valuable lead compound for the development of novel cryoprotectants. Toward this end, we have examined and compared the *in vitro* interactions of native AFGP 8 and analogue **1**.

Materials and Methods

Cell Culture. WRL 68 human embryonic liver cells (ATCC, CL-48) and HEK 293 human embryonic kidney cells (ATCC, CRL-1573) were cultured in Eagle's minimum essential media (MEM) (ATCC, 30-2003) supplemented with 10% fetal bovine serum (FBS) (ATCC, 30-2020) and 1% penicillin−streptomycin (GIBCO, 15140). Cells were incubated in a 37 °C incubator supplied with 5% CO_2. Passage 85 to 87 of WRL 68 cells and passage 34 of HEK 293 cells were used in this study. Rainbow trout gill epithelial cells (Rtgill-W1) (ATCC, CRL-2523) were cultured in Leibovitz's L-15 medium (LLM) (ATCC, 30-2008) supplemented with 10% FBS and 1% penicillin−streptomycin. Cells were incubated in a 19 °C incubator. All cells were trypsinized with a trypsin−EDTA solution (0.25% trypsin, 1 mM EDTA·4Na) (GIBCO, 25200) and incubated in half-area 96-well plates with a density of around 5×10^3 cells/well until they reached 100% confluence for experiments. No evidence of overgrowth or morphological changes consistent with apoptosis was observed.

AFGP Cytotoxicity and MTT Assay. Cells were treated with various amounts of AFGP 8 (isolated from the Greenland cod *Gadus ogac*, A/F Protein, Inc.) or *C*-linked AFGP analogue **1** in a solution of culture medium and subsequently incubated at 37 °C (for human liver and human kidney cells) or 19 °C (for trout gill cells) for 20 h. MTT assays were performed to assess cellular viability. After incubating cells with AFGP 8 or the *C*-linked AFGP analogue, plates were centrifuged at 1500 rpm for 3 min before the culture medium was replaced with 50 μL of MTT (sigma, M5655) solution (5 mg/mL) in Hank's balanced salt solution (HBSS, Invitrogen). Plates were then incubated at 37 °C with 5% CO_2 for 2−4 h. Fifty microliters of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in isopropanol) was added to each well, and each well was aspirated until all of the formazan precipitate was dissolved. The absorbance of each well was read at wavelengths of 570 and 650 nm with a multiwell plate reader (AD 340C Absorbance Detector, Beckman Coulter, Inc.). High optical density (OD) readings indicate greater cell viability. All experiments were repeated three times, and 12 parallel wells were used for each condition. All MTT assay data were analyzed with the Student's *t*-test.

Caspase-3/7 Assay. This study was performed using the APO-ONE homogeneous Caspase-3/7 assay kit (Promega, G7790). The assay was performed in black 96-well plates (Nunclon Surface, Nalge Nunc International). When the cells reached 100% confluence, the culture medium was replaced with fresh culture medium and additional treatments. 2% DMSO (Sigma) was used to improve the solubility of staurosporine in the culture medium and was, therefore, added to all wells. No cells were seeded in the blank control wells. In the negative control, no additional compounds were added into the cell culture. The positive control contained 20 μM staurosporine (Sigma, from *Streptomyces* sp., S4400) as a solution in MEM (Eagle's minimum essential media). The experimental treatment consisted of 5 mg/mL AFGP 8 or *C*-linked AFGP analogue **1** as a solution in MEM. After incubating cells at 37 °C with 5% CO_2 for 20 h, the caspase substrate was added into each well, and the plate was incubated at room temperature on a plate shaker (200 rpm). The level of fluorescence was read at 4, 6, 10, 24, and 48 h using a Spectra MAX GeminiXS fluorescence plate reader (Molecular Device) with excitation at 499 nm (emission at 521 nm).

Vybrant Apoptosis Assay. This study was performed using Vybrant apoptosis assay kit # 7 (Molecular Probes, V-23201). WRL 68 cells were cultured in a 35 mm petri dish (10 mm microwell, MatTek) at 37 °C with 5% CO_2. After cells grew to 50% confluence, the culture medium was replaced with fresh medium and additional treatments. In the negative control, no additional compounds were added into the cell culture. The positive control consisted of 20 μM staurosporine (Sigma, from *Streptomyces* sp., S4400) as a solution in MEM. The experimental treatment consisted of 5 mg/mL AFGP 8 as a solution in MEM. After 4 h of additional incubation, the solution was removed, and the cells were washed three times with PBS. A solution containing 8.1 μM Hoechst 33324, 0.1 μM YO-PRO-1, and 1.5 μM propidium iodide in PBS was added to the cells, which were incubated at 0 °C for 30 min. The solution was removed from the petri dish, and the cells were washed three times with PBS. The cells were then imaged with a Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) inverted laser scanning microscope.

Confocal Microscopy. AFGP 8 was labeled with FluoReporter Oregon Green 488 protein labeling kit (Molecular Probes, F-6153) according to product guidelines. The labeled AFGP 8 (F-AFGP 8) was purified with a D-Salt Polyacrylamide 1800 Desalting Column (PIERCE, 43426). The concentration of purified AFGP 8 in HBSS solutions was measured with a UV spectrophotometer (Hewlett Packard 8453). WRL 68 cells were pretreated with a 0.5 mg/mL proteinase K (Roche, 1 373 196) solution in PBS (pH 7.95) and incubated at 37 °C under 5% CO_2 atmosphere for 1 h before staining with F-AFGP 8. Cells with or without proteinase K treatment were incubated in the culture medium with 0.89 mg/mL F-AFGP 8 as a solution in MEM and 250 nM MitoTracker Orange CMTMRos (Molecular Probes, M-7510), 75 nM LysoTracker Red DND-99 (Molecular Probes, L-7528), or 10 μM membrane probe RH-237 (Molecular Probes, S-1109) as solutions in MEM at 0, 19, or 37 °C for 10, 20, or 50 min, respectively. The cells were then washed three times with HBSS and imaged with a Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) inverted laser scanning microscope.

Transmission Electron Microscopy. WRL 68 cells were incubated with 4 mg/mL AFGP 8 as a solution in MEM for 4 or 12 h. Fixation was carried out with 2.5% glutaraldehyde in a solution of 0.067 M Sorensen phosphate buffer containing 0.015% $CaCl_2$ and 0.05 M sucrose (pH 7.2) at 37 °C for 1 h. Cells were then seeded at room temperature for 2 h. After the removal of the buffer, the sample was fixed for 2 h with a 1% OsO_4 solution containing the Sorensen buffer without sucrose. After dehydration in ice-cooled ethyl alcohol solutions (50, 70, 85, and 95%), the sample was washed three times with 100% ethyl alcohol. The sample was embedded in Epon resin, polymerized at 60 °F, and sectioned using a diamond knife with a Reichert Ultracut ultramicrotome. The sections were stained in a 2% aqueous uranyl acetate solution for 20 min, followed by Reynolds lead stain for another 7 min. Finally, the sections were observed with a Hitachi 7000 TEM at 70 kV using a 20 μm objective aperture.

Results and Discussion

Novel *C*-linked AFGP Analogues. We have previously reported the rational design and synthesis of functional *C*-linked AFGP analogue **1**.[22] This particular low molecular weight AFGP

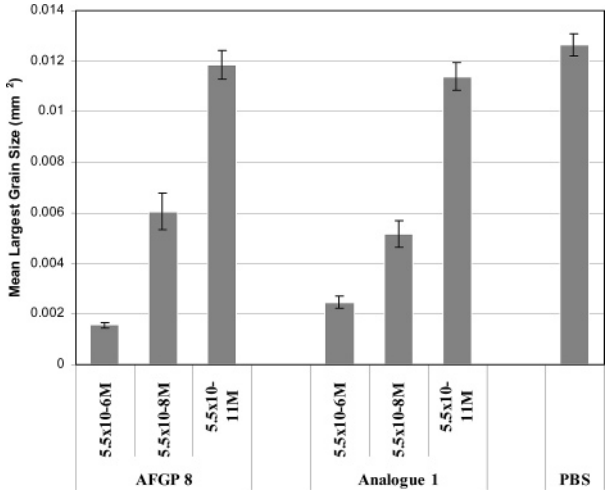

Figure 2. Recrystallization−inhibition (RI) activity of AFGP 8 and *C*-linked AFGP analogue **1**. Mean largest grain size represents the average surface area of ice grains in a field of view. Both AFGP 8 and *C*-linked AFGP analogue **1** were tested at different concentrations in PBS to exclude nonspecific RI effects. The error bars indicate SEM.

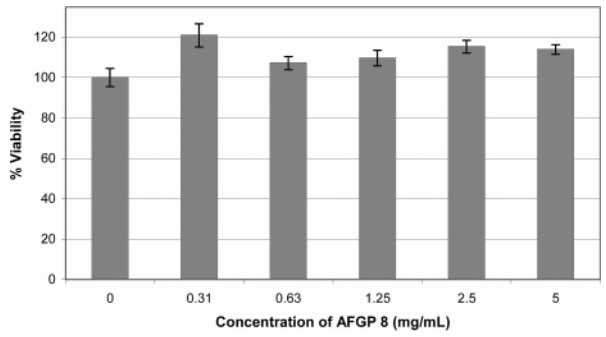

Figure 3. MTT assay of rainbow trout gill epithelial cells (Rtgill-W1) with AFGP 8. Rtgill-W1 cells were incubated at 19 °C for 20 h with AFGP 8 in MEM. Optical density (OD) was measured at 570 nm. High and low OD values represent increased and decreased cell viability, respectively. The error bars indicate SEM.

analogue does not possess many of the structural features previously thought to be essential for antifreeze activity. Furthermore, **1** does not exhibit any thermal hysteresis activity and is an extremely potent inhibitor of recrystallization. Shortly after the publication of this result, the RI activity was thoroughly investigated and determined to be comparable to that of native AFGP 8 (Figure 2).

Assessing the Cytotoxicity of AFGP 8 and *C*-Linked AFGP Analogue 1. AFGP 8 was isolated from Greenland cod (*Gadus ogac*). It is the lowest molecular weight fraction and exhibits the least amount of antifreeze activity. Cytotoxic effects of AFGP 8 were determined by assessing cell viability using the MTT assay[23] in three different cell lines: a gill epithelial cell line (Rtgill-W1) indigenous to rainbow trout (*Oncorhynchus mykiss*), a human embryonic liver cell line (WRL 68), and a human embryonic kidney cell line (HEK 293). As illustrated in Figure 3, trout gill cells suffered no cytotoxic effects after 20 h of incubation with 0.31−5.0 mg/mL AFGP 8 at 19 °C. This is not a surprising result, given that AFGP 8 is indigenous to fish.[16]

However, incubating AFGP 8 with human liver cells produced a very different result (Figure 4). Solutions of greater than 2 mg/mL AFGP 8 showed significant toxicity after 20 h of exposure at 37 °C ($P < 0.05$).

An identical trend was observed in human kidney cells (Figure 5), where significant toxicity was shown for all

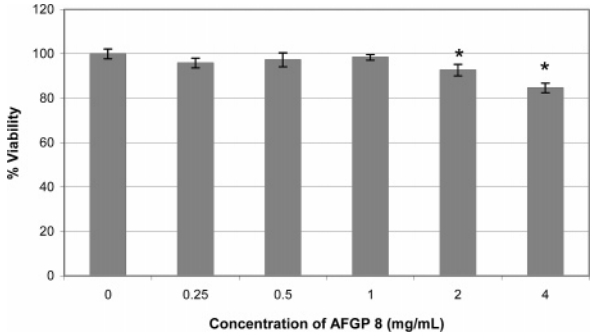

Figure 4. MTT assay of AFGP 8 in human embryonic liver cells (WRL 68). WRL 68 cells were incubated at 37 °C for 20 h with different concentrations of AFGP 8 in MEM. Optical density (OD) was measured at 570 nm. High and low OD values represent increased and decreased cell viability, respectively. The error bars indicate SEM. The asterisks indicate the significant difference ($P < 0.05$) from cells without AFGP 8 treatment.

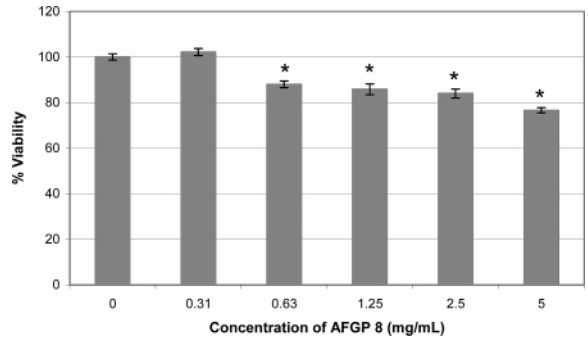

Figure 5. MTT assay of human embryonic kidney cells (HEK 293) with AFGP 8. HEK 293 cells were incubated at 37 °C for 20 h with different concentrations of AFGP 8 in MEM. Optical density (OD) was measured at 570 nm. High and low OD values represent increased and decreased cell viability, respectively. The error bars indicate SEM. The asterisks indicate the significant difference ($P < 0.05$) from cells without AFGP 8 treatment.

treatments with AFGP 8 concentrations greater than 0.63 mg/mL ($P < 0.05$).

It is interesting to note that AFGP 8 did not exhibit cytotoxic effects in cultures of human liver cells at concentrations less than 1 mg/mL (Figure 4, $P > 0.05$). Similarly, AFGP 8 concentrations of less than 0.31 mg/mL failed to exhibit cytotoxic effects in human kidney cells (Figure 5, $P = 0.31$). These results suggest that liver cells may have a higher tolerance to the cytotoxicity of AFGP 8 than kidney cells. The broad implications of this observation are that the cytotoxicity of AFGP 8 is not only concentration-dependent but also cell-specific. Although this result may significantly limit medical applications of native AFGP 8, low concentrations of AFGP 8 might still be useful as a cryoadjuvant, provided that solutions less than 1 mg/mL are capable of conferring cryoprotection. Rubinsky and co-workers have reported a similar observation when assessing the cryotoxicity of AFP type I for cryosurgical applications.[15b] Prior studies using AFGP 8 as a cryoprotectant typically employed concentrations greater than 10 mg/mL. To the best of our knowledge, there is only one report where the toxicity of AFGPs was observed.[20] This work described toxicity in spinach thylakoid membranes, but mammalian cells were not examined. Irrespective of the commercial limitations posed by the cytotoxicity of AFGP 8 in human liver and kidney cells, the fact that a cytotoxic response is observed in human cell lines is fundamentally interesting.

C-Linked AFGP analogue **1** did not appear to be cytotoxic in human liver cells even at high concentrations (Figure 6). This

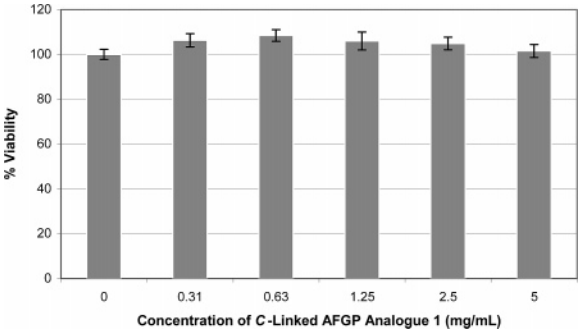

Figure 6. MTT assay of human embryonic liver cells (WRL 68) with *C*-linked AFGP analogue **1**. WRL 68 cells were incubated at 37 °C for 20 h with different concentrations of AFGP 8 in MEM. Optical density (OD) was measured at 570 nm. High and low OD values represent increased and decreased cell viability, respectively. The error bars indicate SEM.

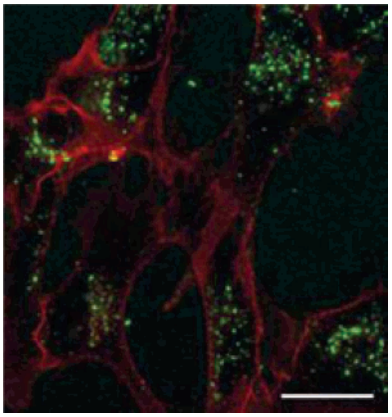

Figure 7. Internalization study of fluorescently labeled AFGP 8 (F-AFGP 8) in fish gill epithelial cells (Rtgill-W1). Rtgill-W1 cells were incubated with 1.5 mg/mL F-AFGP 8 and 10 μM membrane probe RH-237 in MEM at 19 °C for 10 min. F-AFGP 8 appears green, and RH-237 appears red. The scale bar = 50 μm.

lack of apparent cytotoxicity was very encouraging because one of the greatest limitations with common cryoprotectants (eg., DMSO) is that they are cytotoxic, and care must be taken to remove the cryoprotectant during the thawing cycle.[24] In theory, such limitations will not be imposed upon *C*-linked AFGP analogue **1**.

Internalization of AFGP 8 and *C*-Linked AFGP Analogue 1. To assess whether the difference in cytotoxicity in human cells was due to selective internalization, we studied the internalization of AFGP 8 in human liver cells and fish cells using fluorescently labeled glycoproteins and confocal microscopy. Fluorescently labeled AFGP 8 (F-AFGP 8) and *C*-linked AFGP analogue **1** (F-**1**) were prepared by labeling the *N*-terminus of the glycopeptide with Oregon Green 488. In some experiments, cells were treated with Proteinase K prior to incubation with F-AFGP 8 or F-**1** in order to remove cell membrane receptors. Human liver cells or trout gill cells were incubated with F-AFGP 8 or F-**1**, together with MitoTracker orange or RH-237 as counter stains at 0, 19, or 37 °C for 10, 20, and 50 min and imaged with the confocal microscope. Trout gill cells and human liver cells were both successfully stained with F-AFGP 8 at physiological temperatures (Figures 7 and 8a), indicating that both cell types readily internalized AFGP 8. Similar results were obtained upon the incubation of human liver cells with F-**1** (Figure 8b). However, there was no evidence of internalization when human liver cells were treated with F-AFGP 8 (Figure 8c) or F-**1** (data not shown) at 0 °C.

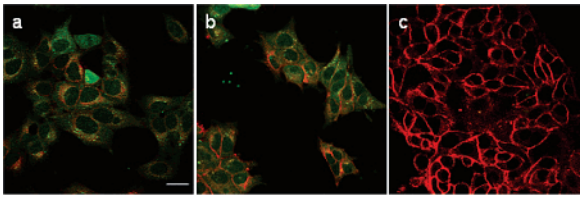

Figure 8. Internalization study of fluorescently labeled AFGP 8 (F-AFGP 8) and fluorescently labeled *C*-linked AFGP analogue **1** (F-**1**) in human embryonic liver cells (WRL 68). WRL 68 cells were incubated with 10 μM RH-237 membrane probe solution in MEM and 1.5 mg/mL F-AFGP 8 or F-**1** solution in MEM. F-AFGP 8 and F-**1** appear green, and RH-237 appears red. (a) WRL 68 cells were incubated with F-AFGP 8 at 37 °C for 20 min. (b) WRL 68 cells were incubated with F-**1** at 37 °C for 10 min. (c) WRL 68 cells were incubated with F-AFGP 8 at 0 °C for 50 min. The scale bar = 25 μm.

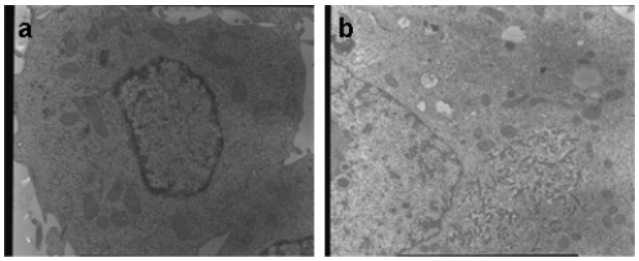

Figure 9. TEM images of WRL 68 cells incubated with AFGP 8. (a) Negative control: WRL 68 cells without AFGP 8 treatment. (b) WRL 68 cells were incubated with 4 mg/mL AFGP 8 solution in MEM at 37 °C for 12 h. Both images were taken with 10,000× magnification.

These experiments confirm that F-AFGP 8 is readily internalized into the cytoplasmic space for both cell lines after only 10 min of incubation as evidenced by the appearance of bright green vesicles in the cytoplasmic space (Figures 7 and 8). Given the size of these glycoproteins and their hydrophilic nature, it seemed unlikely that internalization was occurring via passive diffusion across the cell membrane. The fact that discrete vesicles were observed suggests that internalization occurs via endocytosis.

It has been documented that ATP-dependent endocytosis can be inhibited at 0 °C.[25,26] The fact that the glycoproteins were rapidly internalized by trout gill cells at 19 °C and human liver cells at 37 °C but could not be internalized by human liver cells at 0 °C confirmed that internalization is an ATP-dependent process.[27] It has also been shown that receptors highly specific for galactose residues exist on the liver cell membrane and mediate the binding and cellular internalization of glycoproteins presenting galactose residues.[28,29] Futhermore, the treatment of cells with Proteinase K removes the cell-surface receptors responsible for intracellular signaling events and internalization.[25,30] However, the treatment of human liver cells with Proteinase K prior to incubation with F-AFGP 8 did not result in any noticeable differences (data not shown) in internalization, suggesting that the internalization process is not receptor-mediated.

Co-Localization of AFGP 8 and *C*-Linked AFGP Analogue 1. Transmission electron microscopy (TEM) of human embryonic liver cells (WRL 68) treated with 4 mg/mL AFGP 8 revealed increased numbers of microtubules and secondary lysosomes relative to the negative control (Figure 9).

Further observation revealed that the microtubules were attached to many vesicles (Figure 10). The role of microtubules in endocytotic trafficking has been well documented,[31] and cellular internalization is facilitated via the endosomal pathway, where cytoplasmic vesicles are transformed into lysosomes.[32,33]

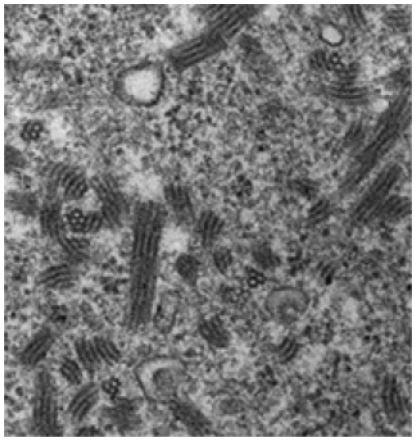

Figure 10. TEM image of WRL 68 cells incubated with AFGP 8 showing vesicles attaching to microtubules. The image was taken with 10,000× magnification.

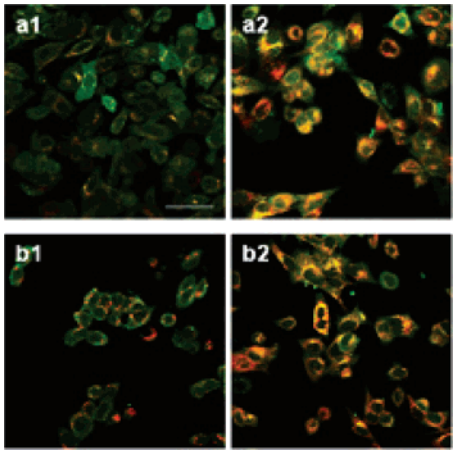

Figure 11. Co-localization of fluorescently labeled AFGP 8 (F-AFGP 8) and fluorescently labeled *C*-linked AFGP analogue **1** (F-**1**) with LysoTracker Red DND-99 in human embryonic liver cells (WRL 68). WRL 68 cells were incubated with 75 nM LysoTracker Red DND-99 in MEM and 1.5 mg/mL F-AFGP 8 or F-**1** solution in MEM at 37 °C. F-AFGP 8 and F-**1** appear green, and LysoTracker Red DND-99 appears red. The co-localization of LysoTracker Red DND-99 with F-AFGP 8 or F-**1** is evidenced by yellow emission. (a1) WRL 68 cells were incubated with AFGP 8 for 25 min. (a2) WRL 68 cells were incubated with F-AFGP 8 for 80 min. (b1) WRL 68 cells were incubated with F-**1** for 10 min. (b2) WRL 68 cells were incubated with F-**1** for 50 min. The scale bar = 50 μm.

In order to confirm the cellular localization of the peptides in lysosomes after cellular internalization via endocytosis, F-AFGP 8 and F-**1** were added to the cell medium along with Lysotracker Red DND-99 as a counter stain. The fluorescently labeled glycoproteins fluoresce green, whereas Lysotracker Red fluoresces intensely red when it is exposed to acidic environments such as that inside the lysosomes. Co-localization of the green fluorescent glycopeptide and red fluorescent Lysotracker Red was indicated by intense yellow emissions. As illustrated in Figure 11, co-localization occurred in the lysosomes, and the amount of co-localization increased with time.

Investigating the Mechanism of Cytotoxicity. Cell death can be generally classified into two categories: necrosis and apoptosis.[34] During live cell imaging, we noticed changes in cell morphology consistent with an apoptotic mechanism of cell death. These changes included nuclear blebbing and the punctate appearance of cell membranes.[35] To confirm whether cell death was occurring via apoptosis, we performed a caspase-3/7 assay that specifically identifies whether effector caspases 3 and 7 are activated (Figure 12). In these studies, staurosporine, a known inducer of apoptosis, was used as a positive control. The blank readings represent wells containing only the culture medium, whereas the negative control contained cells as well as the culture medium. The latter control served to quantify caspase-3/7 activity in normal cells as a function of time. 2% DMSO was required to solubilize staurosporine and was, therefore, added to all wells. The added DMSO contributed to background cytotoxicity in the negative controls. The incubation of human liver cells for 18 h with 5 mg/mL AFGP 8 resulted in significantly increased caspase-3/7 activity relative to the negative control (Figure 12). As expected, this activity increased with time, confirming that effector caspases 3 and 7 are activated upon exposure to AFGP 8 and that an apoptotic process is being initiated.

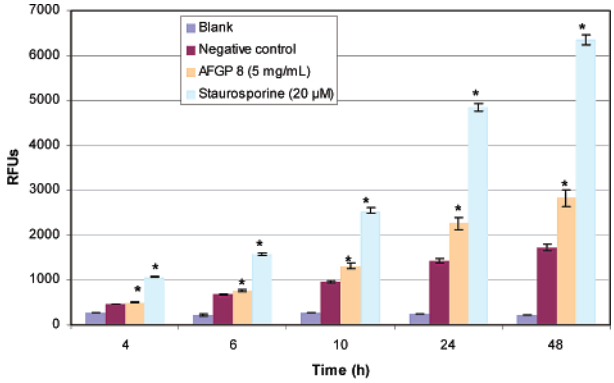

Figure 12. Caspase-3/7 assay of WRL 68 incubated with AFGP 8. No WRL 68 cells were seeded in the blank. WRL 68 cells incubated with vehicle treatment (2% DMSO in MEM) served as the negative control. The experimental treatment shows WRL 68 cells incubated with 5 mg/mL AFGP 8 solution at 37 °C for 20 h. The positive control shows WRL 68 cells incubated with 20 μM staurosporine solution at 37 °C for 20 h. Relative fluorescence units (RFUs) were measured with excitation at 499 nm and emission at 521 nm. The error bars indicate SEM. High or low RFUs represent activated or depressed caspase-3/7 activity, respectively. The difference in the fluorescence produced in AFGP 8-treated cells and the negative control is significant ($P < 0.05$) for measurements at all incubation times with the caspase-3/7 substrate ($P = 0.027$ for 4 h, 8.4×10^{-3} for 6 h, 5.7×10^{-4} for 10 h, 4.6×10^{-4} for 24 h, and 4.3×10^{-4} for 48 h). The asterisks indicate the significant difference ($P < 0.05$) from the negative control.

The above result was independently confirmed using the fluorescent Vybrant apoptosis assay. In this assay, human liver cells were incubated with three different fluorescent nucleic acid stains: YO-PRO-1, propidium iodide, and Hoechst 33342. YO-PRO-1 stains apoptotic cells green, propidium iodide stains only dead or late apoptotic cells red, and Hoechst 33342 stains both apoptotic cells and live, healthy cells blue. Apoptotic cells are confirmed visually with both green and blue fluorescent stains. Staurosporine was used as a positive control. After the incubation of human liver cells with 5 mg/mL AFGP 8 for 4 h, most cells were stained green and blue, which indicated that the cells had undergone apoptosis (Figure 13). In the negative control, cells were only stained by the blue Hoechst 33342 dye. As for the staurosporine-treated control, a large number of cells were stained green and blue as well as red, identifying them as late apoptotic or dead cells.

Caspase-3/7 levels were also assessed after the treatment of human liver cells with *C*-linked AFGP analogue **1** (Figure 14). As illustrated in Figure 14, caspase-3/7 levels in WRL 68 cells did not climb above the negative controls. In fact, caspase-3/7 levels remained significantly lower in cells treated with AFGP analogue **1** compared to the levels in untreated cells.

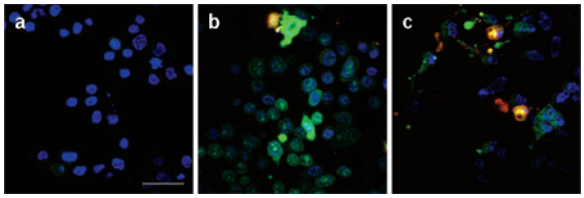

Figure 13. Vybrant apoptosis assay of WRL 68 cells incubated with AFGP 8. WRL 68 cells were incubated with 8.1 μM Hoechst 33342, 1.5 μM propidium iodide, and 0.1 μM YO-PRO-1 solutions in MEM at 0 °C for 30 min. Hoechst 33342 stains the nuclei of all cells blue. Propidium iodide stains dead cells and late apoptotic cells red. YO-PRO-1 stains early apoptotic cells green. (a) Negative control: WRL 68 cells were preincubated with MEM. (b) WRL 68 cells were preincubated with 5 mg/mL AFGP 8 solution in MEM at 37 °C for 4 h. (c) WRL 68 cells were preincubated with 20 μM staurosporine solution in MEM at 37 °C for 4 h. The scale bar = 50 μm.

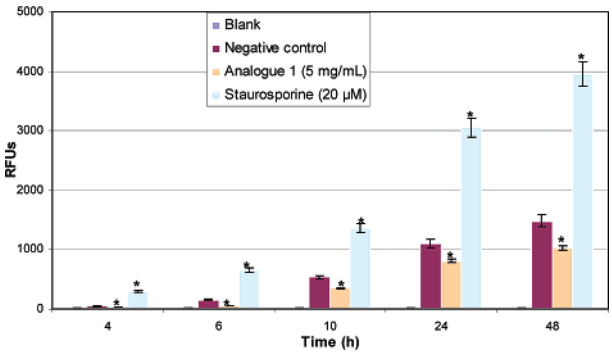

Figure 14. Caspase-3/7 assay of WRL 68 cells incubated with *C*-linked AFGP analogue **1**. No WRL 68 cells were seeded in the blank. WRL 68 cells incubated with vehicle treatment (2% DMSO in MEM) served as the negative control. The experimental treatment shows WRL 68 cells incubated with 5 mg/mL *C*-linked AFGP analogue **1** solution at 37 °C for 20 h. The positive control shows WRL 68 cells incubated with 20 μM staurosporine solution in MEM at 37 °C for 20 h. Relative fluorescence units (RFUs) were measured with excitation at 499 nm and emission at 521 nm. The error bars indicate SEM. High or low RFUs represent activated or depressed caspase-3/7 activity, respectively. The asterisks indicate the significant difference ($P < 0.05$) from the negative control.

The mechanism of cell death upon the exposure of human cells to AFGP 8 is of fundamental interest because AFGPs are not indigenous to mammalian systems. Caspases 3 and 7 are key intermediates in the apopotic signaling cascade that are not activated during necrosis. Our results indicate that the treatment of human liver cells with AFGP 8 resulted in the conversion of pro-caspases 3 and 7 into active caspases 3 and 7, suggesting that AFGP 8 is initiating an apoptotic process (Figure 12). Given that internalization of AFGP 8 occurs, it is likely that apoptosis results from an intrinsic activation pathway.[35,36] At this time, it is not known whether the cytotoxic effect is due to AFGP 8 or some metabolite of AFGP because the escape of substances from the lysosomal degradation pathway is precedented.[37]

In contrast, the treatment of human liver cells with *C*-linked AFGP analogue **1** did not result in increased caspase-3/7 activity. This was not surprising because this analogue failed to display any *in vitro* cytotoxicity. However, it was surprising that caspase-3/7 activity in human liver cells treated with *C*-linked AFGP analogue **1** was actually depressed well below that of background activity. Furthermore, this effect occurred in the presence of 2% DMSO. This exciting result suggests that analogue **1** possesses caspase-inhibitory activity. Recent studies have shown that the addition of caspase inhibitors can prevent cold-induced apoptosis from occurring upon low-temperature preservation of cells.[34,38] The fact that **1** is a potent inhibitor of recrystallization and also appears to suppress caspase activity is extremely encouraging and further illustrates the potential of **1** as a cryoprotectant.

Conclusions

In summary, we have demonstrated that the addition of high concentrations of AFGP 8 to human liver and kidney cells increases caspase-3/7 activity, leading to cell death via an apoptotic process. Consistent with previous literature reports, lower concentrations (less than 1 mg/mL for human liver cells and less than 0.31 mg/mL for human kidney cells) failed to cause any cytotoxic effects. This cytotoxic effect is likely due to an intrinsic activation of apoptosis because AFGP 8 is rapidly internalized by human liver cells, likely via ATP-dependent endocytosis. Despite the many useful antifreeze properties of AFGP 8, the *in vitro* cytotoxicity will dramatically affect the future use of native AFGP 8 for cryoprotection and hypothermic storage. In contrast, functional *C*-linked AFGP analogue **1**, possessing recrystallization−inhibition activity nearly identical to that of native AFGP 8 and lacking thermal hysteresis, is not cytotoxic to human liver cells, even at high concentrations. Like AFGP 8, it is rapidly internalized by cells, likely via endocytosis, and it is ultimately localized in lysosomes. *C*-Linked AFGP analogue **1** appears to suppress caspase-3/7 activity *in vitro* and shows tremendous promise as a cryoprotectant because it may also be effective at preventing cold-induced apoptosis. This compound may have other uses for the treatment of conditions where apoptosis plays a key role, such as various bacterial infections, stroke, and spinal cord injury.[39,40] The mechanism-(s) by which *C*-linked AFGP analogue **1** inhibits effector caspases and its effectiveness as a cryoprotectant are currently under investigation in our laboratory. The results from these studies will be reported in due course.

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