Inhibition of infectious haematopoietic necrosis virus in cell cultures with peptide-conjugated morpholino oligomers

M Alonso¹, D A Stein², E Thomann¹, H M Moulton², J C Leong³, P Iversen² and D V Mourich^{1,2}

¹ Department of Microbiology and Center for Fish Disease Research, Oregon State University, Corvallis, OR, USA

² AVI BioPharma, Corvallis, OR, USA

³ Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe, HI, USA

Abstract

Delivery of phosphorodiamidate morpholino oligomers (PMO) into fish cells *in vitro* and tissues *in vivo* was examined. Uptake was evaluated by fluorescence microscopy and flow cytometry after treating cultured cells or live rainbow trout with 3' fluorescein-tagged PMO. Arginine-rich peptide conjugated to the 5' end of the PMO markedly enhanced cellular uptake in culture by 8- to 20-fold compared with non-peptide-conjugated PMO as determined by flow cytometry. Enhanced uptake of PMO conjugated to peptide was also observed in tissues of fish treated by immersion. The efficacy of PMO as inhibitors of infectious haematopoietic necrosis virus (IHNV) replication was determined *in vitro*. Peptide-conjugated PMOs targeting sequences within the IHNV genomic RNA (negative polarity) or antigenomic RNA (positive polarity) significantly inhibited replication in a dose-dependent and sequence-specific manner. A PMO complementary to sequence near the 5' end of IHNV genomic RNA was the most effective, diminishing titre by 97%, as measured by plaque assay and Western blot. These data demonstrate that replication of a negative-stranded non-segmented RNA virus can be inhibited by antisense compounds that target positive polarity viral RNA, or by a compound that targets negative polarity viral RNA.

Keywords: antisense morpholino oligomers, cell culture, infectious haematopoietic necrosis virus, *Novirhabdovirus*, rainbow trout.

Introduction

Infectious haematopoietic necrosis virus (IHNV) has a negative-sense non-segmented RNA genome, and belongs to the genus *Novirhabdovirus*, in the family Rhabdoviridae. Viral genomic RNA acts as a direct template for the RNA synthesis events of transcription and replication. In the process of transcription six monocistronic mRNAs are synthesized, which are then translated into individual proteins. In the process of replication, a full-length positive-strand replicative intermediate RNA is synthesized and this becomes the template for the production of progeny genomic RNAs. 'Antigenomic' RNA refers in this paper to both mRNA and replicative intermediate RNA. The major distinguishing feature of *Novirhabdovirus* is the presence of a non-virion gene between the glycoprotein and the polymerase genes (Kurath, Higman & Bjorklund 1997). The six genes of IHNV are present in the genome in the following order: 3'-N-P(M1)-M(M2)-G-NV-L-5', where N is the nucleocapsid protein, P or M1 is the phosphoprotein, M or M2 is the matrix protein, G is the glycoprotein, NV is the non-virion protein, and L is the polymerase protein (Kurath, Ahern, Pearson & Leong 1985; Morzunov, Winton & Nichol 1995).

Originally enzootic on the west coast of North America, IHNV has been spread by movement of infected fish and eggs to Asia and to Europe, where

Correspondence Marta Alonso, Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, 216 Dryden Hall, Corvallis, 97331-3804 OR, USA (e-mail: hearnma@science.oregonstate.edu)

it has become widely established among populations of rainbow trout, *Oncorhynchus mykiss* (Walbaum). IHNV is an economically important fish pathogen, causing severe acute infections in young salmonid fish and resulting in high mortalities. Depending upon the species of fish, strain of the virus, and environmental conditions, outbreaks of IHNV may result in losses of 80–100% when young fish are infected. Once clinical signs become apparent, the disease is usually irreversible and fatal. Following a disease outbreak, surviving fish may become virus carriers without showing obvious signs (Kim, Dummer, Chiou & Leong 1999). Presently, the only control measure for the spread of IHNV is by destruction of infected stocks. Although effective DNA vaccines to IHNV have been developed and tested under laboratory conditions (Anderson, Mourich, Fahrenkrug, LaPatra, Shepherd & Leong 1996; Alonso, Johnson, Simon & Leong 2003), these are not yet commercially available. The requirements for low cost, ease of application and safety have restricted their commercial development for aquaculture. Such issues will need to be addressed before large-scale application of DNA vaccines on fish farms is adopted. However, taking into consideration aspects of safety and ease of administration, antiviral agents may be a more feasible approach to the control of IHNV infection and disease.

Sequence-specific oligomers designed to inactivate selected messenger RNAs (the sense strands) are commonly called 'antisense' oligos. Antisense oligomers of various structural types have been used to inhibit the gene expression of several viral pathogens (Ma, Rede, Naqvi & Cook 2000). The first antisense compound to receive FDA approval for use in humans, a therapeutic for the treatment of cytomegalovirus (CMV) retinitis, targets the *IE-2* gene of CMV (Green, Roh, Pippin & Drebin 2000). Antisense phosphorodiamidate morpholino oligomers (PMO) are a new class of antisense agents with high specificity and efficacy (Stein, Foster, Huang, Weller & Summerton 1997; Summerton, Stein, Huang, Matthews, Weller & Partridge 1997; Schmajuk, Sierakowska & Kole 1999). They contain purine or pyrimidine bases attached to a backbone composed of 6-member morpholine rings joined by phosphordiamidate inter-subunit linkages and are usually synthesized to be 20–25 subunits long. PMO are sequence-specific, uncharged, water soluble and nuclease resistant (Hudziak, Barofsky, Barofsky, Weller, Huang & Weller 1996;

Summerton & Weller 1996). PMO bind to RNA by Watson-Crick base pairing, and can prevent translation by steric blocking of translation initiation with minimal toxicity (Stein *et al.* 1997; Ghosh, Stein, Weller & Iversen 2000). Regions of mRNA sequence that are likely to be effective targets for PMO antisense agents can be predicted with relative ease and usually involve either RNA splice sites or the region comprising the 5'-untranslated region, AUG translational start codon and the first 20 or so bases of protein coding sequence (Stein *et al.* 1997; Giles, Spiller, Clark & Tidd 1999; Ghosh *et al.* 2000). PMO have demonstrated efficacious, specific, and non-toxic reduction of target protein levels in a number of tissue cultures (Arora, Knapp, Smith, Statfield, Stein, Reddy, Weller & Iversen 2000; Hudziak, Summerton, Weller & Iversen 2000; Nasevicius & Ekker 2000; Kipshidze, Kim, Iversen, Yazdi, Bhargava, New, Mehran, Tio, Haudenschild, Dangas, Stone, Iyer, Roubin, Leon & Moses 2002). Further, these studies have established PMOs as essentially non-toxic agents with favourable pharmacokinetic properties (Iversen, Arora, Acker, Mason & Devi 2003). PMOs have demonstrated the ability to inhibit replication of vesivirus (a calicivirus) (Stein, Skilling, Iversen & Smith 2001), and mouse hepatitis virus (a coronavirus) (Neuman, Stein, Kroeker, Paulino, Moulton, Iversen & Buchmeier 2004). The goals of this study were to determine whether efficient and non-toxic delivery of PMO into fish cells and tissues is possible, and if so, whether replication of IHNV could be inhibited by PMO complementary to either antigenomic or genomic RNA.

Materials and methods

Phosphorodiamidate morpholino oligomers

The PMOs were synthesized at AVI BioPharma (Corvallis, OR, USA) as previously described (Summerton & Weller 1996). The chemical structure of the PMO backbone is shown in Fig. 1a. Purity of full-length oligomers was >95% as determined by reverse-phase high-pressure liquid chromatography and MALDI TOF mass spectroscopy. Membrane-penetrating peptide was covalently conjugated to the 5' end of the PMO with the cross-linker *N*-[γ -maleimidobutyryloxy] succinimide ester (Moulton, Hase, Smith & Iversen 2003). The conjugated peptides used in this study were designated as P002 (RRRQRRKKRC) (Moul-

Figure 1 (a) Chemical structure of phosphorodiamidate morpholino oligomers (PMO). The deoxyribose of DNA is replaced by a 6-membered morpholine ring, and the phosphodiester internucleoside bond is replaced by a phosphorodiamidate linkage in the PMO structure. Arginine-rich peptide, when present, is conjugated to the 5' end of a PMO through the illustrated linker. Arginine-rich peptide sequences used in this work are described in Materials and methods. Fluorescein, when present, is conjugated directly to the nitrogen of the morpholine ring, on the 3' end of the PMO. (b) Targeting localizations in the IHNV genome of the PMOs used in this study.

ton *et al.* 2003) and P003 (RRRRRRRRRFFC) (Moulton, Nelson, Hatlevig, Reddy & Iversen 2004). Both peptides were custom synthesized by Global Peptide Services (Fort Collins, CO, USA) and purified to >90% purity. Details of the conjugation process and purification of peptide-conjugated PMOs has been previously described (Moulton *et al.* 2003, 2004). Table 1 and Fig. 1b show the nomenclature, sequences and targeting locations in the IHNV genome of the PMOs used in this study. PMOs were designed to target the AUG translation initiation regions of the N and G

genes of the Round Butte 1 (RB1) isolate of IHNV (GenBank accession numbers U50401 and U50402, respectively). As the RB1 isolate of IHNV has not been completely sequenced, a PMO to target the L gene translation-initiation region was designed according to the sequence reported for the IHNV WRAC isolate (GenBank accession no. L40883). An additional compound (P003-L 3' PMO) was designed to hybridize to sequence near the 5'-terminus of the IHNV negative-strand genomic RNA. The four PMOs described above were conjugated to P003 peptide. G and L

Table 1 Oligomer sequence and target location in IHNV RNA

Oligomer name	Location of target sequence in IHNV	Oligomer 5'–3' sequence
P003-G PMO	AUG-region of G m/c RNA ^a	P003-5'-GGT GTA CAT TGT TTT GGT GGG-3'
P003-L PMO	AUG-region of L m/c RNA ^a	P003-5'-GAA GTC CAT CTT TCT GTG ATG-3'
P003-L 3'PMO	Near 5'-end of genomic RNA	P003-5'-TTC TTT CCA CTA GGC GAA CAA-3'
P003-N PMO	AUG-region of N m/c RNA ^a	P003-5'-GCT TGT CAT CGT TCG TGA TCC-3'-F1
G PMO	AUG-region of G m/c RNA ^a	5'-GGT GTA CAT TGT TTT GGT GGG-3'
L PMO	AUG-region of L m/c RNA ^a	5'-GAA GTC CAT CTT TCT GTG ATG-3'
L 3' PMO	Near 5'-end of genomic RNA	5'-TTC TTT CCA CTA GGC GAA CAA-3'
P003-N Scramble PMO		P003-5'-TGC TCA CTG CTG TCA GTG TCT-3'
P003-irrelevant sequence PMO		P003-5'-TGT TCA TCT GTC CTG TCT GTC-3'
P003-PMO-FITC		P003-5'-CCT CTT ACC TCA GTT ACA-3'-F1
P002-PMO-FITC		P002-5'-CCT CTT ACC TCA GTT ACA-3'-F1
PMO-FITC		5'-CCT CTT ACC TCA GTT ACA-3'-F1

^a m/c RNA-messenger and complementary RNA.

Highlighted codons are the translation-initiation codons of each corresponding IHNV gene.

translation-initiation region PMO, without P003 peptide conjugation, were also prepared. A scrambled-sequence version of the P003-N PMO and a second irrelevant sequence control compound were also synthesized and conjugated to P003 peptide in an identical manner as the antisense compounds, to serve as controls for any generic non-sequence-specific activity of the P003-PMO chemistry. In order to permit analysis of the uptake of PMO forms into fish cell lines by fluorescent microscopy and flow cytometry, carboxyfluorescein was conjugated to the 3'-end of a random-sequence PMO (PMO-FITC). The same fluorescein-tagged random sequence PMO was also 5'-conjugated to P002 peptide (P002-PMO-FITC) and P003 peptide (P003-PMO-FITC). All the lyophilized PMOs used in this study were dissolved in sterile water prior to use in culture cells.

Viruses and treatment of cell cultures with PMO

Initial studies were designed to evaluate PMO uptake in cell lines capable of supporting IHNV infections. Two fish cell lines were evaluated, epithelioma papulosum cyprini (EPC) cells (Fijan 1983) and a Chinook salmon embryonic cell line (CHSE-214) (Lannan, Winton & Fryer 1984), in order to investigate cell-to-cell variation. The EPC cell line was obtained from Dr Sara Perez (Centro de Investigaciones Biológicas, Spain) and the CHSE-214 cells from the ATCC. To investigate the ability of PMO to inhibit IHNV infection, CHSE-214 cells were grown in duplicate 6-well plates at 17 °C in minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine. Prior to treatment with PMO compounds, confluent cell monolayers were rinsed twice with RPMI supplemented with 10% FBS and 2 mM L-glutamine. Following the removal of the second rinse, 2 mL of RPMI-10% FBS containing an appropriate PMO or corresponding volume of dH₂O was added. Cells and medium were then incubated overnight at 17 °C. The next day, log dilutions of IHNV (RB1 isolate) in MEM-2% FBS (200 µL per well) were added. After 1 h of adsorption at 15 °C, virus inoculum was removed and quantification of virus was performed by plaque assay following the method of Burke & Mulcahy (1980). After 10 days of incubation at 15 °C to allow IHNV plaque formation, the monolayers were fixed and stained with a crystal violet solution [0.25% crystal violet, 10% ethanol and 2% formaldehyde in

phosphate-buffered saline (PBS)] for 48 h, and the number of plaque-forming units were scored. CHSE-214 cells infected with log dilutions of IHNV but receiving mock treatment with water only, as well as PMO-treated cells without IHNV infection, were included as controls.

Fluorescent microscopy

Confluent monolayers of EPC and CHSE-214 cells in 24-well plates were treated with 10 µM fluorescein-conjugated PMO. After overnight incubation at 17 °C in a dark box, the cells were washed with PBS three times. The cells were then visualized with a Nikon Diaphot 300 fluorescence microscope (Nikon, Tokyo, Japan), and images were captured with an Olympus digital camera and MagnaFire software (Optronics, Goleta, CA, USA).

Flow cytometry

The extent of PMO uptake in the two fish cell lines was quantified using flow cytometry. Confluent monolayers of EPC and CHSE-214 cells in 24-well plates were washed twice with RPMI-10% FBS and then treated with 10 µM fluorescein-conjugated PMO. After overnight incubation at 17 °C in a dark box, cells were washed with PBS three times, resuspended, spun down and then suspended in 500 µL of PBS. The flow data were collected using a BD FACSCaliburTM cytometer (Becton Dickinson, Mountain View, CA, USA). Data were analysed using FlowJoTM Software (Tree Star, San Carlos, CA, USA). Cells that underwent the same treatment in the absence of fluorescein-conjugated PMO were used to establish light scattering and autofluorescence parameters for comparison with fluorescein-positive cells.

Western immunoblots

Equal volumes of infected cell lysates from each well of a given treatment were resuspended in PBS, combined, and evaluated for protein concentration by spectrophotometry (Bio-Rad Laboratories, Hercules, CA, USA). Approximately 10 µg of each sample was homogenized in an equal volume of SDS-PAGE sample buffer. After boiling for 5 min, the reaction mixtures were loaded onto a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and run in a Bio-Rad Miniprotein II Electrophoresis Cell at 150 V for

1 h. Protein standards were the MagicBench SDS-PAGE standards (BenchmarkTM, Grand Island, NY, USA). Separated proteins were electrophoretically transferred to a polyvinylidene fluoride transfer membrane (ISC BioExpress, Kaysville, UT, USA) in cold Towbin buffer for 1 h at 100 V using a Bio-Rad Mini Transblot electrophoretic transfer cell. The blot was blocked for 12 h at ambient temperature in TBS containing 5% non-fat dried milk. The membrane was then incubated for 1 h at room temperature with 136J monoclonal antibody (mAb) in TBST. 136J is a mAb raised against the 67-kDa glycoprotein of IHNV (Xu, Mourich, Engelking, Ristow, Arnzen & Leong 1991). The membrane was subsequently washed three times for 10 min each with TBST and then incubated for 1 h with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad) at a 1:3000 dilution in TBS. Finally, the membrane was washed repeatedly with TBST, incubated with ECL Western blotting detection system (Amersham, Arlington Heights, IL, USA) and exposed to Kodak Biomax film (Eastman Kodak, Rochester, NY, USA). Duplicate blots were probed for β -actin levels with mAb AC-40 (Sigma, St Louis, MO, USA) to confirm equal amounts of protein loading per lane.

Treatment of fish with fluorescently labelled PMOs

The rainbow trout fry used in these studies were obtained from Roaring River Hatchery (Scio, OR, USA) and held in 100-L tanks at the Salmon Disease Laboratory, Oregon State University, until the fish mean weight was 0.3 g. Several experimental groups of five fish were compared in these laboratory trials, including an untreated group and groups immersed in either PMO-FITC, P002-PMO-FITC or P003-PMO-FITC. The fish were treated by static immersion for 20 min in 50 mL of water at 17 °C with stated PMO compound present at 10 μ M, or water without compound, and then transferred to a 2-L beaker containing non-chlorinated pathogen-free water at 17 °C. After 1 h, fish were killed by anaesthetic overdose in 200 μ g mL⁻¹ of MS-222. Gills were excised and immediately imaged with a fluorescence microscope. The remaining gills and visceral tissues of each fish were homogenized in 1 mL of tissue lysis buffer (Promega, Madison, WI, USA) using a homogenizer. The lysates were then centrifuged at 10 000 *g* for 10 min to pellet large cellular debris, and 600 μ L of the supernatant fluid

were examined for total fluorescence using a model F-2000 fluorometer (Hitachi, Tokyo, Japan).

Results

Cellular uptake of fluorescently labelled PMOs

Intracellular delivery of PMO-FITC, P002-PMO-FITC and P003-PMO-FITC into two different lines of cultured fish cells was evaluated. The images taken of CHSE-214 and EPC cells treated with PMO-FITC show no discernable fluorescence, indicating the absence of compound uptake (data not shown). Fluorescence was visible when either of the two fish cell lines were treated with P002-PMO-

Figure 2 Microscopy of CHSE-214 and EPC cells incubated O/N with 10 μ M fluorescein-conjugated PMOs. Left-hand side panel is bright-field image and right-hand side is the same field under fluorescent illumination. (a) CHSE-214 cells incubated with P002-PMO-FITC. (b) CHSE-214 cells incubated with P003-PMO-FITC. (c) EPC cells incubated with P002-PMO-FITC. (d) EPC cells incubated with P003-PMO-FITC. Compound names/sequences are as in Table 1.

FITC or P003-PMO-FITC (Fig. 2). CHSE-214 cells (Fig. 2a,b) appeared to show a slightly higher number of fluorescent cells than did the EPC cells (Fig. 2c,d). For both cell lines, the fluorescent signal in the P003-PMO-FITC-treated cells was markedly higher than in the P002-PMO-FITC-treated cells.

Flow cytometry was used to quantify the uptake of PMO compounds in CHSE-214 and EPC cells. Fig. 3 represents the flow cytometric data from the fish cell lines treated with PMO-FITC, P002-PMO-FITC or P003-PMO-FITC. CHSE-214 cells treated with P003-PMO-FITC exhibited the highest mean fluorescent intensity (m.f.i.) of 86.07 in the fluorescein detection channel (Fig. 3a) and this was slightly lower for EPC cells (63.1 m.f.i.) (Fig. 3b) resulting in approximately a 20- and 13-fold, respectively, increase in uptake over cells treated with the PMO-FITC. Uptake of the P002-PMO-FITC into CHSE-214 and EPC cells was less compared with the P003 conjugate but still 15- and 8-fold, respectively, more than PMO-FITC. These results confirmed the

differential delivery of PMO to the two cell lines and demonstrate the considerable PMO delivery-enhancing capacity of both peptides.

Inhibition of IHNV amplification by PMOs

Having established by fluorescence-assay that P003-PMO is able to enter fish cells, we investigated whether PMOs could functionally inhibit IHNV replication. PMOs complementary to viral genomic or antigenomic RNA were tested for their ability to inhibit IHNV amplification in cell culture. The effect of the PMOs on the titre of IHNV in CHSE-214 cells is shown in Fig. 4a,b. A simple average percentage inhibition is presented for ease of comparison. Several other similar experimental trials were conducted that yielded results consistent with those of Fig. 4. For the experiment reported, PMOs were added to CHSE-214 cell cultures at a final concentration of 15 μ M in RPMI-10% FBS. Significant inhibition of viral titre was observed with the P003-L PMO (Fig. 4a; lane 2), P003-L 3'

Figure 3 Flow cytometric analysis of cells treated with fluorescein-conjugated PMOs. Cultured cells; (a) CHSE-214 or (b) EPC were treated with either 10 μ M fluorescein-conjugated PMO-FITC (solid histogram), P002-PMO-FITC (—) or P003-PMO-FITC (---) for 1 h. The cells were analysed by flow cytometry after trypsinization and washing. (c) The mean fluorescent intensity (m.f.i.) for each cell type and treatment are calculated from the histogram shown.

Figure 4 Inhibition of IHNV titre by PMOs. Representative experiments from the several trials carried out to determine the effect of different PMO compounds on viral titre. The data are presented as two separate graphs, as these two series of samples were tested at different times and the mock-treated controls achieved differing final titre readings. Single wells of CHSE-214 cells were treated with the designated PMOs at a concentration of 15 μ M overnight and then infected with IHNV. Final viral titres were assessed by plaque assay as described in Materials and methods. (a) Lanes: 0, No PMO; 1, P003-G PMO; 2, P003-L PMO; 3, P003-L 3' PMO; 4, G PMO; 5, L PMO; 6, L 3' PMO. (b) IHNV viral titres in CHSE-214 treated with N-targeted PMOs. Lanes: 0, No PMO; 7, P003-N PMO; 8, P003-N Scramble PMO and 9, P003-irrelevant sequence PMO. In the lower portion of panels a and b, the results are expressed as the percentage inhibition of titre of treated cells compared with untreated cells. (c) Immunoblots of CHSE-14 cells receiving PMO treatment and subsequent IHNV infection as above were harvested 10 days post-inoculation. Cell lysates were prepared and used for immunoblots. Blots were probed with an antibody against the 67-kDa IHNV glycoprotein (upper blot). The lower blot shows the 43-kDa band generated with a β -actin antibody. Sample designations that appear above the lanes apply to both blots and correspond to the sample numbers above in Fig. 4a.

PMO (Fig. 4a; lane 3) and P003-N PMO (Fig. 4b; lane 7); with 49, 54 and 64% inhibition, respectively. P003-G PMO failed to show a large effect on the IHNV viral replication (23.3% inhibition) (Fig. 4a; lane 1). PMO-G and PMO-L (both non-peptide-conjugated) were also used to further assess the contribution of P003 peptide to cellular delivery. No effect on viral replication was observed with any of the non-peptide conjugated PMOs (Fig. 4a; lanes 4–6). Furthermore, IHNV growth was not inhibited by the P003-N Scramble PMO (Fig. 4b; lane 8) or by the P003-irrelevant sequence PMO (Fig. 4b; lane 9), suggesting a lack of cytotoxicity by this form of peptide-conjugated

PMO compound under the conditions tested, and indicating that the IHNV antisense P003-PMOs used in the study were specific.

Western blot analysis of infected cells treated with L or G-targeted PMOs was conducted to compare the viral titre observations with protein expression. Lysates from CHSE-214 cells treated with various PMOs and then infected with IHNV were subjected to Western-immunoblotting and probed with either IHNV glycoprotein (Fig. 4c, top panel) or β -actin monoclonal antibodies (Fig. 4c, bottom panel). This analysis showed diminished signal from the samples treated with a 20 μ M concentration of P003-L 3' PMO (lane 3) or

P003-L PMO (lane 2). The level of signal from the sample of infected cells treated with P003-L 3'PMO was nearly undetectable, indicating that this particular PMO compound caused a potent inhibition of IHNV amplification. In contrast, a clearly discernible 67 kDa band (representing IHNV glycoprotein) was evident in lysates from cells incubated with P003-G PMO, or any of the three non-peptide-conjugated PMOs (lanes 1, 4–6). These results are consistent with the previous observations of reduced viral titre shown in Fig. 4a,b. Immunoblotting results of identical samples probed with the β -actin antibody show no significant differences between samples. These data confirm the consistent level of total protein in the cell lysates that were loaded on the gel from which the immunoblot was made.

Dose–response study with active PMO compounds

In order to investigate the issue of efficacy vs. specificity over a range of PMO doses in cell culture, various concentrations of PMOs were used and the effects on IHNV titre were evaluated. Dose–response studies were performed with the three PMOs that had shown significant inhibition of viral titre at 15 μ M in the previous experiment; P003-L PMO, P003-L 3' PMO and P003-N PMO. CHSE-214 cells were infected with log dilutions of IHNV in the absence or in the presence of the above PMOs at final concentrations of 0.2, 1, 2, 10 and 20 μ M, and virus titres were determined as above. P003-L PMO treatment resulted in little inhibition of IHNV viral titre except at 20 μ M (Fig. 5a). Similar results were obtained with the P003-N PMO (Fig. 5c). Treatment with P003-L 3' PMO yielded no inhibition at 0.2, 1 or 2 μ M, moderate inhibition at 10 μ M and marked inhibition (97%) of IHNV infection at 20 μ M (Fig. 5b). Treatments (20 μ M) of P003-L PMO, P003-L 3' PMO or P003-N PMOs were not toxic to mock-infected cells, as assessed visually. Cells remained attached to the plate and no change in the colour of the culture medium was observed. Additionally, cells treated with P003-N Scramble PMO at 20 μ M showed no inhibition of IHNV titre (Fig. 5c).

In vivo uptake of fluorescently labelled PMOs

To study the uptake of PMO into living fish tissues, groups of five fish were treated by static

Figure 5 Plaque assay results from PMO dose–response challenge of IHNV-infected cells. Details of experimental time-line as for Fig. 4a,b and as described in text. Duplicate wells of CHSE-214 cells treated with various concentrations of PMOs were infected with log dilutions of IHNV and the final infected titres were evaluated by plaque assay. Each bar graph represents cells treated with a different PMO: (a) P003-L PMO, (b) P003-L 3' PMO, and (c) P003-N PMO. Dose designations appear under the x-axis of the graph.

immersion in a 10 μ M solution of PMO-FITC, P002-PMO-FITC or P003-PMO-FITC. After 20 min of treatment and a subsequent 1 h of rinse-immersion, fish from each treatment group were killed by anaesthetic overdose and the gills excised, observed visually on a fluorescence microscope and photographed (Fig. 6). P003-PMO-FITC was diffusely distributed throughout the gill tissue and generated intense fluorescent staining (Fig. 6c). In contrast, the fluorescence throughout the gill tissues of the PMO-FITC-

Figure 6 Fluorescence microscopy of gills from rainbow trout. Approximately 0.3 g fish were exposed to 10 μM of the stated compounds by immersion for 20 min as described in Materials and methods. (a) PMO-FITC treatment, (b) P002-PMO-FITC and (c) P003-PMO-FITC treatment. The left-hand side images are bright field and the right-hand side images are the identical fields under fluorescent illumination.

treated fish (Fig. 6a) or P002-PMO-FITC-treated fish (Fig. 6b) was very weak. The viscera of animals from each treatment group were homogenized and examined for whole-body fluorescence using a fluorometer. When data from the homogenates of the P003-PMO-FITC replicate fish were pooled, a 565 mean value of fluorescence signal was obtained. In contrast, a 502 value of fluorescence signal was detected from the homogenates of PMO-FITC-treated fish. A background autofluorescence signal of 395 was recorded for untreated fish. These results indicate that PMO and peptide-conjugated PMO can apparently associate with and/or be taken up by fish tissues and that the P003 peptide is an effective PMO delivery enhancer.

Discussion

The study presented here was carried out in order to examine the potential of PMO antisense compounds to act as effective chemotherapeutic agents against a viral disease that has a sizable impact on the aquaculture industry. IHNV is responsible for large fish losses in the US trout industry. At the present time, there is no commercially available vaccine or chemotherapeutic for IHNV.

In order to bring about the reduction of an undesirable protein in a living cell, an antisense compound must be able to enter the cellular compartment(s) where its target genetic sequence is located, specifically the cytosolic and/or nuclear compartments. In the present study, peptide-conjugated PMOs tagged with fluorescein were efficiently delivered into two fish cell types in culture, under standard incubation conditions, as shown by fluorescence microscopy and flow cytometry analysis. The data showed that CHSE-214 cells exhibited higher levels of fluorescence than EPC cells when incubated with these compounds. Both cell lines showed individual cells with diffuse, apparently cytoplasmic signal, as well as fluorescent nuclei, indicating delivery was extensive into both major cellular compartments. The highest amount of intracellular fluorescence was observed in the P003-PMO-FITC treated cells. The ability of the P003 peptide to deliver PMO across biological membranes was characterized previously in HeLa cells (Moulton *et al.* 2004). Our results indicate that the P003 peptide also significantly enhances delivery and activity of PMO in fish cell lines. The uptake of P003-PMO was clearly mediated by P003, as incubation of either cell line with PMO lacking peptide conjugation yielded no intracellular fluorescent signal.

In the present work, P003-PMO directed against either IHNV RNA polymerase or nucleoprotein were shown to inhibit viral amplification. The effects observed were sequence specific and dose dependent. Considerable inhibition of viral titre was observed when infected cells were incubated with 15 μM of P003-N, P003-L 3' or P003-L PMOs with 64, 54 and 49% of inhibition, respectively. When the cells were incubated with 20 μM concentration of the same PMOs 69, 97 and 60% inhibitions were recorded, indicating a concentration-dependent inhibition of IHNV amplification by these compounds. Treatment with P003-G PMO at a dose of 15 μM resulted in only 23% inhibition of the

IHNV titre. We assume that the minimal impact on viral titre by this compound reflects either (i) inaccessibility of the target sequence to the P003-G PMO compound, (ii) poor avidity of duplexing between compound and RNA target sequence, or (iii) successful hybridization, but a lack of effect on IHNV amplification by the resultant lowered amounts of G-gene product. A P003-PMO (P003-N Scramble PMO) with no significant homology to any IHNV or salmonid cellular sequences, showed no inhibition of IHNV growth at concentrations up to 20 μM , providing evidence that the IHNV antisense-PMOs used in this study were not cytotoxic and had biological impact solely through a target-specific antisense mode of action.

P003-L 3' PMO, which is complementary to the 5'-end of the negative-strand viral genome, was shown to be the most potent inhibitor of IHNV replication tested, almost completely blocking IHNV amplification at a concentration of 20 μM . The reduced intensity of the 67-kDa G-protein band on a Western blot supports the observations of reduced viral titre. This compound was designed somewhat inadvertently, but fortuitously is complementary to sequence located approximately 155–175 nucleotides (nt) from the 5' terminus of the genomic (negative) strand. This location is within the L gene, the 5' end of which is at approximately 150 nt before the 5' terminus of the genomic strand. The sequence that comprises the 59 5' terminal nt of the genomic strand is known as the 'trailer region', and its integrity has been demonstrated as critical to proper transcription (Whelan & Wertz 1999b) and virion assembly (Whelan & Wertz 1999a) in vesicular stomatitis virus, a related rhabdovirus. Fig. 7 shows an M-fold secondary structure diagram of 140 nt of IHNV genomic RNA that comprises the P003-L 3' PMO binding region and surrounding sequence. This diagrammed region shows a series of stable stem loops and indicates that the P003-L 3' PMO binding region is located in a region that is particularly highly ordered. The diagram shown had the highest ΔG value for any of the predicted configuration variations offered by the folding program (data not shown). We speculate that disruption of this putative stem loop located near the 5' terminus of the genomic RNA may have contributed to the high inhibition of IHNV amplification observed. PMOs complementary to IHNV antigenomic RNA such as P003-N PMO, P003-G PMO or P003-L PMO also showed inhibition of IHNV replication, but to

Figure 7 Predicted secondary structure of the P003-L 3' PMO binding region and surrounding sequence in the 5' end of the viral RNA-negative strand. The P003-L 3' PMO sequence target is designated as a black line bordering the super-structure.

a much lesser extent. Although translational arrest certainly represents an important mechanism of action for antisense drugs, our results suggest that PMO interaction with the 5' end of the viral negative strand profoundly affects IHNV replication in cell culture, and could represent a potent antiviral compound. The success of an antisense PMO probably depends, to some extent, on its molar ratio to its target RNA. It may be that the superior efficacy of the PMO with a negative-strand target compared with that of a PMO with a positive-strand target was due to the lower molar amount of negative-strand genomic RNA target compared with the amount of positive strand targets (i.e. many mRNA molecules for a given target, in addition to full-length positive-strand replicative-intermediate RNA). In addition, the strong antiviral effect of the genomic-targeted PMO may be due, in part, to the fact that it affects viral transcription early in the viral replication cycle.

This is the first report of PMO challenge of a rhabdovirus. The efficacy afforded by PMOs targeting sequences in the antigenomic L- and N-genes, and sequences in the terminal portion of the L-gene genomic RNA, may represent productive targeting guidance for use of this technology against other negative-stranded non-segmented RNA viruses.

Acknowledgements

We wish to thank the chemistry group at AVI BioPharma for the synthesis, purification, and characterization of the PMO compounds used in these studies. This work was supported by AVI BioPharma (Corvallis, Oregon) and by grants R/BT-28-NSI, R/BT-35 and 04-058-04 from the Oregon Sea Grant and grant 3610006041 (Animal Health and Disease Research) from the United States Department of Agriculture to Dr Jo-Ann Leong.

References

- Alonso M., Johnson M., Simon B. & Leong J.A. (2003) A fish specific expression vector containing the interferon regulatory factor 1A (IRF1A) promoter for genetic immunization of fish. *Vaccine* **21**, 1591–1600.
- Anderson E.D., Mourich D.V., Fahrenkrug S.C., LaPatra S., Shepherd J. & Leong J.A. (1996) Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Molecular Marine Biology and Biotechnology* **5**, 114–122.
- Arora V., Knapp D.C., Smith B.L., Statfield M.L., Stein D.A., Reddy M.T., Weller D.D. & Iversen P.L. (2000) c-Myc antisense limits rat liver regeneration and indicates role for c-Myc in regulating cytochrome P-450 3A activity. *Journal of Pharmacology and Experimental Therapeutics* **292**, 921–928.
- Burke J.A. & Mulcahy D. (1980) Plaquing procedure for infectious hematopoietic necrosis virus. *Applied and Environmental Microbiology* **39**, 872–876.
- Fijan N. (1983) Some properties of the *epithelioma papulosum cyprini* (EPC) cell line from common carp *Cyprinus carpio*. *Annals of Virology* **134E**, 207–220.
- Ghosh C., Stein D., Weller D. & Iversen P. (2000) Evaluation of antisense mechanisms of action. *Methods in Enzymology* **313**, 135–143.
- Giles R.V., Spiller D.G., Clark R.E. & Tidd D.M. (1999) Antisense morpholino oligonucleotide analog induces missplicing of c-myc mRNA. *Antisense and Nucleic Acid Drug Development* **9**, 213–220.
- Green D.W., Roh H., Pippin J. & Drebin J.A. (2000) Antisense oligonucleotides: an evolving technology for the modulation of gene expression in human disease. *Journal of the American College of Surgeons* **191**, 93–105.
- Hudziak R.M., Barofsky E., Barofsky D.F., Weller D.L., Huang S.B. & Weller D.D. (1996) Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. *Antisense and Nucleic Acid Drug Development* **6**, 267–272.
- Hudziak R.M., Summerton J., Weller D.D. & Iversen P.L. (2000) Antiproliferative effects of steric blocking phosphorodiamidate morpholino antisense agents directed against c-myc. *Antisense and Nucleic Acid Drug Development* **10**, 163–176.
- Iversen P.L., Arora V., Acker A.J., Mason D.H. & Devi G.R. (2003) Efficacy of antisense morpholino oligomer targeted to c-myc in prostate cancer xenograft murine model and a Phase I safety study in humans. *Clinical Cancer Research* **9**, 2510–2519.
- Kim C.H., Dummer D.M., Chiou P.P. & Leong J.A. (1999) Truncated particles produced in fish surviving infectious hematopoietic necrosis virus infection: mediators of persistence? *Journal of Virology* **73**, 843–849.
- Kipshidze N.N., Kim H.S., Iversen P., Yazdi H.A., Bhargava B., New G., Mehran R., Tio F., Haudenschild C., Dangas G., Stone G.W., Iyer S., Roubin G.S., Leon M.B. & Moses J.W. (2002) Intramural coronary delivery of advanced antisense oligonucleotides reduces neointimal formation in the porcine stent restenosis model. *Journal of the American College of Cardiologists* **39**, 1686–1691.
- Kurath G., Ahern K.G., Pearson G.D. & Leong J.C. (1985) Molecular cloning of the six mRNA species of infectious hematopoietic necrosis virus, a fish rhabdovirus, and gene order determination by R-loop mapping. *Journal of Virology* **53**, 469–476.
- Kurath G., Higman K.H. & Bjorklund H.V. (1997) Distribution and variation of NV genes in fish rhabdoviruses. *Journal of General Virology* **78**, 113–117.
- Lannan C.N., Winton J.R. & Fryer J.L. (1984) Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro* **20**, 671–676.
- Ma D.D., Rede T., Naqvi N.A. & Cook P.D. (2000) Synthetic oligonucleotides as therapeutics: the coming of age. *Biotechnology Annual Reviews* **5**, 155–196.
- Morzunov S.P., Winton J.R. & Nichol S.T. (1995) The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. *Virus Research* **38**, 175–192.
- Moulton H.M., Hase M.C., Smith K.M. & Iversen P.L. (2003) HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers. *Antisense and Nucleic Acid Drug Development* **13**, 31–43.
- Moulton H.M., Nelson M.H., Hatlevig S.A., Reddy M.T. & Iversen P.L. (2004) Cellular uptake of antisense morpholino oligomers conjugated to arginine-rich peptides. *Bioconjugated Chemicals* **15**, 290–299.
- Nasevicius A. & Ekker S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. *Nature Genetics* **26**, 216–220.
- Neuman B.W., Stein D.A., Krocker A.D., Paulino A.D., Moulton H.M., Iversen P.L. & Buchmeier M.J. (2004) Antisense morpholino-oligomers directed against the 5' end of the genome inhibit coronavirus proliferation and growth. *Journal of Virology* **78**, 5891–5899.
- Schmajuk G., Sierakowska H. & Kole R. (1999) Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. *Journal of Biological Chemistry* **274**, 21783–21789.
- Stein D., Foster E., Huang S.B., Weller D. & Summerton J. (1997) A specificity comparison of four antisense types: morpholino, 2'-O-methyl RNA, DNA, and phosphorothioate DNA. *Antisense and Nucleic Acid Drug Development* **7**, 151–157.
- Stein D.A., Skilling D.E., Iversen P.L. & Smith A.W. (2001) Inhibition of Vesivirus infection in mammalian tissue culture

- with antisense morpholino oligomers. *Antisense and Nucleic Acid Drug Development* **11**, 317–325.
- Summerton J. & Weller D. (1997) Morpholino antisense oligomers: design, preparation, and properties. *Antisense and Nucleic Acid Drug Development* **7**, 187–195.
- Summerton J., Stein D., Huang S.B., Matthews P., Weller D. & Partridge M. (1997) Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. *Antisense and Nucleic Acid Drug Development* **7**, 63–70.
- Whelan S.P. & Wertz G.W. (1999a) The 5' terminal trailer region of vesicular stomatitis virus contains a position-dependent cis-acting signal for assembly of RNA into infectious particles. *Journal of Virology* **73**, 307–315.
- Whelan S.P. & Wertz G.W. (1999b) Regulation of RNA synthesis by the genomic termini of vesicular stomatitis virus: identification of distinct sequences essential for transcription but not replication. *Journal of Virology* **73**, 297–306.
- Xu L., Mourich D.V., Engelking H.M., Ristow S., Arnzen J. & Leong J.C. (1991) Epitope mapping and characterization of the infectious hematopoietic necrosis virus glycoprotein, using fusion proteins synthesized in *Escherichia coli*. *Journal of Virology* **65**, 1611–1615.

Received: 19 January 2005

Revision received: 26 April 2005

Accepted: 27 April 2005