UNIVERSIDAD MIGUEL HERNÁNDEZ DE ELCHE

INSTITUTO DE BIOLOGÍA MOLECULAR Y CELULAR

CARACTERIZACIÓN DE LA ACTIVIDAD ANTIVIRAL Y CAPACIDAD INMUNOMODULADORA DE LAS DEFENSINAS EN PECES UTILIZANDO EL MODELO VHSV/TRUCHA ARCO IRIS

JUAN ALBERTO FALCÓ GRACIÁ

TESIS DOCTORAL 2008

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Don **José Manuel González Ros**, Catedrático de Bioquímica y Biología Molecular y Director del Instituto de Biología Molecular y Celular,

INFORMA, que la Tesis Doctoral titulada "**CARACTERIZACIÓN DE LA ACTIVIDAD ANTIVIRAL Y CAPACIDAD INMUNOMODULADORA DE LAS DEFENSINAS EN PECES UTILIZANDO EL MODELO VHSV/TRUCHA ARCO IRIS**", que para optar al grado de Doctor en Ciencias presenta D. **Juan Alberto Falcó Graciá**, ha sido realizada en el Instituto de Biología Molecular y Celular de la Universidad Miguel Hernández.

Elche, 3 de Marzo de 2008.

Fdo: Prof. Dr. José Manuel González Ros

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Dña. **Amparo Estepa Pérez**, Profesora Titular de Bioquímica de la Universidad Miguel Hernández, y Don **Julio Coll Morales**, Investigador del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA),

INFORMAN, que la Tesis Doctoral titulada "**CARACTERIZACIÓN DE LA ACTIVIDAD ANTIVIRAL Y CAPACIDAD INMUNOMODULADORA DE LAS DEFENSINAS EN PECES UTILIZANDO EL MODELO VHSV/TRUCHA ARCO IRIS**", que para optar al grado de Doctor en Ciencias presenta D. **Juan Alberto Falcó Graciá**, ha sido realizada bajo nuestra dirección. Considerando que esta tesis se halla concluida, autorizamos su presentación para que pueda ser juzgada por el tribunal correspondiente.

Elche, 3 de Marzo de 2008.

Fdo: Dra. Amparo Estepa Pérez Fdo: Dr. Julio Coll Morales

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PREFACIO

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Tal y como requiere la normativa interna de la Universidad Miguel Hernández, la memoria correspondiente a la Tesis Doctoral titulada "Caracterización de la actividad antiviral y capacidad inmunomoduladora de las defensinas en peces utilizando el modelo VHSV/trucha arco iris", que se presenta con un conjunto de publicaciones, consta de los siguientes apartados:

-Introducción general donde se presentan los trabajos y se justifica la unidad temática.

-Resumen global de los resultados obtenidos, de la discusión de éstos y de las conclusiones finales.

-Anexo con las publicaciones (presentadas en el idioma original de publicación).

> **Publicación 1.** *Falco A, Mas V, Tafalla C, Perez L, Coll JM, Estepa A. 2007.Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response.* ***Antiviral Research****, 76(2):111-23.*
>
> **Publicación 2.** *Falco A, Brocal I, Pérez L, Coll JM, Estepa A, Tafalla C. 2007. In vivo modulation of the rainbow trout (Oncorhynchus mykiss) immune response by the human alpha defensin 1, HNP1.* ***Fish and Shellfish Immunology****, 24(1):102-12.*
>
> **Publicación 3.** *Falco A, Chico V, Marroquí L, Perez L, Coll JM, Estepa A. 2008. Expression and antiviral activity of a beta-defensin-like peptide identified in the rainbow trout (Oncorhynchus mykiss) EST sequences.* ***Molecular Immunology****, 45(3):757-65.*

En la sección de resultados y discusión, para hacer referencia a las figuras que se muestran en las publicaciones, se utilizará siempre el número que la figura tiene en la publicación seguido del número que hace referencia a la publicación donde ésta se encuentra. Por ejemplo, para citar la figura 2 de la publicación 1 se indicará como Fig. 2, P1.

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INTRODUCCIÓN

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1.-IDENTIFICACIÓN DEL PROBLEMA

La protección de las poblaciones de peces acuicultivados frente a las enfermedades causadas por bacterias, virus y parásitos es uno de los retos más importantes que tiene aún por resolver la industria de la acuicultura ya que, las pérdidas ocasionadas por ellas, limitan en gran medida su rentabilidad y tasa de producción. Como no existen vacunas frente a la mayoría de estas enfermedades, tan sólo el uso de antibióticos ha mitigado en parte el problema aunque con las restricciones impuestas por, i) las cada vez más frecuentes resistencias que inducen, ii) los residuos que dejan en los animales tratados y que pueden ser perjudiciales a medio-largo plazo para la salud del consumidor, iii) el negativo impacto medioambiental que su administración a través del agua ocasiona y iv) su limitada eficacia ya que no son activos frente a virus, y hay que tener en cuenta que en la actualidad las enfermedades de origen vírico son las que mayor impacto negativo tienen en la acuicultura.

Como alternativa al uso de los antibióticos y no sólo en la acuicultura, se está proponiendo desde hace más de dos décadas el tratamiento de las enfermedades infecciosas con péptidos antimicrobianos endógenos (AMPs), uno de los componentes más importantes de las defensas innatas de todas las formas de vida conocidas (Hancock & Scott, 2000). Entre otras, las ventajas que poseen los AMPs respecto a los antibióticos convencionales son, i) no generan resistencias, ii) tienen un espectro de actividad más amplio y muchos de ellos poseen una potente actividad antiviral y iii) llevan asociada a su actividad antimicrobiana la capacidad para modular la respuesta inmune. Como desventajas hay que señalar los elevados costes económicos derivados de su producción, porque, aunque son de pequeño tamaño (15-70 aminoácidos (aas)), su síntesis química es complicada. Sin embargo, y teniendo en cuenta las grandes pérdidas económicas que las enfermedades infecciosas, sobre todo las de origen vírico, causan en acuicultura y los pocos o nulos avances realizados en los últimos años en relación con el desarrollo de vacunas eficaces y seguras para uso en acuicultura, la identificación

de moléculas que al mismo tiempo posean actividad antimicrobiana y propiedades inmunoestimuladoras para ser utilizadas directamente como agentes terapéuticos o como modelo para el diseño de fármacos eficaces y al mismo tiempo respetuosos con la salud del consumidor y el medio ambiente, es de suma importancia.

2.-FINALIDAD DEL TRABAJO

Dentro de este contexto, durante **el desarrollo de esta Tesis Doctoral** se **ha pretendido y conseguido** la **identificación de AMPs con actividad antiviral** frente a **rabdovirus** de peces **utilizando como modelo** el binomio rabdovirus de la septicemia hemorrágica vírica **(VHSV)/ trucha** arco iris (*Oncorhynchus mykiss)*. Entre otras, las razones que nos han llevado a elegir este modelo son,

1.-Los rabdovirus son, entre los virus que infectan a peces acuicultivados, los que causan a nivel mundial mayores pérdidas a la industria de la acuicultura, hasta el punto de poner en más de una ocasión en serias dificultades la viabilidad y sostenibilidad de este sector en muchos países de nuestro entorno. En Europa, actualmente las pérdidas directamente asociadas a las rabdovirosis se cifran en unos 60 millones de € anuales (Olesen, 1997) y en el año 2000 tan sólo en Dinamarca se perdieron unas 165 toneladas de trucha con un valor comercial de 211 000 € (Skall et al, 2005a). Teniendo en cuenta estas cifras no es de extrañar que los brotes de estos virus sean de obligada declaración a la Oficina Internacional de Epizootias (OIE).

2.-Los salmónidos, especies piscícolas más susceptibles a estos virus, son aún la base de la piscicultura en España y la Unión Europea (UE). Además, está demostrado que el VHSV también puede infectar a especies acuicultivadas marinas de alto valor económico como el rodaballo, cuya producción es cada vez de mayor importancia en la industria de la acuicultura española (Coll, 1999; Coll, 2001).

3.-La producción acuícola española depende en gran medida de la importación de huevos, alevines y juveniles de países donde estos virus son endémicos y, además, se han detectado recientemente (año 2006) brotes de VHSV en países de la UE con los que España mantiene importantes relaciones comerciales, como es el caso del Reino Unido y Rumania.

4.-Aunque en la actualidad España está considerada como una zona libre de rabdovirus, en 2005 se aisló el virus de la necrosis hematopoyética infecciosa (IHNV) de una piscifactoría de Granada.

5.-La trucha arco iris es, entre los peces teleósteos, una especie modelo para la realización de estudios relacionados con enfermedades infecciosas que afectan a peces acuicultivados y por ello su sistema inmune es uno de los que hasta el momento está mejor caracterizado.

6.-La experiencia que el grupo de investigación IBMC tiene con este modelo de trabajo ya que algunos de sus miembros llevan casi 15 años trabajando en diversos aspectos relacionados con la respuesta inmune de salmónidos frente a rabdovirus así como en la caracterización de las etapas del ciclo de multiplicación del VHSV y búsqueda de compuestos capaces de inhibirlas.

3.-DESCRIPCIÓN DEL MODELO DE TRABAJO

3.1 Los rabdovirus de peces y el VHSV

Hasta el momento se han aislado de peces más de 20 rabdovirus (Frerichs, 1989), pero sólo unos pocos, principalmente los que infectan peces teleósteos, han sido suficientemente caracterizados y asignados dentro de alguno de los géneros de la familia *rhabdoviridae* (**Tabla 1**).

Tabla 1. Clasificación de los rabdovirus que infectan a peces teleósteos (Essbauer & Ahne, 2001)

Rabdovirus de peces teleósteos	
Miembros del género Novirabdovirus	Virus de la necrosis hematopoyética infecciosa (IHNV) Virus de la septicemia hemorrágica vírica (VHSV) Rabdovirus Hirame (HIRRV)
Miembros tentativos del género Novirabdovirus	Rabdovirus de Snakehead (SHRV) Virus B12 de la anguila (EEV-B12) Virus C26 de la anguila (EEV-C26)
Miembros tentativos del género Vesiculovirus	Virus de la viremia primaveral de la carpa (SVCV) Rabdovirus pike fry (PFR) Virus americano de la anguila (EVA) Rabdovirus de la enfermedad ulcerativa (UDRV)

Los rabdovirus de peces están generalmente asociados con epizootias y elevadas pérdidas en la industria de la acuicultura y entre todos ellos destacan, por su mayor impacto económico en la piscicultura, el VHSV y el IHNV. Ambos virus pertenecen al género *Novirhabdovirus* dentro de la familia *Rhabdoviridae* que agrupa a los rabdovirus de peces cuyo genoma codifica para una proteína

adicional no estructural denominada Nv que mapea entre los genes de las proteínas G y L (Biacchesi et al, 2000; Walker & Kongsuwan, 1999) (**Figura 3**). Aunque VHSV e IHNV tienen un rango de huésped similar y causan una enfermedad de síntomas clínicos parecidos, no existen entre ellos reacciones cruzadas de seroneutralización.

En la actualidad no existen tratamientos terapéuticos eficaces frente a VHSV o IHNV aunque sí vacunas DNA eficaces que están basadas en el gen de la glicoproteína G (gpG) de superficie de estos virus. Por motivos relacionados con la seguridad no están comercializadas (Boudinot et al, 1998; Boudinot et al, 2001; DeKinkelin et al, 1995; Fernandez-Alonso et al, 2001; LaPatra et al, 2001; Leong et al, 1995; Lorenzen et al, 2002a; Lorenzen et al, 2002b) con excepción de la vacuna DNA frente a IHNV (APEX-IHN, Vical-Aqua Health Ltd of Canada) autorizada en Canadá desde 2005 (Salonius et al, 2007).

El VHSV es el agente causal de la **enfermedad de la septicemia hemorrágica viral (VHS)**. Entre las especies más susceptibles a este virus está la trucha arco iris (*Oncorhynchus mykiss*), especie de donde fue aislado por primera vez en Dinamarca en 1963 (Jensen, 1963). Posteriormente, el VHSV ha sido aislado de numerosas especies marinas y en la actualidad, se cifran en al menos 48 el número de especies distintas de donde se ha aislado el virus. Todas estas especies están distribuidas por el Hemisferio Norte (EEUU, Canadá, Japón Corea y Europa) (**Figura 1**) (Skall et al, 2005b). Entre ellas están el salmón Chinook (Hopper, 1989), salmón coho (Eaton et al, 1991), rodaballo (Schlotfeldt et al, 1991), bacalao (Meyers et al, 1992), y la platija japonesa (Isshik et al, 2001). Además, se han demostrado infecciones experimentales en trucha de río, salmón atlántico y salvelino (Rasmussen, 1965), lubina (Castric & DeKinkelin, 1984) y rodaballo (Snow & Smail, 1999).

En la década de los 80 se aislaron y caracterizaron en España cinco brotes de VHSV (Basurco & Coll, 1989; Basurco & Coll, 1992). Según consta en la base

de datos sobre enfermedades de los animales acuáticos (http://www.collabcen.net/toWeb/aq2.asp) de la OIE, desde 1994 no se han vuelto a detectar brotes de VHSV en España, de manera que, en la actualidad, España es considerada por la UE como una zona libre de VHSV aunque en 2005 se aisló IHNV en una piscifactoría de Granada.

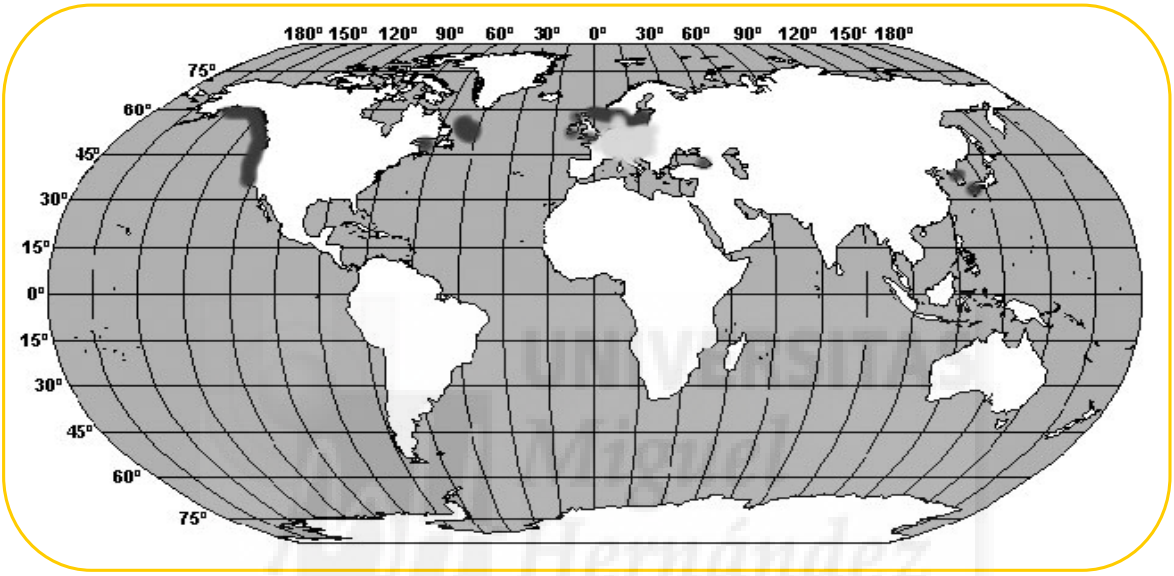

Figura 1. Distribución geográfica del VHSV (Skall et al, 2005b). Los colores oscuros señalan áreas de donde el VHSV ha sido aislado de especies marinas. Los colores claros señalan las regiones de donde proceden los aislados de trucha (regiones de donde se consideraba que era endógeno el VHSV hasta que en 1988 fue aislado en USA).

El VHSV afecta a peces de todas las edades aunque la mortalidad es mucho más elevada en alevines, en un rango de temperaturas de 4 a 18°C, siendo los brotes más frecuentes a 10°C (Meier et al, 1994). La transmisión del virus es horizontal (Wolf, 1988) y aunque la transmisión vertical no ha sido probada sí se ha detectado VHSV en la superficie del huevo (Peters & Neukirch, 1986). Aunque durante mucho tiempo se ha sugerido que las células endoteliales del intestino y branquias y los leucocitos eran los sitios de entrada/replicación primaria del virus (Chilmonczyk, 1980; DROLET, 1994; Wolf, 1988) en la actualidad se ha demostrado que la base de las aletas son el primer portal de entrada para VHSV

en salmónidos y que a tiempos cortos post-infección sólo allí puede detectarse replicación del virus (Harmache et al, 2006). Sin embargo, aún no se han identificado los mecanismos implicados en la transmisión del virus desde las aletas al resto del cuerpo. Tras 48 horas post-infección puede detectarse el virus en riñón (Chilmonczyk, 1980) y bazo (Chilmonczyk, 1980) y a tiempos posteriores en branquias, timo, hígado, bazo, corazón, píloro, riñón anterior, riñón posterior y cerebro. El riñón anterior, principal órgano hematopoiético de los peces, es en todos los casos el órgano más afectado (De Kinkelin, 1979)

La importancia de la sintomatología de esta enfermedad varía de las infecciones agudas a las crónicas (DeKinkelin et al, 1980). La sintomatología aguda, asociada siempre a una alta y rápida mortalidad, se caracteriza por peces letárgicos, oscuros, exoftálmicos (**Figura 2A**) y anémicos. Las hemorragias son evidentes en los ojos, piel, branquias y la base de las aletas. Internamente, se observan hemorragias puntiformes en los tejidos perioculares, músculo esquelético y vísceras (**Figura 2B**). El hígado aparece moteado y hemorrágico y los riñones se manifiestan enrojecidos y más delgados de lo normal (Ghittino, 1965).

Figura 2. Peces infectados con VHSV. (**A**) Trucha arco iris mostrando exoftalmia y (**B**) hemorragias internas puntiformes en órganos internos.

Finalmente se observa una natación incontrolada y un aumento de la frecuencia de los movimientos respiratorios. En infecciones latentes la mortalidad es muy baja y los peces tienen una apariencia normal aunque pueden estar hiperactivos. Clínicamente, tanto los peces infectados como los portadores son reservorios de VHSV, que es diseminado a través de las heces, orina y fluidos sexuales (Essbauer & Ahne, 2001).

Los **viriones de VHSV** (**Figura 3**) son envueltos, tienen un tamaño medio de ~ 170 x 80 nm y su genoma, como el de todos los rabdovirus, está constituido por una molécula de RNA monocatenario de polaridad negativa que en este caso tiene ~11 000 bases (Hill et al, 1975) y un peso molecular de 5-6.4 x 10^6 kDa (Enzmann et al, 1981). En la actualidad se conoce la secuencia completa del genoma, tanto de VHSV (GenBanK Accession number Y18263) (Heike, 1999; Nishizawa, 2002; Schutze et al, 1999) como de IHNV (Morzunov et al, 1995; Nichol et al, 1995; Oshima et al, 1995; Schutze et al, 1995) lo que ha permitido, entre otras cosas, realizar los análisis filogenéticos necesarios para determinar la existencia de varios genotipos entre los diferentes aislados de estos virus. En el caso concreto de VHSV se han podido establecer tres genotipos: los genotipos I, II y III que, incluyen, respectivamente aislados del continente europeo, de las Islas Británicas y de Norteamérica. Sin embargo, los anticuerpos neutralizantes anti-gpG no son capaces de distinguirlos. A su vez, los aislados de VHSV del genotipo I de acuerdo con sus patrones de neutralización cruzada, han sido asignados a cuatro serotipos distintos (Castric et al, 1992; LeBerre et al, 1977; Vestergaard-Jorgensen, 1972) que se corresponden parcialmente con la diversidad genética encontrada entre los aislados del continente europeo (Benmansour et al, 1997).

El genoma de VHSV codifica para 5 proteínas estructurales y 1 no estructural (**Figura 3**): L (polimerasa RNA dependiente de RNA, 190kDa), gpG (glicoproteína de superficie, 65kDa), N (proteína de la nucleocápsida, 40kDa), P o también denominada M1 o NS dependiendo del género al que pertenezca el rabdovirus (fosfoproteína, 19kDa), M2 (proteína de matriz, 25kDa) y Nv (proteína

no estructural, 36kDa). Además, se ha descrito una proteína relacionada antigénicamente con la N, la Nx (Basurco & Coll, 1991). La envoltura es una bicapa lipídica procedente de la última célula que ha parasitado el virus, que tiene insertadas ~400 espículas (homotrímeros de gpG) (Einer-Jensen et al, 2004; Estepa & Coll, 1996a; Estepa et al, 2001; Nunez et al, 1998; Rocha et al, 2004a; Thiery et al, 2002).

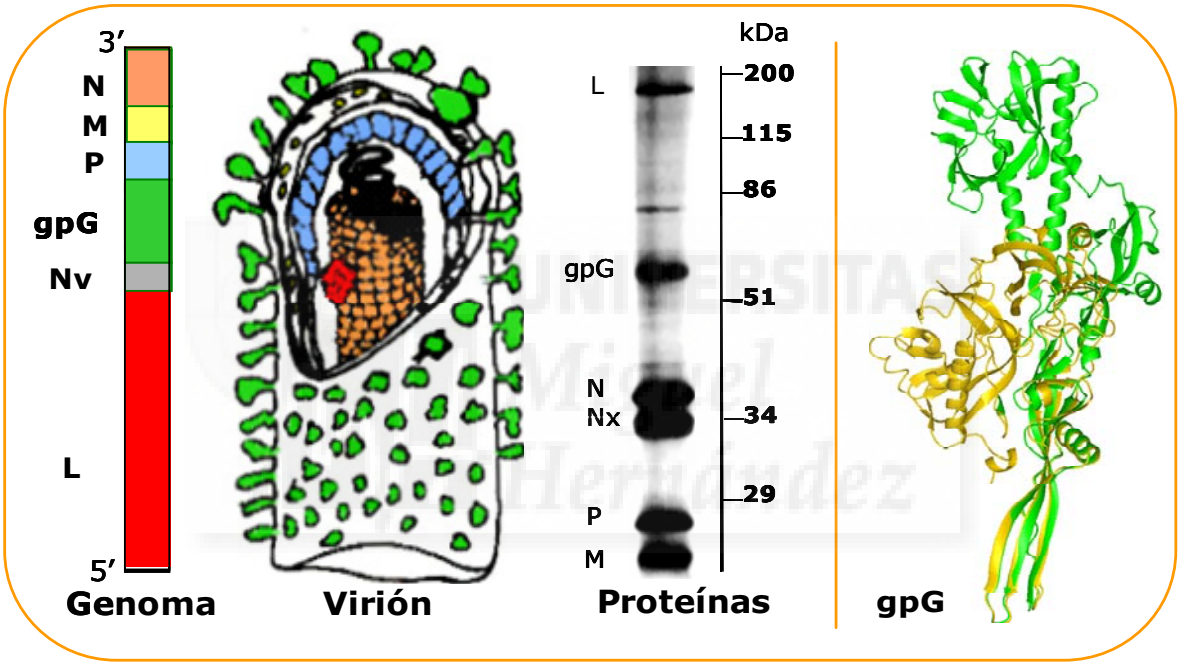

Figura 3. Estructura del VHSV y de su gpG. Dentro de la partícula vírica, el RNA está empaquetado por la proteína N formando la nucleocápsida. La proteína L es una RNA polimerasa dependiente de RNA que posee actividad tanto de replicasa como de transcriptasa (Banerjee & Chattopadhyay, 1990; Schnell & Conzelmann, 1995) que se asocia con la nucleocápsida y la fosfoproteína P para formar los complejos de replicación (Chen et al, 1997; Hwang et al, 1999) y transcripción (Chen et al, 1997; Pattnaik et al, 1997). La proteína P, además, juega un papel activo durante la morfogénesis y ensamblaje del virus (Das & Pattnaik, 2005). La proteína de la matriz, M, está localizada dentro de la partícula, entre la membrana y la nucleocápsida e interviene en los procesos de morfogénesis. Nv, proteína no estructural (Basurco & Benmansour, 1995; Essbauer & Ahne, 2001; Schutze et al, 1996; Schutze et al, 1999) con funciones aún no claramente descritas (Thoulouze et al, 2004). La estructura del monómero de gpG del VHSV en su conformación nativa (amarillo) y de fusión (verde) modelada de acuerdo con la estructura de la gpG del rabdovirus de la estomatitis vesicular (VSV) publicada recientemente (Roche et al, 2006; Roche et al, 2007).

De entre todas las proteínas del VHSV, la gpG es muy probablemente la más estudiada por lo relevante de los procesos en los que interviene durante el ciclo de multiplicación del virus (**Figura 4**), además de por ser la diana de los anticuerpos neutralizantes inducidos en el huésped (Benmansour et al, 1991; Lorenzen et al, 1990) durante una infección por VHSV. La gpG de VHSV tiene 507 aas y posee todos los rasgos de secuencia (sitios de N-glicosilación, puentes disulfuro, zonas análogas a heptadas hidrofóbicas repetidas, una región transmembrana, una cola citoplasmática carboxi-terminal, un péptido señal, etc.) previamente descritos para las gpGs de los rabdovirus de mamíferos aunque sólo comparta con ellas un 18-26% de homología de secuencia (Roche et al, 2006; Walker & Kongsuwan, 1999). En estos momentos aún no se ha resuelto la estructura tridimensional de la gpG de VHSV, pero se dispone de muchos datos sobre la localización estructural de los dominios de fusión y propiedades fusogénicas de esta proteína, todos ellos derivados de ensayos bioquímicos y biofísicos así como del uso de anticuerpos monoclonales (MAb) anti-gpG y mutantes (Einer-Jensen et al, 1998; Estepa & Coll, 1996a; Estepa & Coll, 1996b; Rocha et al, 2004a; Rocha et al, 2004b).

Las etapas del **ciclo de multiplicación del VHSV**, las mismas que las de todos los rabdovirus: unión al receptor, endocitosis y fusión de membranas virus-vesícula celular, replicación/expresión génica y ensamblaje/salida de los nuevos viriones, se esquematizan en la **figura 4**. La infección comienza con la unión del virus a su receptor celular específico a través de la gpG (Bearzotti et al, 1999; Schlegel et al, 1983). Una vez unido, el virus se internaliza utilizando un mecanismo conocido como endocitosis mediada por receptor. Tras la endocitosis, y mediada también por la gpG del virus, ocurre la fusión dependiente de pH ácido (~ pH 5,8-6) entre la membrana del virión y la de los cuerpos multivesiculares (vesículas internas) del endosoma. Seguidamente, y mediante un segundo proceso de fusión, mediado ya por proteínas celulares, entre la membrana exterior del endosoma y la membrana de los cuerpos multivesiculares, se produce la liberación del virión en el citoplasma de la célula diana y la disociación de la

proteína M para dejar libre las ribonucleocápsidades (RNPs, nucleocápsidas asociadas a las proteínas L y P). Una vez que las RNPs están libres en el citoplasma celular, se dispara la transcripción primaria del genoma del virus.

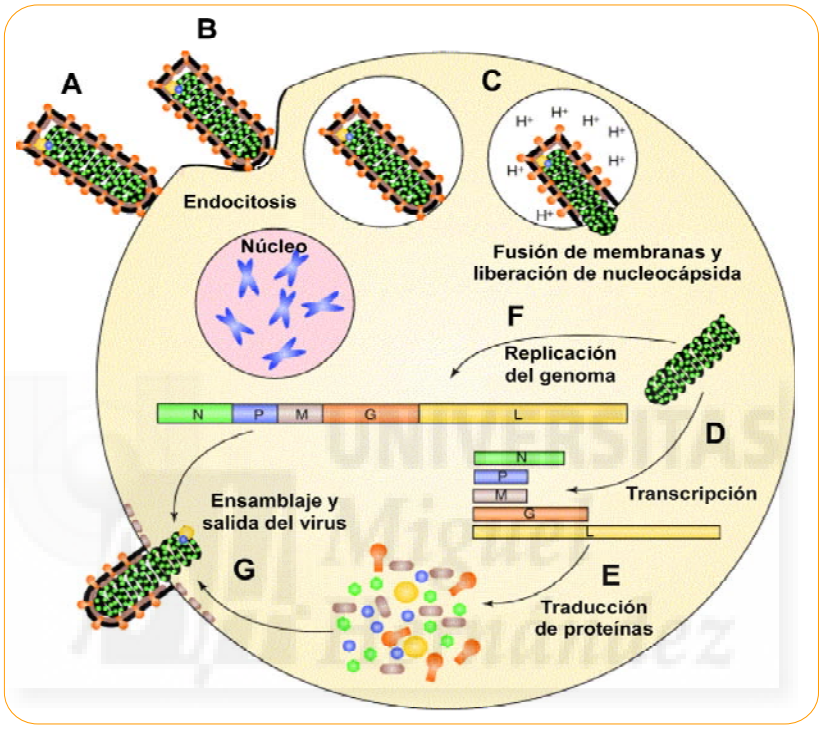

Figura 4. Esquema del ciclo de replicación de un rabdovirus. Adsorción y entrada que engloba: unión al receptor celular (**A**), endocitosis mediada por receptor (**B**) y fusión de membranas y liberación de las nucleocápsidas en el citoplasma (**C**). (**D**, **E** y **G**) Expresión génica y replicación que incluye: transcripción (**D**), traducción de las proteínas víricas (**E**) y replicación del genoma (**F**). (**G**) Ensamblaje y salida del virus.

Tras la traducción de los mRNA virales y la acumulación de las proteínas del virus, comienza la replicación del genoma también en el citoplasma de la célula. Por último, las proteínas recién sintetizadas y los nuevos genomas se ensamblan en las proximidades de la membrana plasmática y el virus sale de la célula por gemación, provocando la lisis celular en estados avanzados de la infección (Wagner, 1975; Wagner, 1987).

3.2 La respuesta inmune en peces teleósteos

Los ambientes acuáticos naturales contienen habitualmente altas concentraciones de organismos patógenos y, por lo tanto, los peces viven en íntimo contacto con altas concentraciones de bacterias, virus y parásitos. Para los virus, se ha estimado recientemente que tanto en agua dulce como salada, el número medio de partículas por litro es de 10^{10} (Tort, 2004; Wilhelm, 1999).

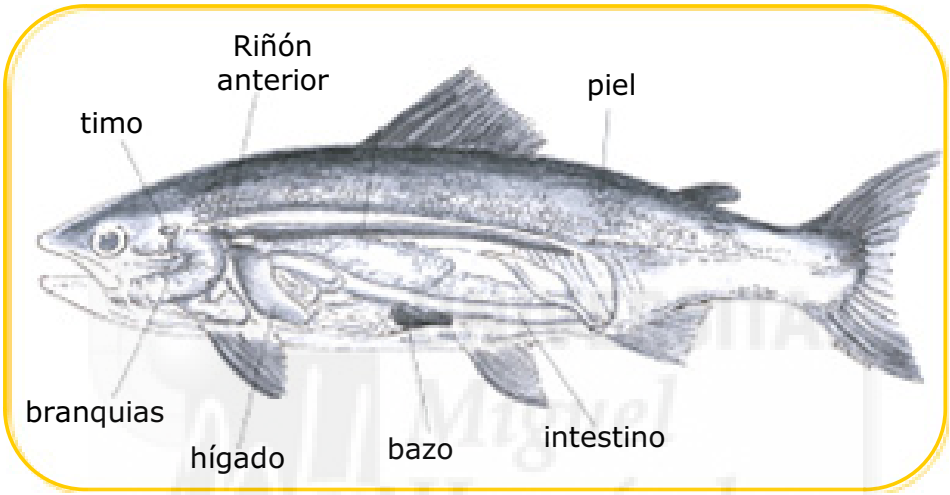

Figura 5. Órganos con funciones inmunes en peces teleósteos (Tort, 2004).

A pesar de ello, en condiciones normales, los peces mantienen un buen estado de salud defendiéndose de los virus y otros invasores potenciales mediante una compleja red de mecanismos de defensa que en el caso de los peces óseos o teleósteos, los primeros vertebrados que ya poseen un sistema inmune adaptativo (DeLuca et al, 1983), incluye tanto elementos de respuesta inespecíficos constitutivos e inducibles (barreras físicas, citoquinas, AMPs y sistema de complemento e interferón (IFN) principalmente) como específicos de antígeno (células T y B, moléculas del complejo mayor de histocompatibilidad (MHC), etc) (Du Pasquier et al, 1998; Plouffe et al, 2005) (**Figura 5**).

En peces, el **sistema inmune no específico o innato** es el que parece jugar el papel más importante en el control y propagación de los agentes

infecciosos (Bly & Clem, 1991; Douglas et al, 2003b; Tort, 2004), principalmente porque actúa de manera rápida y eficaz mediante mecanismos de reconocimiento no específicos y relativamente independientes de temperatura (Du Pasquier, 1982; Ellis, 2001; Magnadottir, 2006). En su conjunto, la respuesta inmune innata frente a patógenos está iniciada, conducida y coordinada por receptores celulares (PRRs) capaces de reconocer patrones moleculares asociados a patógenos (PAMPs) (Janeway & Medzhitov, 2002). Los PAMPs incluyen, colectivamente, lípidos, carbohidratos, péptidos y ácidos nucleicos específicos de determinados grupos de microorganismos. La activación de estos receptores conlleva finalmente a la expresión de distintas moléculas de secreción que diversifican la respuesta antimicrobiana y aumentan la eficacia de la misma. Dentro de las moléculas de secreción, los AMPs funcionan como importantes efectores de la inmunidad innata (Boman, 2003). El papel de estas dos partes del sistema inmune innato (PRRs y AMPs) en el control de infecciones virales ha sido descrito recientemente (Bowie & Haga, 2005).

En relación con la **respuesta inmune adaptativa** de los peces frente a virus, hay que señalar que, aunque más específica y con memoria, es más lenta y dependiente de temperatura (Ellis, 2001; Magnadottir, 2006) que la innata y, así por ejemplo en salmónidos, la respuesta de anticuerpos puede tardar en elaborarse más de seis semanas.

Al igual que en mamíferos, en peces son los anticuerpos los que confieren la especificidad al reconocimiento. Sin embargo, en el caso de los peces parece existir un repertorio de anticuerpos menos diversos (Palm et al, 1998) y fundamentalmente constituido por inmunoglobulinas (Ig) M, aunque recientemente, un nuevo isotipo de Ig, la IgT ha sido descrito en trucha (Hansen et al, 2005). Además, en algunos casos, por ejemplo en truchas supervivientes a una infección por rabdovirus, no pueden ser detectados anticuerpos neutralizantes frente al virus (Estepa et al, 1994; Olesen et al, 1991), lo que indica que a parte de los anticuerpos, deben de existir otros mecanismos de defensa específicos

implicados en la protección (Lorenzen et al, 2002a). Las células T citotóxicas específicas (Tc) parecen ser este mecanismo (Fischer et al, 1998; Hasegawa & N., 1998; Manning & London., 1996; Nakanishi et al, 2002; Stuge, 2000) aunque la falta de marcadores específicos de subpoblaciones de linfocitos en salmónidos no ha permitido hasta el momento la adecuada caracterización de estas células ni de las respuestas mediadas por ellas. Sin embargo, las células y respuestas Tc están siendo caracterizadas en estos organismos a nivel genético y de hecho en los últimos años se han identificado en los genomas de peces muchas secuencias homólogas a las que poseen en mamíferos las moléculas de CD8 (Hansen & Strassburger, 2000), MHC I y II, receptor de células T (TCR) (Partula et al, 1995; Partula et al, 1996), β2-microglobulina (β2 μ) (Rodrigues et al, 1998; Shum et al, 1996), transportadores asociados al procesamiento de antígeno (Fischer et al, 2006), etc.

En el caso concreto de la trucha arco iris, se han encontrado diferencias en cuanto a la afinidad del TCR por un epítopo determinado en los antígenos víricos ("respuesta Tc-privada") resultado que directamente sugirió que los peces son capaces de elaborar una respuesta Tc específica frente a virus (Boudinot et al, 2001). Posteriormente, experimentos *in vitro* han demostrado que la eliminación específica de células infectadas por IHNV en truchas vacunadas frente a este virus, es al igual que en mamíferos, dependiente de moléculas MHC I (Nakanishi et al, 2002). En 2007, los experimentos descritos por Utke *et al* (Utke et al, 2007a; Utke et al, 2007b) utilizando un clon específico de truchas que posee un MHC I (secuencia Onmy-UBA*501, número de acceso GenBank AF287488) compatible con la línea celular RTG2 (fibroblastos de trucha) (Wolf & Quimby, 1962) ponen claramente de manifiesto la existencia de Tc específicos frente a VHSV en truchas infectadas con VHSV así como en truchas inmunizadas genéticamente con el gen de la gpG de VHSV. A pesar de ello, la importancia relativa de la interdependencia entre los mecanismos de defensa humorales y

celulares en la protección de la trucha arco iris frente a infecciones con VHSV está aún por determinar.

En los últimos años, se ha realizado un tremendo esfuerzo en profundizar en el sistema inmune de los peces teleósteos, sobre todo a nivel molecular. Así, por ejemplo, se han identificado y clonado las secuencias de muchas citoquinas previamente descritas en mamíferos. Las citoquinas son proteínas de bajo peso molecular producidas principalmente por células del sistema inmune y que actúan de forma autocrina o paracrina regulando las funciones inmunes. Entre las citoquinas que se han identificado en peces se encuentran varias isoformas del IFN de tipo I, el IFN de tipo II (IFNα), interleuquinas (IL) como la IL1β, IL6, (Secombes et al, 2001), IL8 (Laing et al, 2002b), IL10, IL12 (Yoshiura et al, 2003), factores de necrosis tumoral (TNFα) y factores de crecimiento transformante (TGFβ), etc. También se han identificado varias quimioquinas (citoquinas con actividad quimiotáctica) en distintas especies. Sin embargo, son muy pocos los ensayos de actividad que se han realizado hasta el momento con estas moléculas, por lo que aún se desconoce el papel que tienen en el sistema de defensa antiviral.

4.-LOS PÉPTIDOS ANTIMICROBIANOS

Los AMPs son pequeños polipéptidos que forman parte de las defensas innatas de todos los organismos y son expresados por diversos tipos celulares incluidos monocitos/macrófagos, neutrófilos, células epiteliales, queratinocitos y mastocitos. En general, tienen un amplio espectro de acción ya que no sólo son activos frente a bacterias (Gram-positivas y negativas) sino también frente a hongos e incluso virus (Ganz, 2003; Lehrer, 2004; Mookherjee & Hancock, 2007; Yang et al, 2004; Zanetti, 2004).

Los AMPs descritos hasta el momento, aunque muy diferentes en cuanto a secuencia y estructura (**Tabla 2**), poseen una serie de propiedades comunes como ser de pequeño tamaño (10-46 aas), naturaleza catiónica (carga básica a pH fisiológico por poseer un elevado número de residuos de lisina y arginina) y anfipáticos.

Tabla 2. Clasificación de algunos de los principales péptidos antimicrobianos según su estructura química

Estructura	Péptido	Organismo (*Nombre científico*)
Lineal helicoidal	Cecropinas	Mariposa de la seda (*Hyalophora cecropia*)
	Bombinina	Sapo de panza amarilla (*Bombina variegata*)
	Dermaseptinas	Rana arbórea (*Phyllomedusa sauvageii*)
	Magaininas	Rana (*Xenopus laevis*)
Lineal no helicoidal	Drosocina	Mosca de la fruta (*Drosophila malanogaster*)
	Indolicidina	Buey (*Bos taurus*)
Cíclica	Taquiplesinas	Cangrejo (*Tachypleus tridentatus*)
	Criptidinas	Ratón (*Mus musculus*)
	Protegrinas	Cerdo (*Sus scrofa*)

Aunque la **permeabilización y desestabilización** (**Figura 6**) de las membranas de los agentes infecciosos es un **mecanismo** habitual que utilizan casi todos los **AMPs** para neutralizar directamente a los patógenos, en la actualidad se sabe que para ellos existen dianas alternativas (Hancock &

Patrzykat, 2002) como por ejemplo las glicoproteínas de superficie de los virus (Klotman & Chang, 2006; Leikina et al, 2005) o los receptores celulares que algunos agentes infecciosos utilizan para infectar las células (Jenssen et al, 2006).

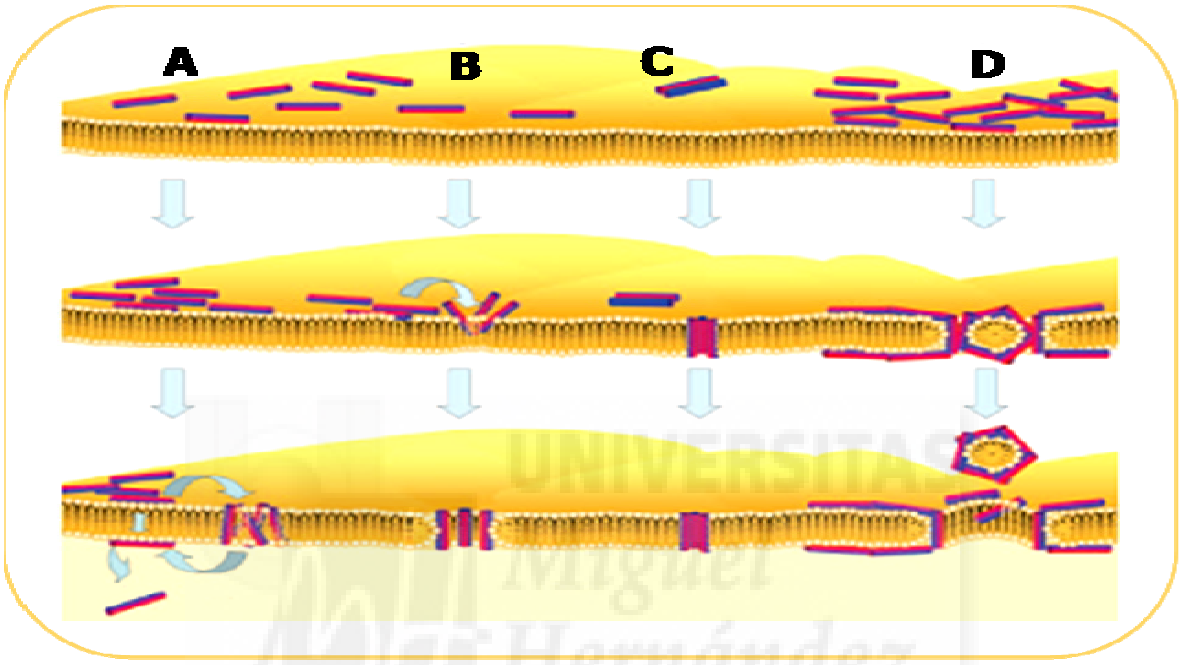

Figura 6. Mecanismo de acción de los péptidos antimicrobianos (Jenssen et al, 2006). Su naturaleza catiónica y anfipática es lo que permite a los AMPs interaccionar con las membranas de los microorganismos que en general tienen un elevado número de cargas negativas en su superficie. Esta interacción electrostática inicial conduce posteriormente a la inserción y/o penetración de los AMPs en la membrana mediante mecanismos variados. (**A**) Los péptidos se agregan en la superficie de la membrana sin adoptar ninguna orientación específica formando complejos tipo micela entre los lípidos y proteínas; (**B**) modelo del poro-toroidal: los péptidos se insertan en la membrana en una disposición perpendicular al plano de la bicapa con sus regiones hidrofílicas e hidrofóbicas interaccionando con las cabezas polares y los ácidos grasos de los fosfolípidos, respectivamente. En el modelo mostrado en (**C**), "barril", los péptidos también se insertan en la membrana en una disposición perpendicular al plano de la bicapa, pero en este caso formando una estructura con apariencia de barril. Las regiones hidrofílicas de los péptidos se disponen hacia el lumen del poro y las hidrofóbicas interaccionan con la bicapa lipídica. El modelo "alfombra" (**D**), propone que los péptidos se agregan en una disposición paralela a la bicapa cubriendo, como si fueran "una alfombra", una determinada zona de la membrana. A una determinada concentración crítica, los péptidos actúan de manera similar a un detergente, causando la formación de micelas y poros. En amarillo se representa la membrana de cualquier patógeno. Los bastoncillos bicolor (azul-magenta) son los péptidos antimicrobianos. Azul, regiones hidrofóbicas; magenta, regiones hidrofílicas.

Aunque minoritarios, también se han identificado AMPs cuya actividad microbicida no está relacionada con la desestabilización de membranas (Gallo & Huttner, 1998; Jenssen et al, 2006). Mediante mecanismos aún no conocidos, estos AMPs son capaces de atravesar las membranas de los patógenos e interaccionar posteriormente con dianas intracelulares (**Figura 7**). Sin embargo, lo más probable es que en el contexto de una infección, un AMP ejerza su acción empleando de manera simultánea varios mecanismos, por ejemplo desestabilización de membranas en combinación con la inhibición/interferencia de dianas intracelulares tales como DNA girasas, proteínas Dna K, etc, para controlar a un patógeno. Además, un AMP concreto puede inhibir a un mismo patógeno por diferentes mecanismos en función del tejido donde ocurre la infección, la ausencia o presencia de otros mecanismos inmunes, la fase de crecimiento del patógeno, etc (Yount et al, 2006). Esta variada, especial y compleja combinación de mecanismos de acción que para neutralizar los patógenos utilizan los AMPs, son la clave de la casi nula existencia de microorganismos resistentes a la acción de estos péptidos (Jenssen et al, 2006). Además, los péptidos antimicrobianos de células eucariotas, se expresan como preproproteínas utilizando rutas biosintéticas de extremada complejidad que sólo poseen este tipo de células, por lo que es muy improbable la adquisición de resistencias genéticas frente a ellos por parte de microorganismos (David Andreu, 1998).

Junto a su actividad antimicrobiana directa (eliminación de patógenos), los **AMPs** poseen también capacidad para **modular la respuesta inmune.** De hecho se ha demostrado que en condiciones fisiológicas y durante una infección, la capacidad inmunomoduladora de muchos AMPs es más importante que su actividad microbicida (Bowdish et al, 2006; Mookherjee & Hancock, 2007), lo que sugiere que en muchos casos, las propiedades anti-infectivas de los AMPs están directamente asociadas a su actividad como reguladores de la respuesta inmune.

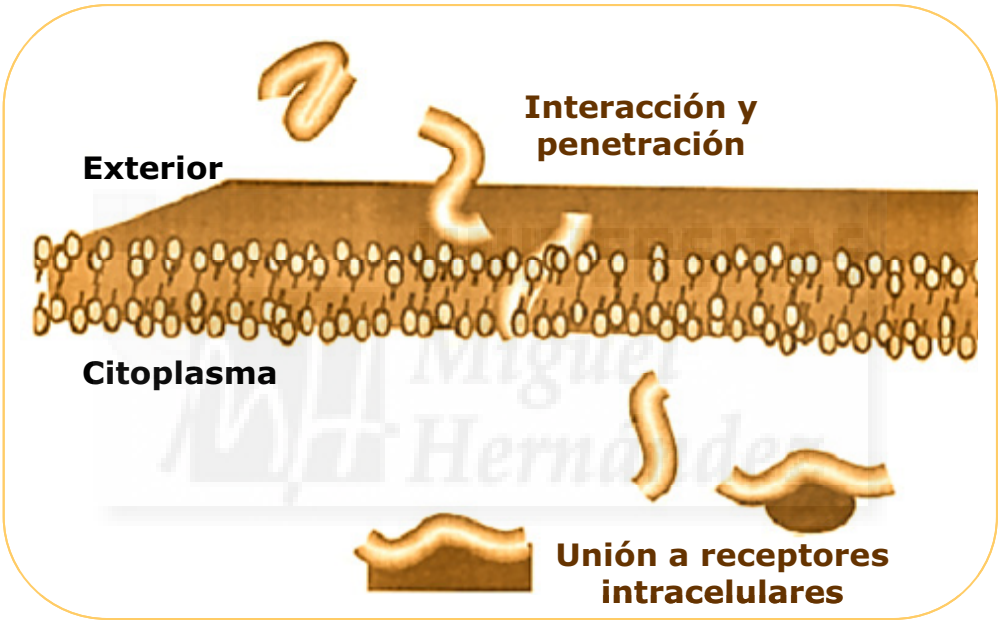

Figura 7. Mecanismo de acción de los péptidos antimicrobianos (Gallo & Huttner, 1998). Penetración de un AMP lineal a través de una bicapa lipídica y su posterior interacción con un receptor intracelular.

Como ya se ha indicado anteriormente, y contrariamente a lo que se creía en un principio, muchos AMPs tienen una potente actividad antiviral. De hecho, en respuesta a una infección vírica la inmunidad innata responde inmediatamente, y las células infectadas además de citoquinas y quimioquinas, producen también AMPs para controlar la replicación de los virus y dar tiempo suficiente a que la inmunidad adaptativa elabore su respuesta.

Entre los AMPs caracterizados, las defensinas son muy probablemente los antimicrobianos endógenos con el espectro de actividad antiviral más amplio, ya que han demostrado ser activos tanto frente a virus con envuelta como desnudos (revisado en (Klotman & Chang, 2006)). Además, durante una infección las defensinas juegan un papel crucial ya que junto a su actividad antimicrobiana directa participan activamente en la elaboración de la respuesta inmune tanto innata como adaptativa (Bowdish et al, 2006; Froy, 2005; Ganz, 1999; Ganz, 2003; Ganz & Lehrer, 1994; Ganz & Lehrer, 1995; Ganz et al, 1990; Hertz et al, 2003; Klotman & Chang, 2006; Lehrer & Ganz, 1992; Lehrer & Ganz, 2002; Lehrer et al, 1993; Lillard et al, 1999; Oppenheim et al, 2003; Raj & Dentino, 2002; Sakamoto et al, 2005; Schutte & McCray, 2002; Selsted & Ouellette, 2005; Van Wetering et al, 1997; Yang et al, 2000; Yang et al, 1999).

Las **defensinas** son una familia de péptidos antimicrobianos (29-47 aas) de naturaleza catiónica, ricos en residuos de cisteínas (6-8 por molécula que forman 3-4 puentes disulfuro) y con estructura en hoja β (2-3 antiparalelas). Están ampliamente distribuidas en la naturaleza y su presencia ha sido descrita en plantas, insectos, invertebrados y vertebrados (Froy & Gurevitz, 2003; Ganz, 2003; Klotman & Chang, 2006; Selsted & Ouellette, 2005; Thomma et al, 2002). Sin embargo, péptidos tipo defensina aún no han sido aislados en peces aunque sí AMPs pertenecientes a otras familias (**Tabla 3**). Hasta la publicación de alguno de los resultados obtenidos durante el desarrollo de esta Tesis Doctoral, tan sólo se habían identificado algunas secuencias génicas en pez zebra, fugu y tetraodon que podrían codificar para péptidos tipo β-defensinas (Zou et al, 2006).

Dependiendo de la localización y conectividad de sus cisteínas, las defensinas se pueden clasificar en tres grupos (que coinciden con los grupos de organismos en los que se expresan): defensinas de plantas, defensinas de invertebrados y defensinas de vertebrados superiores. A su vez, las defensinas de vertebrados superiores, las mejor caracterizadas hasta ahora, se clasifican en tres subfamilias de acuerdo con la distribución de los enlaces disulfuro en la molécula:

α-defensinas (Cys: 1-6, 2-4, 3-5) (**Figura 8A**), β-defensinas (Cys: 1-5, 2-4, 3-6) (**Figura 8B**) y θ-defensinas (**Figura 8C**) (cíclicas). De las tres subfamilias, las de mayor representación en la naturaleza son la α y la β. Las θ-defensinas sólo se han identificado y aislado de leucocitos de mono Rhesus (Tang et al, 1999).

Tabla 3. Familias de péptidos antimicrobianos descritas en peces

AMP	Tipo	Estructura	Tamaño aa	Especie	Referencia
Hepcidina	Cíclico***	Lámina β	26-19	*Morone chrysops x Morone saxatilis, Chrysophrys major, Paralichthys olivaceus*	Chen et al., 2005; Hirono et al., 2005; Lauth et al., 2005; Shike et al., 2002
Catelicidinas	Lineal	Variable	71-53	*Myxine glutinosa, Oncorhynchus mykiss, Salmo salar*	Chang et al., 2005; Chang et al., 2006; Uzzell et al., 2003
Oncorrincinas	Lineal	?	65-70	*Oncorhynchus mykiss*	Fernandes et al., 2003; Fernandes et al., 2002; Fernandes et al., 2004
Pleurocidinas	Lineal	Helicoidal	26-22	*Pleuronectes americanus*	Cole et al., 1997; Douglas et al., 2001
Piscidinas*	Lineal	Helicoidal	22	*Morone saxatilis x Morone chrysops*	Lauth et al., 2002; Silphaduang et al, 2001
Parasina**	Lineal	Lámina β	19	*Parasilurus asotus*	Park et al., 1998
Histona H1	-	Helicoidal	>100	*Salmo salar*	Luders et al., 2005; Richards et al., 2001
LBP/BPI	-	Lámina β	45-50	*Oncorhynchus Mykiss, Gadus morhua, Cyprinus carpio*	Inagawa et al., 2002; Kono et al, 2003; Solstad et al., 2007
Apolipoproteinas	-	Helicoidal	100-200	*Cyprinus carpio, Oncorhynchus mykiss*	Concha et al., 2003; Concha et al., 2004; Villarroel et al., 2007

* También conocidas como moronecidinas
** Derivados de la histona H2B
*** Posee 3 enlaces disulfuro

En general, las defensinas están presentes en células y tejidos implicados en la defensa frente agentes infecciosos. En animales, se expresan principalmente en leucocitos y células de Paneth y se acumulan en gránulos dentro del citoplasma de estas células. Además, expresan defensinas de manera constitutiva (Valore et al, 1998) o en respuesta a una infección (Harder et al, 2001) células de algunos epitelios.

Las α-defensinas son codificadas y expresadas como prepropéptidos. El prepropéptido contiene un péptido señal aminoterminal (N-terminal) (~19 aa), una propieza aniónica (~45 aa) y un péptido maduro catiónico carboxiterminal (C-terminal) (~45 aa) (Daher et al, 1988; Ganz, 2003; Ganz, 2005; Valore & Ganz, 1992). El procesamiento post-transduccional del prepropéptido varía en función de la especie y del tipo celular que exprese la defensina (**Tabla 4**).

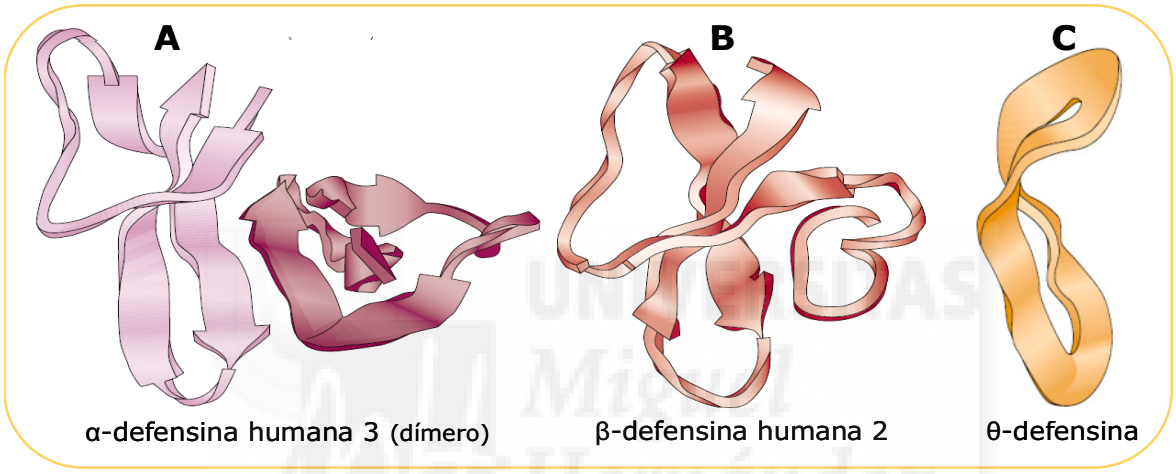

Figura 8. Estructura de algunas defensinas de vertebrados representativas de cada subfamilia (Ganz, 2003). Las estructuras secundarias de lámina β se representan con flechas. (**A**) α-defensina humana 3 que en disolución acuosa forma un dímero rico en láminas β. (**B**) Monómero de la β-defensina humana 2 (HB2) en solución en el que también predomina la estructura en lámina β pero contiene un pequeño fragmento N-terminal con hélice α. (**C**) Estructura cíclica de las θ-defensinas.

Las α-defensinas humanas 1, 2, 3 y 4 (HNP1, 2, 3 y 4) se expresan principalmente en neutrófilos (Ganz, 2003) y suponen el 5-7% de las proteínas totales de estas células. Además, expresan HNPs otras células relacionadas con funciones inmunes en las superficies mucosas y otros tejidos (Cunliffe, 2003; Fellermann & Stange, 2001; Hein et al, 2002) e incluso, algunas células las pueden internalizar (Ganz, 1987; Mackewicz et al, 2003; Zaharatos et al, 2004), aunque el significado biológico de la endocitosis de las defensinas aún es desconocido.

Las HNP1 y 3 están codificadas por los genes HDEFA1 y HDEFA1A, respectivamente (Daher et al, 1988), cuyas secuencias sólo difieren en tres pares de bases (pb). Sin embargo, como estas diferencias están localizadas en la región que determina el comienzo de la transcripción, ambos polipéptidos difieren en el primer aa (Linzmeier et al, 1999) (**Figura 9**). La HNP2 (**Figura 9**) (sin representación génica) es el resultado del procesamiento post-transduccional de parte de la HNP3 expresada por los neutrófilos (Daher et al, 1988). La HNP4 supone menos del 2% de las proteínas totales de neutrófilos y, al igual que las HNP1 y 3, está codificada genéticamente y posee una secuencia de aas diferente a las anteriores (Wilde et al, 1989) (**Figura 9**).

Tabla 4. Procesamiento post-transduccional de las defensinas

Defensina	Especie molecular que se acumula	Lugar de procesamiento	Enzimas de procesamiento	Referencias
HNP 1-3	Péptido maduro	Golgi y gránulos maduros de promielocitos	Desconocidas	Ganz et al., 1985; Valore et al, 1992
HD5	Propéptido	Durante o después de la liberación de las células de Paneth	Tripsina en las células de Paneth	Ghosh et al., 2002; Porter et al., 2005
Criptidinas (ratón)	Péptido maduro	Golgi y gránulos maduros	Matrilisina*	Wilson et al., 1999
HBD2	Péptido maduro	Retículo endoplásmico	Peptidasa señal	

* Es una metaloproteína

Las α-defensinas de leucocitos están conservadas evolutivamente y han sido aisladas en humanos, conejos, ratas, cerdos de Guinea y hamsters. Sin embargo, los leucocitos de ratón carecen de ellas aunque en las células intestinales de Paneth se expresan péptidos tipo α-defensina llamados criptidinas (Ganz, 2003; Tang et al, 1999).

Las α-defensinas humanas 5 y 6 (HD5 y 6) se expresan principalmente en las células intestinales de Paneth (Ganz, 2003) así como en glándulas salivares, tracto genital femenino e intestino delgado en situaciones de inflamación (Fahlgren et al, 2003; Fellermann & Stange, 2001; Quayle et al, 1998; Svinarich et al, 1997). Además, se ha descrito la presencia de HD5 en las secreciones uretrales de hombres con infección. A diferencia de las HNPs, HD5 se libera como un propéptido y es procesada extracelularmente (Ghosh et al, 2002; Porter et al, 2005) (**Tabla 5**).

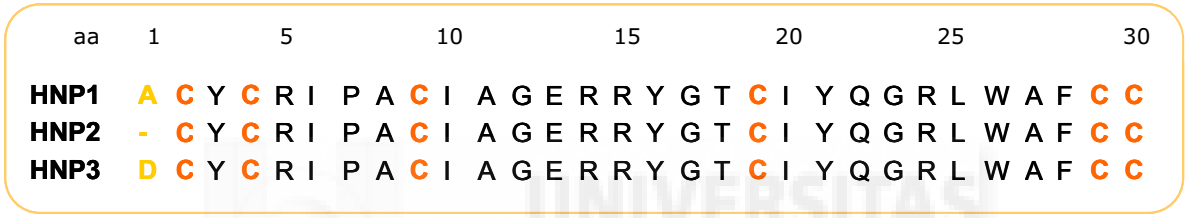

Figura 9. Secuencia de aas de las HNP1, 2 y 3. Sólo se muestra la secuencia del péptido maduro. Cada residuo de aa está representado según el código abreviado de una letra. En amarillo se señalan los residuos de aa en los que se diferencian los tres péptidos y en naranja los residuos de cisteína. Las posiciones que ocupan los aas en la secuencia se indican en la parte superior.

La estructura de los precursores de las β-defensinas es más simple que en las α-defensinas y consiste en un péptido señal N-terminal, una corta o en ocasiones inexistente propieza y un péptido maduro C-terminal. En humanos se han identificado 28 β-defensinas (HBD) (Schutte et al, 2002), seis de las cuales, HBD1-6, son casi específicas de células epiteliales (Ganz, 2003; Garcia et al, 2001; Yang et al, 2004). Sin embargo, se ha detectado expresión de HBD1 y 2 en monocitos, macrófagos y células dendríticas (DCs) y de HBD4 en los testículos y cavidad gástrica.

Las θ-defensinas tienen una estructura circular formada por la unión de dos precursores tipo α-defensina de 9 aa cada uno. La unión de los precursores ocurre tras un proceso post-transduccional y la interacción es tipo cabeza-cola (Leonova et al, 2001; Porter et al, 2005). Hasta el momento, las θ-defensinas (RTD) sólo se

han encontrado en leucocitos de macaco Rhesus. Concretamente, se han identificado tres isoformas, RTD1, 2 y 3. En medula ósea de humanos se han encontrado mRNAs de secuencia homóloga a los de las θ-defensinas que, como contienen un codón de stop prematuro, presuntamente no se traducen *in vivo*. Al péptido que resultaría de la traducción de este mRNA encontrado en humanos, se le ha denominado retrociclina, y se ha comprobado que *in vitro* posee actividad antiviral (Nguyen et al, 2003).

En relación con la actividad antiviral (**Tabla 5**) e inmunomoduladora de las defensinas no se van a hacer referencias en esta introducción para evitar solapamientos y duplicaciones con la introducción de las publicaciones que figuran en el anexo I.

Tabla 5. Actividad antiviral de las defensinas (Klotman & Chang, 2006)

Defensinas	Virus	Efecto en la infectividad
α-defensinas		
HNP1, 2 y 3	HIV1, HSV1, HSV2, VSV, influenza, CMV, adenovirus y papillomavirus	Inhibición
HNP1	Echovirus, reovirus y virus vaccinia	Ninguno
HNP4	HIV1	Inhibición
HD5	Papillomavirus	Inhibición
RMAD4	HIV1	Inhibición
NP1, cerdo de Guinea	HIV1	Inhibición
NP1, rata	HIV1	Inhibición
NP1, conejo	HIV1 y HSV2	Inhibición
Criptidina 3	HIV1	Incremento
β-defensinas		
HBD1	HIV1 y virus vaccinia	Ninguno
HBD2	HIV1 y adenovirus	Inhibición
	Rhinovirus y virus vaccinia	Ninguno
HBD3	HIV1 y virus influenza	Inhibición
HBD6	PIV3 (in vivo)	Incremento
B-defensina 4, oveja	PIV3 (in vivo)	Inhibición
θ-defensinas		
Retrociclina 1 y 2	HIV-1, HSV-2 y virus influenza	Inhibición
RTD1, 2 y 3	HIV1 y HSV2	Inhibición

Virus de la inmunodeficiencia humana (HIV), virus del herpes (HSV), citomegalovirus (CMV), virus parainfluenza (PIV), α-defensina mieloide de Rhesus (RMAD), péptido de neutrófilo (NP).

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RESULTADOS Y DISCUSIÓN

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1.-ACTIVIDAD ANTIVIRAL E INMUNOREGULADORA DE LA HNP1 *IN VITRO*

Los resultados de este trabajo (**P1** de esta Tesis Doctoral) claramente demuestran que, i) la inhibición de virus de vertebrados inferiores, como es el caso del VHSV que afecta a peces teleósteos, por una α-defensina humana (HNP1) ocurre a través de más de un mecanismo, ii) la actividad de las defensinas, como ya se había sugerido, es interespecífica y iii) la HNP1 tiene capacidad para regular la respuesta inmune en peces ya que leucocitos de riñón anterior de trucha tratados *ex vivo* con HNP1 modifican los patrones de expresión de algunos genes relacionados con la respuesta inmune temprana

La capacidad de la HNP1 para inhibir, tanto *in vivo* como *in vitro*, la infectividad/replicación de virus con y sin envoltura se conoce desde hace ya algunas décadas (Daher et al, 1986). Sin embargo y a pesar de los numerosos estudios que a este respecto se ha realizado hasta el momento, los mecanismos que subyacen a la actividad antiviral de la HNP1, y que se sabe incluyen tanto actuación directa sobre las partículas víricas como sobre la células que soportan la infección y el sistema inmune innato del organismo infectado, no son suficientemente conocidos (Klotman & Chang, 2006). En el caso concreto de la inhibición de la infectividad del VHSV, la HNP1 ejerció su actividad antiviral mediante dos mecanismos distintos:

-Inactivación directa de los viriones cuando de manera previa a la infección VHSV es incubado con HNP1 (**Figs. 1 y 4**, **P1**).

-Inactivación indirectamente mediante la activación de mecanismos de defensa antiviral en las células susceptibles de ser infectadas por este virus (**Figs. 2 y 3**, **P1**).

1.1 Inactivación directa de VHSV por HNP1

En primer lugar y antes de comenzar los ensayos de actividad antiviral se comprobó que el rango de concentraciones de HNP1 que se pretendía usar (0-20µg/ml) no era tóxico para ninguna de las dos líneas celulares de origen piscícola, EPC (epitelioma papiloso de carpa) y RTG2 (fibroblastos de gónada de trucha arco iris), que se iban a emplear en los ensayos de infectividad. A continuación y para evaluar la capacidad de la HNP1 para inactivar directamente al VHSV, 10^3 ffu de VHSV se preincubaron durante 12h a 14ºC con concentraciones crecientes de HNP1 en 25µl de medio de cultivo sin suero (suero fetal bovino, FCS). Se utilizó medio sin FCS para las incubaciones porque estudios previos encontrados en la bibliografía demostraban que la presencia de suero inhibía la capacidad de la HNP1 para interaccionar con los viriones (Chang et al, 2005; Daher et al, 1986; Mackewicz et al, 2003). Tras el tratamiento, la infectividad del VHSV se valoró 24h post-infección en las líneas celulares EPC y RTG2 utilizando un ensayo de inmuno-detección de focos de infección previamente puesto a punto en nuestro laboratorio (Lorenzo et al, 1996; Mas et al, 2002; Micol et al, 2005; Perez et al, 2002). Como se muestra en la **figura 1A** (**P1**), el tratamiento de VHSV con HNP1 inhibe la infectividad del virus y esta inhibición es proporcional a la concentración de HNP1 utilizada e independiente de la línea celular (tanto en la línea EPC como en la línea celular RTG2 se obtuvieron resultados semejantes). La máxima inhibición, superior al 90% en ambas líneas celulares, se observo con 20µg/ml de HNP1. Resultados similares a los encontrados en este trabajo habían sido ya previamente descritos para la inhibición de los virus HSV1 y 2 (Daher et al, 1986), HIV1 (Chang et al, 2003; Chang et al, 2005) y VSV (Daher et al, 1986) por la HNP1.

En las condiciones experimentales utilizadas en el ensayo anterior, la mezcla de infección (HNP1-VHSV), que se añadía a las monocapas celulares y que contenían HNP1 (diluida 5 veces), se mantenía durante todo el periodo de infección. Sin embargo, cuando el exceso de HNP1 y virus no unido se retiraba 2h

después de la infección y a continuación se lavaban las células infectadas con PBS, se añadía medio fresco con 2% de FCS y se dejaba transcurrir la infección durante 22h más, la capacidad de la HNP1 para inactivar/inhibir la infectividad del VHSV se reducía ~30% (**Fig. 1B**, **P1**). Así pues, el exceso de HNP1 no unida al virus parece contribuir en un 30%, posiblemente actuando sobre las células, a la inhibición inicialmente observada (**Fig. 1A**, **P1**).

Si la HNP1 inactiva directamente a VHSV en ausencia de FCS, algún tipo de interacción virus-péptido tiene que ocurrir. Para detectar esta interacción se realizaron ensayos de unión en fase sólida en los que diferentes concentraciones de HNP1 eran inmovilizadas en pocillos de placas de ELISA de 96 pocillos. En estas condiciones de ensayo y utilizando el MAb 2C9 anti-proteína N de VHSV (Sanz & Coll, 1992) se comprobó que la HNP1 inmovilizada une partículas de VHSV y que la unión/interacción aumenta con la concentración de HNP1 (**Fig. 4A**, **P1**). Además, con este ensayo también se demostró que la interacción VHSV-HNP1 depende de la correcta conformación de la HNP1 ya que el tratamiento del péptido con DTT (agente reductor de enlaces disulfuro) reducía considerablemente la unión/interacción. De la misma manera, el efecto directo de HNP1 y de las θ-defensinas sobre los viriones de HSV1 y HIV se anula cuando se reducen los enlaces disulfuro que mantienen la estructura de la HNP1 con agentes reductores (Daher et al, 1986; Wang et al, 2004).

Aunque en principio parece posible que la inhibición de la infectividad de VHSV por la HNP1 pudiera deberse a una pérdida de la capacidad del virus para unirse a las células (adsorción) tras el tratamiento, mediante ensayos de inmunotransferencia (western blot) se comprobó que tanto el VHSV no tratado como el tratado con HNP1 (20μg/ml) se unían a las células en igual proporción (**Fig. 2B**, **P1**). Dado que la adsorción no se modifica, la posible alteración que sobre la partícula vírica pudiere causar la HNP1 debe afectar a una etapa posterior de la entrada del virus. Las únicas moléculas expuestas en la superficie de VHSV son la gpG y los fosfolípidos de la envoltura que procede de la membrana

plasmática de la última célula a la que ha parasitado el virus. Como ya había sido descrito que la HNP1 interaccionaba con las glicoproteínas de membrana de otros virus envueltos, y que esta interacción reducía la capacidad de infección (Klotman & Chang, 2006; Sinha et al, 2003; Wang et al, 2004; Yasin et al, 2004), se evaluó la funcionalidad de la gpG de VHSV tras el tratamiento de los viriones con la HNP1, mediante un ensayo de formación de sincitios (Estepa et al, 2001; Mas, 2004) que valora la capacidad de la gpG para inducir fusión de membranas a pH ácido. Para ello, células EPC, que expresan de forma estable la proteína verde fluorescente (eGFP), se infectaron con VHSV y 24h post-infección (p.i.) se trataron con HNP1 (20µg/ml) durante 1h a 14ºC. Transcurrida la incubación las células se lavaron para retirar el exceso de péptido y se indujo la fusión con medio de fusión a pH 6 (Estepa et al, 2001; Mas, 2004). Finalmente, y para detectar la presencia de la gpG en las células fusionadas, se utilizó el MAb I10 anti-gpG. En las células infectadas y no tratadas con HNP1, como era de esperar, se pudo observar un elevado porcentaje de núcleos en sincitio (~19% y ~35% a moi 10^{-2} y 10^{-3}, respectivamente) así como la presencia de la gpG en los sincitios (**Fig. 4B y 4C**, **P1**). Sin embargo, en las células no infectadas (datos no mostrados) y en las infectadas que habían sido tratadas con HNP1 (**Fig. 4B**, **P1**) tan sólo se detectaron algunos sincitios (3-4 núcleos/sincitio) inespecíficos (no inducidos por la gpG ya que el marcaje de la gpG nunca se localizó en los sincitios) (**Fig. 4D**, **P1**). Estudios recientes han demostrado que las defensinas, incluida la HNP1, inactivan virus envueltos interaccionando, de manera similar a como lo hacen las lectinas, con los *O*- o *N*-glicanos de las glicoproteínas de la superficie de estos virus (Gallo et al, 2006; Hazrati et al, 2006; Klotman & Chang, 2006; Leikina et al, 2005; Wang et al, 2004; Yasin et al, 2004) de manera que la capacidad de estas glicoproteínas para unirse a los receptores o fusionar con las membranas de la célula huésped queda total o parcialmente inhibida. Si la HNP1 interacciona con la gpG de VHSV de manera similar a una lectina o es otro el tipo de interacción que ocurre está aún por estudiar.

1.2 Inactivación indirecta de VHSV por HNP1

Los resultados mostrados en la **figura 1B** (**P1**) parecían sugerir que la presencia de HNP1 durante el periodo de infección también era capaz de disminuir la infectividad del VHSV. Para comprobar si esta suposición era cierta, células EPC en medio de cultivo con 2% de FCS se infectaron con VHSV en presencia o ausencia de diferentes concentraciones de HNP1. La **figura 2A** (**P1**) muestra que la HNP1 también inhibe la infectividad de VHSV cuando se añade en el momento de la infección y su presencia se mantiene durante todo el periodo de infección. El patrón de inhibición obtenido en este caso fue muy similar al observado cuando la HNP1 se incubaba con el virus de manera previa a la infección (**Fig. 1A**, **P1**)**.** Así pues, además de por inactivación directa de viriones, la HNP1 es capaz de inhibir la infectividad del VHSV en células de peces por algún otro mecanismo.

En células de mamífero, está descrito que el tratamiento con HNP1 es capaz de inducir un "estado antiviral" en las células tratadas (Klotman & Chang, 2006). Como *a priori* parecía difícil suponer que la HNP1 indujera en células de pez una respuesta similar, se realizaron ensayos encaminados a estudiar esta posibilidad. Células EPC y RTG2 se trataron de manera previa a la infección, durante 24h y en presencia de suero, con distintas concentraciones de HNP1. Tras la incubación, las células se lavaron para retirar la HNP1 y se infectaron con VHSV. 24h p.i. se analizaron los resultados, y tal y como se observa en la **figura 3A** (**P1**), la permisividad de las células tratadas con HNP1 a la infección por VHSV con respecto a las no tratadas, es menor y depende de la concentración de HNP1. La disminución de los niveles de mRNA de las proteínas N (**Fig. 3B**, **P1**) y gpG (datos no mostrados) de VHSV estimados por PCR cuantitativa (qPCR) en los cultivos infectados en estas condiciones confirmaron los resultados de la **figura 3A** (**P1**).

En su conjunto, los datos obtenidos en los ensayos anteriores indican claramente que el tratamiento con HNP1 induce en las células de peces al igual que en las células de mamífero (Chang et al, 2005; Klotman & Chang, 2006)

algún mecanismo intracelular de defensa antiviral. En un intento por determinar la naturaleza del mecanismo antiviral inducido por la HNP1, células RTG2 se trataron con HNP1 (20ug/ml) durante 24h para posteriormente extraer el RNA y analizar por RT-PCR (semi-cuantitativa) la expresión de genes relacionados con la respuesta innata antiviral como por ejemplo IFN tipo I. Como controles positivo y negativo se utilizaron células RTG2 tratadas con poly I:C, un potente inductor de IFN tipo I, o con pleurocidina, un AMP de peces planos que no afecta a los niveles de expresión del IFN ni de los genes relacionados con él (Chiou et al, 2006). La respuesta de IFN en los cultivos de células RTG2 tratadas con HNP1, poly I:C o no tratadas se valoró analizando los niveles de expresión del gen de la proteína Mx cuya transcripción es inducida por IFN. De las tres isoformas de la proteína Mx presentes en trucha, Mx1, Mx2 y Mx3, se eligió la Mx3 como marcador de la respuesta de IFN porque estudios previos habían demostrado que esta es la isoforma que se induce preferentemente en células RTG2 en respuesta a diferentes inductores (Tafalla et al, 2007). Además, en estos mismos cultivos se analizó la expresión del receptor tipo Tol 3 (TLR3). Los niveles de expresión tanto del gen de la proteína Mx3 como del de TLR3 aumentaron en las células RTG2 (**Fig. 5**, **P1**) en respuesta al tratamiento con poly I:C o con HNP1, aunque el aumento fue siempre mayor en repuesta a poly I:C.

Los efectos de la HNP1 pueden variar en función de la célula diana (Chang et al, 2003; Chang et al, 2005; Klotman & Chang, 2006). Por ello, para evaluar si la inducción de IFN así como de genes relacionados con IFN en respuesta a la HNP1 ocurría sólo en la línea celular RTG2 (fibroblastos), o era un mecanismo más general de actuación, leucocitos totales de riñón anterior de trucha arco iris se trataron *ex vivo* con HNP1 y 24h más tarde se analizó la expresión de algunos genes relacionados con la respuesta inmune inespecífica de especial relevancia (Mx1, Mx2, Mx3, TLR3, Vig1 (gen inducido por VHSV), IL1β e iNOS (sintetasa inducible de óxido nítrico)). En respuesta a HNP1, los niveles de expresión de todos los genes analizados aumentó en los leucocitos de riñón anterior de trucha

(**Fig. 6**, **P1**). Resultados similares se obtuvieron en respuesta al tratamiento con poly I:C con excepción del gen de iNOS, cuyos niveles de expresión, en comparación con los de leucocitos no tratados, no se modificó.

Que los niveles de expresión de 4 genes cuya inducción es dependiente de IFN tipo I aumenten significativamente en leucocitos de riñón anterior tratados con HNP1 confirma claramente que, al menos en los tipos celulares utilizados en este trabajo, la HNP1 induce una respuesta antiviral dependiente de la inducción de IFN. Además, como los genes de IL1β e iNOS también fueron inducidos, es posible que la HNP1 sea capaz de modular la respuesta inmune en peces. En mamíferos también se ha descrito la inducción por HNP1 de citoquinas proinflamatorias (Froy, 2005; Lehrer & Ganz, 2002; Yang et al, 2002) e IFNγ (Chang et al, 2005; Klotman & Chang, 2006; Selsted & Ouellette, 2005) pero no de IFN tipo I (α/β). Sólo estudios *in vivo* podrán confirmar los resultados encontrados en este trabajo utilizando leucocitos de riñón anterior de trucha arco iris.

2.-ACTIVIDAD INMUNOMODULADORA DE LA DE HNP1 *IN VIVO*

Los resultados obtenidos *in vitro* (**P1**) con leucocitos de riñón anterior de trucha indicaban que la HNP1 podía tener capacidad para modular la respuesta inmune en peces. Para averiguarlo, se analizó el efecto de la HNP1 *in vivo* sobre los niveles de expresión de genes relacionados con el sistema inmune en trucha arco iris.

Tres grupos de alevines de trucha arco iris (12 peces por grupo) se inyectaron intramuscularmente con: grupo 1, HNP1 (1µg/100µl de PBS/pez); grupo 2, pleurocidina de limanda (LmPle) (1µg/100µl de PBS/pez) y grupo 3, PBS (100µl/pez). Uno, 3 y 7 días post-inyección se sacrificaron 4 peces por grupo. A todos los peces sacrificados se les extrajo músculo esquelético (de la zona de inyección), riñón anterior y sangre para, tras extraer el RNA, analizar por RT-PCR la expresión de genes correspondientes a:

-IL1β, IL8 y TNFα1, citoquinas proinflamatorias.

-CK5B, CK6 y CK7A, quimioquinas de la familia CC.

-Mx1-3, IRF3 (factor regulador de IFN 3), sistema de IFN tipo I.

-iNOS y IFNγ.

-MHC I y II, complejo de histocompatibilidad.

La LmPle, un AMP lineal de naturaleza catiónica y formado por 25 aa, típico de peces planos, se utilizó como control para asegurar que los posibles efectos de la HNP1 sobre la respuesta inmune se debían a las características y propiedades de este péptido, y no simplemente a la inyección de un péptido foráneo, independientemente de cual fuere su secuencia o estructura. En respuesta a LmPLe, sólo se indujeron los genes de las citoquinas proinflamatorias (datos no mostrados).

2.1 Efecto de la HNP1 en la expresión de las citoquinas proinflamatorias

El efecto de HNP1 sobre la respuesta inflamatoria temprana, y la potencial utilización de la HNP1 como adyuvante en peces, se evaluó analizando la expresión de los genes de IL1β, IL8 y el TNFα1 (**Fig. 1**, **P2**). En músculo, se observó un aumento en los niveles de expresión de los genes de las tres citoquinas. En mamíferos, el efecto de las HNPs sobre la expresión de la IL1β es controvertido (Sakamoto et al, 2005) y parece dependiente del tipo celular. En nuestro caso, la expresión de IL1β se incrementó en respuesta a HNP1 pero también, y como ya había sido descrito (Chiou et al, 2006), en respuesta a LmPle (datos no mostrados). Resultados similares se observaron para TNFα1. Por lo tanto, parece que el efecto de HNP1 sobre IL1β y TNFα1 es inespecífico y se corresponde con una respuesta inflamatoria frente a un péptido extraño. Por el contrario, los niveles de expresión de IL8 inducidos por HNP1 son significativamente mayores, en consonancia con lo que ya se había descrito previamente en mamíferos (Sakamoto et al, 2005; Van Wetering et al, 1997). Aunque la IL8 es característica de la respuesta inmune temprana y pertenece a la familia de las quimioquinas CXC (Laing et al, 2002a), esta citoquina puede ser catalogada tanto dentro de las citoquinas proinflamatorias como de las quimioquinas. Este carácter dual de la IL8 podría explicar el efecto diferente que tiene HNP1 sobre esta citoquina en relación con el observado sobre IL1β y TNFα1. Existe una relación muy estrecha entre péptidos antimicrobianos y quimioquinas. Aunque con reserva por parte de algunos investigadores, se ha propuesto que algunos péptidos antimicrobianos evolucionaron a quimioquinas (Laing & Secombes, 2004a). De hecho, algunas quimioquinas tienen actividad antimicrobiana (Yang et al, 2003) y muchos péptidos antimicrobianos tienen actividad quimiotáctica (Chertov et al, 1996).

En riñón anterior, únicamente el nivel de expresión de la IL8 se incrementó significativamente a día 1 post-inyección. En leucocitos de sangre se observó un aumento de los niveles de IL1β a día 3 y una disminución de TNFα1 a día 7.

2.2 Efecto de la HNP1 en la expresión de quimioquinas de la familia CC

Para investigar si hay alguna relación entre HNP1 y quimioquinas en peces también se estudió el efecto de HNP1 sobre la expresión de las algunas quimioquinas pertenecientes a la familia CC (CK5B, CK6 y CK7A). En trucha arco iris, se han encontrado dos isoformas (A y B) para CK5 y CK7 (Laing & Secombes, 2004b), pero su función biológica es todavía desconocida y, por ello, en este estudio se analizó sólo una de las dos isoformas para cada una de estas quimioquinas (**Fig. 2**, **P2**).

En riñón anterior y en respuesta a HNP1 sólo aumentan los niveles de expresión del gen de CK7A y en leucocitos de sangre no se observan cambios apreciables en la expresión de ninguno de los tres genes. En músculo, sin embargo, los resultados muestran una fuerte inducción de la expresión de los genes de CK5B y CK7A el día 3.

2.3 Efecto de la HNP1 en la expresión de genes relacionados con el sistema de IFN tipo I

El efecto de HNP1 sobre la expresión de genes relacionados con el sistema del IFN tipo I se evaluó analizando la expresión de los genes de las tres isoformas de Mx y de IRF3 (**Fig. 3**, **P2**).

En riñón anterior, HNP1 induce la expresión de las tres isoformas de Mx pero en especial de Mx1 a día 1 y 3 post-inyección y no produce cambios en los niveles de expresión de IRF3. En sangre, HNP1 no indujo la expresión de ninguno de los

genes relacionados con el sistema IFN tipo I analizados. En músculo, de las tres isoformas de Mx, únicamente Mx3 se incrementó significativamente (días 1 y 3) de acuerdo con datos previos que señalan una inducción preferencial de Mx3 en músculo independientemente del inductor de IFN empleado. En este tejido también se detecta inducción de la expresión de IRF3 a día 7.

2.4 Efecto de la HNP1 en la expresión de iNOS, IFNγ y MHC

En riñón anterior y sangre, HNP1 no tuvo ningún efecto en la expresión de iNOS y IFNγ, efectores de la respuesta inmune típicos de macrófagos. Por el contrario, en músculo, se observa un aumento significativo de iNOS el día 3 (**Fig. 4**, **P2**).

Como ocurre en respuesta a cecropina y Ple (Chiou, 2006), los niveles de expresión de MHC II no se modificaron por la HNP1 en ningún órgano. Sin embargo, sí se observó un ligero aumento de la expresión de MHC I en músculo a día 7 (**Fig. 5**, **P2**) aunque de dudoso significado biológico.

2.5 Actividad quimiotáctica de la HNP1 sobre leucocitos de peces

Como HNP1 aumentó los niveles de expresión de las quimioquinas CK5B y CK7A, homólogas de las quimioquinas de mamíferos RANTES (*regulated on activation, normal T cells expressed and secreted*) y MCP (proteína quimiotáctica de monocitos), respectivamente, se trató de determinar si las HNP1 poseían actividad quimiotáctica. Los resultados utilizando leucocitos de riñón anterior de trucha tratados con HNP1 (**Fig. 6**, **P2**) indican que HNP1 posee actividad quimiotáctica. Los niveles más altos de migración de leucocitos se encontraron a concentraciones de HNP1 de 0.1 y 1µg/ml.

3.-EXPRESIÓN Y ACTIVIDAD ANTIVIRAL DE UN PÉPTIDO TIPO β-DEFENSINA DE TRUCHA (*Oncorhynchus mykiss*)

Los resultados encontrados en las **publicaciones 1** y **2** que claramente muestran que la replicación del VHSV puede ser controlada y el sistema inmune de trucha inmunoregulado por una defensina, aún siendo de origen humano, junto con la identificación en 2006 de secuencias génicas homólogas a las de β-defensinas en tres especies de pez (pez cebra, fugu y tetraodon) (Zou et al, 2006) nos alentaron a realizar una búsqueda en la base de datos de ESTs (expressed sequence tags) de salmónidos (http://grasp.mbb.sfu.ca) con el fin de encontrar en trucha arco iris alguna/s secuencia/s que pudiera/n codificar para péptido/s tipo defensina.

En primer lugar, se realizó la búsqueda, que no tuvo éxito, usando las secuencias de las α-defensinas humanas conocidas hasta el momento. Por el contrario, cuando la búsqueda se repitió utilizando las secuencias de las β-defensinas humanas (**Tabla 1**, **P3**), se identificó una secuencia de trucha arco iris de 715 pb que guardaba cierta homología con la HBD26 y cuya traducción *in silico* (http://www.expasy.org/tools/dna.html) generaba un péptido de 60 aa al que denominamos omBD1 (β-defensina 1 de *Oncorhynchus mykiss*) (**Fig. 1A, P3**).

3.1 Análisis de la secuencia de aminoácidos de omBD1

Utilizando diferentes herramientas informáticas, el análisis de la secuencia de aas de omBD1 reveló la posible existencia en el N-terminal de un péptido señal con un potencial sitio de corte localizado después del aa 19 (Flecha vertical**, Fig. 1A**, **P3**). Como resultado de la eliminación del péptido señal resultaría un propétido de 41 aas (secuencia subrayada, **Fig. 1A**, **P3)** que conserva el patrón consenso de seis residuos de cisteína presente en las β-defensinas de otros organismos. Tras la alineación de la secuencia de aas del propéptido de omBD1 con las de los propéptidos de hBD26 y de las defensinas tipo-β de peces se

observó que omBD1 en relación con estas secuencias (**Fig. 1B**, **P3**), i) posee un 34.9% de identidad con la HBD26 y un 85.7% con las de peces, ii) presenta aas conservados en las posiciones 10 (residuo aromático), 11 (serina/treonina), 17 (glicina), 20 y 21 (residuo cargado positivamente), 28 (ácido glutámico) y 42 (residuo arómatico) y iii) tiene también carga neta positiva (+2), un punto isoeléctrico ~8 y un peso molecular pequeño (4-5kDa) (**Tabla 2**, **P3**).

El árbol filogenético construido a partir de las secuencias alineadas anteriormente (**Fig. 2**, **P3**) muestra dos grupos diferenciados. La secuencia del propéptido de omBD1 se encuentra en el mismo grupo que las secuencias de los propéptidos BD1 de peces. El resto de secuencias de peces (BD2 y BD3) están en el otro grupo y la secuencia de HBD26 (más distanciada evolutivamente) está fuera de ambos.

3.2 Patrón de expresión de omBD1 *in vivo*

Para evaluar el patrón de expresión de transcritos de omBD1, si es que esta defensina posee en algún tejido expresión constitutiva, se utilizaron alevines sanos de trucha arco iris. La expresión se analizó por RT-PCR (semi-cuantitativa) a partir de RNA extraído de hígado, riñón anterior, bazo y músculo. Además, en el estudio de expresión se incluyó RNA procedente de la línea celular RTG2. Aunque los niveles de expresión constitutiva de omBD1 más altos se detectaron en músculo, en riñón anterior también se pudo detectar su presencia (**Fig. 3**, **P3**). En hígado, bazo y células RTG2 no se detectó expresión de omBD1 (datos no mostrados). La expresión diferencial de omBD1 sugiere la existencia en peces de mecanismos de regulación específicos de tejido para las β-defensinas, cuya existencia ha sido ya descrita en otros vertebrados (Pazgier et al, 2006). En mamíferos, la expresión de las defensinas está más diversificada que en otros grupos animales de manera que las β-defensinas se expresan principalmente en células epiteliales (Ganz, 2003; Klotman & Chang, 2006; Yang et al, 2004) y las α

en células relacionadas con el sistema inmune. Hasta el momento, no hay evidencia de la existencia de α-defensinas en peces, por lo que no sería sorprendente que las β-defensinas en estos organismos pudieran expresarse en un rango más amplio de tejidos u órganos, incluyendo aquellos implicados en la respuesta inmune como es el caso del riñón anterior. De hecho en pez cebra, se ha detectado expresión constitutiva de las secuencias tipo β-defensinas identificadas en este pez en todos los tejidos analizados (Zou et al, 2006). La ausencia de expresión de omBD1 en hígado de trucha podría explicarse por la expresión en este órgano de otros péptidos antimicrobianos como por ejemplo las hepcidinas (Douglas et al, 2003a) y LEAP2 (Zhang et al, 2004a).

3.3 Actividad antiviral de omBD1 *in vitro*

Para averiguar si omBD1 poseía, al igual que algunas de las β-defensinas descritas en otros organismos, actividad antiviral, células EPC se transfectaron con distintas cantidades de un vector de expresión de células eucariotas (pMCV1.4) en el que se había clonado la secuencia del DNA de omBD1 bajo el control del promotor de CMV (pMCV1.4-omBD1). En primer lugar y por RT-PCR se comprobó que este vector expresaba eficientemente el mRNA de omBD1 en las células transfectadas (**Fig. 4A**, **P3**). Una vez confirmada la presencia de transcritos de omBD1 en las células transfectadas, se volvieron a transfectar células EPC con pMCV1.4-omBD1 que 24h post-transfección se infectaron con VHSV. Como se muestra en la **figura 4B** (**P3**), la replicación de VHSV en las células transfectadas es prácticamente nula a concentraciones de plásmido de 1μg/ml o superiores.

Teniendo en cuenta los resultados obtenidos con la HNP1 (**P1**), cabía la posibilidad de que la expresión de omBD1 indujera en las células transfectadas un mecanismo de defensa antiviral mediado por IFN tipo I. Para averiguarlo, se preparó medio condicionado (tratamiento con HCl durante 12h a 4ºC, 50ºC 60min y restablecimiento del pH a 7.6) a partir de células EPC sin transfectar (CM) y de

células EPC transfectadas con pMCV1.4-omBD1 (omBD1-CM), pMCV1.4 (V-CM) o FuGene (F-CM). Con estos medios se trataron células EPC durante 24h que, después de varios lavados, se infectaron con VHSV. Únicamente las células tratadas con medio condicionado procedente de células transfectadas con pMCV1.4-omBD1 mostraron resistencia a la infección por VHSV (**Fig. 5**, **P3**). Dado que el tratamiento aplicado no eliminó la actividad antiviral presente en el medio condicionado de células EPC transfectadas con pMCV1.4-omBD1 y que además en estas células el gen de la proteína Mx1, cuya expresión está directamente regulada por IFN tipo I en carpa (Zhang et al, 2004b), está inducido (**Fig. 6 A y B**, **P3**), es más que probable que en estas células una respuesta antiviral mediada por IFN tipo I sea la responsable de la inhibición del VHSV.

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CONCLUSIONES

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1.-HNP1 tiene actividad interespecífica, ya que es capaz de inhibir a VHSV, un rabdovirus de peces, y modular la respuesta inmune en peces.

2.-HNP1 inhibe a VHSV mediante dos mecanismos: i) inactivación directa de los viriones uniéndose a las partículas víricas y, posiblemente, interaccionando con la gpG y ii) inducción de una respuesta antiviral mediada por IFN en las células.

3.-La actividad inmunomoduladora de la HNP1 también se manifiesta in vivo tras inyección intramuscular y esta respuesta es sistémica ya que no sólo se produce en músculo sino además en sangre y riñón anterior. HNP1 también posee capacidad quimiotáctica en leucocitos circulantes de trucha arco iris.

4.-Se ha identificado por primera vez una β-defensina en trucha arco iris y se ha demostrado que es capaz de inhibir la multiplicación de VHSV induciendo en las células la producción de IFN.

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BIBLIOGRAFÍA

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Banerjee AK, Chattopadhyay D (1990) Structure and function of the RNA polymerase of vesicular stomatitis virus. *Adv Virus Res* **38:** 99-124

Basurco B, Benmansour A (1995) Distant strains of the fish rhabdovirus VHSV mantain a sixth functional cistron which codes for a nonstructural protein of unknown function. *Virology* **212:** 741-745

Basurco B, Coll JM (1989) Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. *Bulletin European Association Fish Pathology* **9:** 92-95

Basurco B, Coll JM (1991) In vitro and in vivo variability of the first viral haemorrhagic septicaemia virus isolated in Spain compared to international reference serotypes.

Basurco B, Coll JM (1992) In vitro studies and in vivo immunisation with the first viral haemorrhagic septicaemia viruses isolated in Spain compared to international reference serotypes. *Research Veterinary Science* **53:** 93-97

Bearzotti M, Delmas B, Lamoureux A, Loustau AM, Chilmonczyk S, Bremont M (1999) Fish rhabdovirus cell entry is mediated by fibronectin. *J Virol* **73**(9)**:** 7703-7709

Benmansour A, Basurco B, Monnier AF, Vende P, Winton JR, de Kinkelin P (1997) Sequence variation of the glycoprotein gene identifies three distinct lineages within field isolates of viral haemorrhagic septicaemia virus, a fish rhabdovirus. *J Gen Virol* **78 (Pt 11):** 2837-2846

Benmansour A, Leblois H, Coulon P, Tuffereau C, Gaudin Y, Flamand A, Lafay F (1991) Antigenicity of rabies virus glycoprotein. *J Virol* **65**(8)**:** 4198-4203

Biacchesi S, Thoulouze MI, Bearzotti M, Yu YX, Bremont M (2000) Recovery of NV knockout infectious hematopoietic necrosis virus expressing foreign genes. *J Virol* **74**(23)**:** 11247-11253

Bly JE, Clem LW (1991) Temperature-mediated processes in teleost immunosuppression induced by in vivo low temperature in channel catfish. *Veterinary Immunology Immunopathology* **28:** 365-377

Boman HG (2003) Antibacterial peptides: basic facts and emerging concepts. *Journal of Internal Medicine* **254**(3)**:** 197-215

Boudinot P, Blanco M, de Kinkelin P, Benmansour A (1998) Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout. *Virology* **249**(2)**:** 297-306

Boudinot P, Boubekeur S, Benmansour A (2001) Rhabdovirus Infection Induces Public and Private T Cell Responses in Teleost Fish. *J Immunol* **167**(11)**:** 6202-6209

Bowdish DM, Davidson DJ, Hancock RE (2006) Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol* **306:** 27-66

Bowie AG, Haga IR (2005) The role of Toll-like receptors in the host response to viruses. *Molecular Immunology* **42**(8)**:** 859-867

Castric J, DeKinkelin P (1984) Experimental study of the susceptibility of two marine fish species, Sea Bass (Dicentrarchus labrax and Turbot) (Scophthalmus maximus), to Viral Haemorrhagic Septicaemia. *Aquaculture* **41:** 203-212

Castric J, Jeffroy J, Bearzotti M, DeKinkelin P (1992) Isolation of viral haemorrhagic septicemia virus (VHSV) from wild elvers anguilla anguilla. *Bulletin European Association Fish Pathology* **12:** 21-23

Coll J (1999) Prevalencia de las rabdovirosis en la Acuicultura Europea. . *AquaTic 6, http://aquaticunizares/n2/art602/rabdoUEhtm*

Coll J (2001) Actualidad y futuro de la Acuicultura española. AquaTic 14, http://aquatic.unizar.es/n3/art1403/acuicultura_sp.htm. *AquaTic 14, http://aquaticunizares/n3/art1403/acuicultura_sphtm*

Cunliffe RN (2003) Alpha-defensins in the gastrointestinal tract. *Mol Immunol* **40**(7)**:** 463-467

Chang TL, Francois F, Mosoian A, Klotman ME (2003) CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition. *J Virol* **77**(12)**:** 6777-6784

Chang TL, Vargas J, Jr., DelPortillo A, Klotman ME (2005) Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity. *J Clin Invest* **115**(3)**:** 765-773

Chen JL, Das T, Banerjee AK (1997) Phosphorylated states of vesicular stomatitis virus P protein in vitro and in vivo. *Virology* **228**(2)**:** 200-212

Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, Longo DL, Taub DD, Oppenheim JJ (1996) Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J Biol Chem* **271**(6)**:** 2935-2940

Chilmonczyk S (1980) Some aspects of trout gill structure in relation to egtved virus infection and defense mechanims. *In: Fish diseases (Ahne W, ed) Springer-Verlag, Berlin* 18-22

Chiou P, Khoo J, Bols NC, Douglas S, Chen TT (2006) Effects of linear cationic alpha-helical antimicrobial peptides on immune-relevant genes in trout macrophages. *Dev Comp Immunol* **30**(9)**:** 797-806

Daher KA, Lehrer RI, Ganz T, Kronenberg M (1988) Isolation and characterization of human defensin cDNA clones. *Proc Natl Acad Sci U S A* **85**(19)**:** 7327-7331

Daher KA, Selsted ME, Lehrer RI (1986) Direct inactivation of viruses by human granulocyte defensins. *J Virol* **60**(3)**:** 1068-1074

Das SC, Pattnaik AK (2005) Role of the hypervariable hinge region of phosphoprotein P of vesicular stomatitis virus in viral RNA synthesis and assembly of infectious virus particles. *J Virol* **79**(13)**:** 8101-8112

David Andreu LR (1998) Animal antimicrobial peptides: An overview. *Peptide Science* **47**(6)**:** 415-433

De Kinkelin P, ChilmonnczyK, S., Dorson, M., Le Berre, M and Baudoy, A.M. (1979) Some pathogenic facets of rhabdoviral infections of salmonid fish. *In: Munich Symposia on Microbiology: mechanims of viral pathogenesis and vrulence PA Bachmann***:** 357-375

DeKinkelin P, Bearzotti M, Castric J, Nougayrede P, Lecocq-Xhonneux F, Thiry M (1995) Eighteen years of vaccination against viral haemorrhagic septicemia in France. *Veterinary Research* **26:** 379-387

DeKinkelin P, Le Berre M, Bernard J (1980) Viral hemorrhagic septicemia of rainbow trout: Selection of a thermoresistant virus variant and comparation of polypeptide synthesis with the wild-type virus strain. *Journal Virology* **36:** 652-658

DeLuca D, Wilson M, Warr GW (1983) Lymphocyte heterogeneity in the trout, Salmo gairdneiri, defined with monoclonal antibodies to IgM. *European Journal Immunology* **13:** 546-551

Douglas SE, Gallant JW, Liebscher RS, Dacanay A, Tsoi SC (2003a) Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. *Dev Comp Immunol* **27**(6-7)**:** 589-601

Douglas SE, Patrzykat A, Pytyck J, Gallant JW (2003b) Identification, structure and differential expression of novel pleurocidins clustered on the genome of the winter flounder, Pseudopleuronectes americanus (Walbaum). *Eur J Biochem* **270**(18)**:** 3720-3730

DROLET JS, ROHOVEC, JJ.S. and LEONG, J. (1994) The route of entry and progression of infectious haematopoietic necrosis virus in Oncorhynchus mykiss (Walbaum): a sequential immunohistochemical study. *Journal of Fish Diseases* **17:** 337-347

Du Pasquier L (1982) Antibody diversity in lower vertebrates--why is it so restricted? *Nature* **296**(5855)**:** 311-313

Du Pasquier L, Wilson M, Greenberg AS, Flajnik MF (1998) Somatic mutation in ectothermic vertebrates: musings on selection and origins. *Curr Top Microbiol Immunol* **229:** 199-216

Eaton WD, Hulett J, Brunson R, True K (1991) The first isolation in North America of infectious hematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV) in coho salmon from the same watershed. *Juournal Aquatic Animal Helath* **3:** 114-117

Einer-Jensen K, Ahrens P, Forsberg R, Lorenzen N (2004) Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *J Gen Virol* **85**(Pt 5)**:** 1167-1179

Einer-Jensen K, Krogh TN, Roepstorff P, Lorenzen N (1998) Characterization of Intramolecular Disulfide Bonds and Secondary Modifications of the Glycoprotein from Viral Hemorrhagic Septicemia Virus, a Fish Rhabdovirus. *J Virol* **72**(12)**:** 10189-10196

Ellis AE (2001) Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol* **25**(8-9)**:** 827-839

Enzmann PJ, Maier B, Bigott K (1981) Biochemical data on the RNA of VHS-V and RVC indicating a possible serological relationship. *Bulletin European Association Fish Pathology* **1:** 37-39

Essbauer S, Ahne W (2001) Viruses of lower vertebrates. *J Vet Med B Infect Dis Vet Public Health* **48**(6)**:** 403-475

Estepa A, Coll JM (1996a) Pepscan mapping and fusion-related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. *Virology* **216**(1)**:** 60-70

Estepa A, Coll JM (1996b) Phosphatidylserine binding to solid-phase rhabdoviral peptides: a new method to study phospholipid/viral protein interactions. *J Virol Methods* **61**(1-2)**:** 37-45

Estepa A, Thiry M, Coll JM (1994) Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leukocyte anamnestic responses in vitro. *J Gen Virol* **75 (Pt 6):** 1329-1338

Estepa AM, Rocha AI, Mas V, Perez L, Encinar JA, Nunez E, Fernandez A, Gonzalez Ros JM, Gavilanes F, Coll JM (2001) A Protein G Fragment from the Salmonid Viral Hemorrhagic Septicemia Rhabdovirus Induces Cell-to-Cell Fusion and Membrane Phosphatidylserine Translocation at Low pH. *J Biol Chem* **276**(49)**:** 46268-46275

Fahlgren A, Hammarstrom S, Danielsson A, Hammarstrom ML (2003) Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol* **131**(1)**:** 90-101

Fellermann K, Stange EF (2001) Defensins -- innate immunity at the epithelial frontier. *Eur J Gastroenterol Hepatol* **13**(7)**:** 771-776

Fernandez-Alonso M, Rocha A, Coll JM (2001) DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. *Vaccine* **19**(23-24)**:** 3067-3075.

Fischer U, Ototake M, Nakanishi T (1998) In vitro cell-mediated cytotoxicity against allogeneic erythrocytes in ginbuna crucian carp and goldfish using a non-radioactive assay. *Dev Comp Immunol* **22**(2)**:** 195-206

Fischer U, Utke K, Somamoto T, Kollner B, Ototake M, Nakanishi T (2006) Cytotoxic activities of fish leucocytes. *Fish & Shellfish Immunology* **20**(2)**:** 209-226

Frerichs GN (1989) Rhabdoviruses of fishes. *Viruses of lower vertebratesAhne,W& Kurstak,EedsSpringer, Berlin* **1:** 27-32

Froy O (2005) Regulation of mammalian defensin expression by Toll-like receptor-dependent and independent signalling pathways. *Cell Microbiol* **7**(10)**:** 1387-1397

Froy O, Gurevitz M (2003) Arthropod and mollusk defensins--evolution by exon-shuffling. *Trends Genet* **19**(12)**:** 684-687

Gallo RL, Huttner KM (1998) Antimicrobial peptides: an emerging concept in cutaneous biology. *J InvestDermatol* **111:** 739-743

Gallo SA, Wang W, Rawat SS, Jung G, Waring AJ, Cole AM, Lu H, Yan X, Daly NL, Craik DJ, Jiang S, Lehrer RI, Blumenthal R (2006) Theta-defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. *J Biol Chem* **281**(27)**:** 18787-18792

Ganz T (1987) Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect Immun* **55**(3)**:** 568-571

Ganz T (1999) Defensins and host defense. *Science* **286**(5439)**:** 420-421

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* **3**(9)**:** 710-720

Ganz T (2005) Defensins and other antimicrobial peptides: a historical perspective and an update. *Comb Chem High Throughput Screen* **8**(3)**:** 209-217

Ganz T, Lehrer RI (1994) Defensins. *Curr Opin Immunol* **6**(4)**:** 584-589

Ganz T, Lehrer RI (1995) Defensins. *Pharmacol Ther* **66**(2)**:** 191-205

Ganz T, Selsted ME, Lehrer RI (1990) Defensins. *Eur J Haematol* **44**(1)**:** 1-8

Garcia JR, Jaumann F, Schulz S, Krause A, Rodriguez-Jimenez J, Forssmann U, Adermann K, Kluver E, Vogelmeier C, Becker D, Hedrich R, Forssmann WG, Bals R (2001) Identification of a novel, multifunctional beta-defensin (human beta- defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. *Cell Tissue Res* **306**(2)**:** 257-264.

Ghittino P (1965) Viral hemorrhagic septicemia (VHS) in rainbow trout in Italy. *Ann N Y Acad Sci* **126**(1)**:** 468-478

Ghosh D, Porter E, Shen B, Lee SK, Wilk D, Drazba J, Yadav SP, Crabb JW, Ganz T, Bevins CL (2002) Paneth cell trypsin is the processing enzyme for human defensin-5. *Nat Immunol* **3**(6)**:** 583-590

Hancock RE, Patrzykat A (2002) Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. *Curr Drug Targets Infect Disord* **2**(1)**:** 79-83

Hancock RE, Scott MG (2000) The role of antimicrobial peptides in animal defenses. *Proc Natl Acad Sci U S A* **97**(16)**:** 8856-8861.

Hansen JD, Landis ED, Phillips RB (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proc Natl Acad Sci U S A* **102**(19)**:** 6919-6924

Hansen JD, Strassburger P (2000) Description of an ectothermic TCR coreceptor, CD8 alpha, in rainbow trout. *J Immunol* **164**(6)**:** 3132-3139

Harder J, Bartels J, Christophers E, Schroder J-M (2001) Isolation and Characterization of Human beta -Defensin-3, a Novel Human Inducible Peptide Antibiotic. *J Biol Chem* **276**(8)**:** 5707-5713

Harmache A, LeBerre M, Droineau S, Giovannini M, Bremont M (2006) Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *J Virol* **80**(7)**:** 3655-3659

Hasegawa S, Nakayasu, C., Yoshitomi, T., Nakanishi, T., and Okamoto,, N. (1998) Specific cell-mediated cytotoxicity against an allogeneic target cell line in isogeneic ginbuna crucian carp. *Fish Shellfish Immunol* **8:** 303–313.

Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Keller MJ, Lehrer RI, Herold BC (2006) Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. *J Immunol* **177**(12)**:** 8658-8666

Heike S, Egbert, M., y Mettenleiter, T. C. (1999) Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes* **19:** 59-65

Hein M, Valore EV, Helmig RB, Uldbjerg N, Ganz T (2002) Antimicrobial factors in the cervical mucus plug. *Am J Obstet Gynecol* **187**(1)**:** 137-144

Hertz CJ, Wu Q, Porter EM, Zhang YJ, Weismuller KH, Godowski PJ, Ganz T, Randell SH, Modlin RL (2003) Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol* **171**(12)**:** 6820-6826

Hill BJ, Underwood BO, Smale CJ, Brown F (1975) Physico-chemical and serological characterization of five rhabdoviruses infecting fish. *Journal General Virology* **27:** 369-378

Hopper K (1989) The isolation of VHSV from salmon at Glenwood Springs, Orcas Island, Washington. *American Fish Society Fish Health Newsletter* **17:** 1

Hwang LN, Englund N, Das T, Banerjee AK, Pattnaik AK (1999) Optimal replication activity of vesicular stomatitis virus RNA polymerase requires phosphorylation of a residue(s) at carboxy-terminal domain II of its accessory subunit, phosphoprotein P. *J Virol* **73**(7)**:** 5613-5620

Isshik T, Nishizawa T, Kobayashi T, Nagano T, Miyazaki T (2001) An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder Paralichthys olivaceus in Japan. *Dis Aquat Organ* **47**(2)**:** 87-99.

Janeway CA, Medzhitov R (2002) INNATE IMMUNE RECOGNITION. *Annual Review of Immunology* **20**(1)**:** 197-216

Jensen MH (1963) Preparation of fish tissue cultures for virus research. *Bulletin Office International Epizooties* **59:** 131-134

Jenssen H, Gutteberg TJ, Rekdal O, Lejon T (2006) Prediction of activity, synthesis and biological testing of anti-HSV active peptides. *Chem Biol Drug Des* **68**(1)**:** 58-66

Klotman ME, Chang TL (2006) Defensins in innate antiviral immunity. *Nat Rev Immunol* **6**(6)**:** 447-456

Laing KJ, Bols N, Secombes CJ (2002a) A CXC chemokine sequence isolated from the rainbow trout Oncorhynchus mykiss resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11. *Eur Cytokine Netw* **13**(4)**:** 462-473

Laing KJ, Secombes CJ (2004a) Chemokines. *Dev Comp Immunol* **28**(5)**:** 443-460

Laing KJ, Secombes CJ (2004b) Trout CC chemokines: comparison of their sequences and expression patterns. *Mol Immunol* **41**(8)**:** 793-808

Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T, Secombes CJ (2002b) Identification and analysis of an interleukin 8-like molecule in rainbow trout Oncorhynchus mykiss. *Developmental & Comparative Immunology* **26**(5)**:** 433-444

LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, Kurath G (2001) Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. *Vaccine* **19**(28-29)**:** 4011-4019

LeBerre M, De Kinkelin P, Metzger A (1977) Identification sérologique des rhabdovirus des salmonidés. *Bulletin Office International Epizooties* **87:** 391-393

Lehrer RI (2004) Primate defensins. *Nat Rev Micro* **2**(9)**:** 727-738

Lehrer RI, Ganz T (1992) Defensins: endogenous antibiotic peptides from human leukocytes. *Ciba Found Symp* **171:** 276-290; discussion 290-273

Lehrer RI, Ganz T (2002) Defensins of vertebrate animals. *Current Opinion in Immunology* **14**(1)**:** 96-102

Lehrer RI, Lichtenstein AK, Ganz T (1993) Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu Rev Immunol* **11:** 105-128

Leikina E, Delanoe-Ayari H, Melikov K, Cho MS, Chen A, Waring AJ, Wang W, Xie Y, Loo JA, Lehrer RI, Chernomordik LV (2005) Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. *Nat Immunol* **6**(10)**:** 995-1001

Leong JC, Bootland L, Anderson E, Chiou PW, Drolet B, Kim C, Lorz H, Mourich D, Ormonde P, Perez L, Trobridge G (1995) Viral vaccines for aquaculture. *Journal Marine Biotechnology* **3:** 16-23

Leonova L, Kokryakov VN, Aleshina G, Hong T, Nguyen T, Zhao C, Waring AJ, Lehrer RI (2001) Circular minidefensins and posttranslational generation of molecular diversity. *J Leukoc Biol* **70**(3)**:** 461-464

Lillard JW, Jr., Boyaka PN, Chertov O, Oppenheim JJ, McGhee JR (1999) Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc Natl Acad Sci U S A* **96**(2)**:** 651-656

Linzmeier R, Ho CH, Hoang BV, Ganz T (1999) A 450-kb contig of defensin genes on human chromosome 8p23. *Gene* **233**(1-2)**:** 205-211

Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE (2002a) DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. *Fish Shellfish Immunol* **12**(5)**:** 439-453

Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE (2002b) Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. *Developmental & Comparative Immunology* **26**(2)**:** 173-179

Lorenzen N, Olesen NJ, Jorgensen PE (1990) Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. *J Gen Virol* **71 (Pt 3):** 561-567

Lorenzo G, Estepa A, Coll JM (1996) Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicaemia with anti-nucleoprotein monoclonal antibody. *J Virol Methods* **58**(1-2)**:** 1-6

Mackewicz CE, Yuan J, Tran P, Diaz L, Mack E, Selsted ME, Levy JA (2003) alpha-Defensins can have anti-HIV activity but are not CD8 cell anti-HIV factors. *Aids* **17**(14)**:** F23-32

Magnadottir B (2006) Innate immunity of fish (overview). *Fish Shellfish Immunol* **20**(2)**:** 137-151

Manning MJ, and Nakanishi, T. (1996). , London. (1996) The specific immune system: Cellular defenses. . *In "Fish Physiology XV: The Fish Immune System Academic Press London***:** 159–205

Mas V, Perez L, Encinar JA, Pastor MT, Rocha A, Perez-Paya E, Ferrer-Montiel A, Gonzalez Ros JM, Estepa A, Coll JM (2002) Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library. *J Gen Virol* **83**(Pt 11)**:** 2671-2681.

Mas V, Rocha, A., Perez, L., Coll, J.M. and Estepa, A. (2004) Reversible Inhibition of Spreading of In Vitro Infection and Imbalance of Viral Protein Accumulation at Low pH in Viral Haemorrhagic Septicaemia Rhabdovirus (VHSV), a Salmonid Rhabdovirus. *Journal of Virology* **78(4)**

Meier W, Schmitt M, Wahli T (1994) Viral haemorrhagic septicemia (VHS) of nonsalmonids. *Annual Review Fish Diseases* **4:** 359-373

Meyers TR, Sullivan J, Emmeneger E, Follet J, Short S, Batts WN, Winton JR (1992) *Identification of viral haemorrhagic septicemia virus isolated from pacific cod, Gadus Macocephalus in prince William Sound, Alaska,USA*. *Diseases Aquatic Organisms* **12:** 167-175

Micol V, Caturla N, Perez-Fons L, Mas V, Perez L, Estepa A (2005) The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). *Antiviral Res* **66**(2-3)**:** 129-136

Mookherjee N, Hancock RE (2007) Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cell Mol Life Sci* **64**(7-8)**:** 922-933

Morzunov SP, Winton JR, Nichol ST (1995) The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. *Virus Research* **38:** 175-192

Nakanishi T, Fischer U, Dijkstra JM, Hasegawa S, Somamoto T, Okamoto N, Ototake M (2002) Cytotoxic T cell function in fish. *Developmental & Comparative Immunology* **26**(2)**:** 131-139

Nguyen TX, Cole AM, Lehrer RI (2003) Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **24**(11)**:** 1647-1654

Nichol ST, Rowe JE, Winton JR (1995) Molecular epizootiology and evolution of the glycoprotein and non-vrion protein genes of infectious hematopoietic necrosis virus, a fish rhabdovirus. *Virus Research* **38:** 159-173

Nishizawa T, Iida, H., Takano, R., Isshiki, T., Nakajima, K., y Muroga, K. (2002) Genetic relatedness among Japanese, American y European isolates of viral hemorrhagic septicemia virus (VHSV) based on partial G y P genes. *Diseases Aquatic Organisms* **48:** 143-148

Nunez E, Fernandez AM, Estepa A, Gonzalez-Ros JM, Gavilanes F, Coll JM (1998) Phospholipid interactions of a peptide from the fusion-related domain of the glycoprotein of VHSV, a fish rhabdovirus. *Virology* **243:** 322-330

Olesen N, J, Lorenzen N, Vestergaard-Jorgensen PE (1991) Detection of rainbow trout antibody to Egtved virus by enzyme- linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT. *Diseases Aquatic Organisms* **10:** 31-38

Olesen NJ, and H. Korsholm. (1997) Control measures for viral diseases in Aquaculture: eradication of VHS and IHN. *Bulletin European Association Fish Pathologists* **17:** 229-233

Oppenheim JJ, Biragyn A, Kwak LW, Yang D (2003) Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Ann Rheum Dis* **62 Suppl 2:** ii17-21

Oshima KH, Arakawa CK, Higman KH, Landolt ML, Nichol ST, Winton JR (1995) The genetic diversity and epizootiology of infectious hematopoietic necrosis virus. *Virus Research* **35:** 123-141

Palm RC, Jr., Landolt ML, Busch RA (1998) Route of vaccine administration: effects on the specific humoral response in rainbow trout Oncorhynchus mykiss. *Dis Aquat Organ* **33**(3)**:** 157-166

Partula S, DeGuerra A, Fellah JS, Charlemagna J (1995) Structure and diversity of the T cell antigen receptor beta-chain in a teleost fish. *Journal Immunology* **155:** 699-706

Partula S, DeGuerra A, Fellah JS, Charlemagne J (1996) Structure and diversity of the TCR alpha-chain in a teleost fish. *Journal Immunology* **157:** 207-212

Pattnaik AK, Hwang L, Li T, Englund N, Mathur M, Das T, Banerjee AK (1997) Phosphorylation within the amino-terminal acidic domain I of the phosphoprotein of vesicular stomatitis virus is required for transcription but not for replication. *J Virol* **71**(11)**:** 8167-8175

Pazgier M, Hoover D, Yang D, Lu W, Lubkowski J (2006) Human β-defensins. *Cellular and Molecular Life Sciences (CMLS)* **63**(11)**:** 1294-1313

Perez L, Mas V, Coll J, Estepa A (2002) Enhanced detection of viral hemorrhagic septicemia virus (a salmonid rhabdovirus) by pretreatment of the virus with a combinatorial library-selected peptide. *Journal of Virological Methods* **106**(1)**:** 17-23

Peters F, Neukirch M (1986) Transmission of some fish pathogenic viruses by the heron,Ardea cinerea. *Journal Fish Diseases* **9:** 539-544

Plouffe DA, Hanington PC, Walsh JG, Wilson EC, Belosevic M (2005) Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantation* **12**(4)**:** 266-277

Porter E, Yang H, Yavagal S, Preza GC, Murillo O, Lima H, Greene S, Mahoozi L, Klein-Patel M, Diamond G, Gulati S, Ganz T, Rice PA, Quayle AJ (2005) Distinct defensin profiles in Neisseria gonorrhoeae and Chlamydia trachomatis urethritis reveal novel epithelial cell-neutrophil interactions. *Infect Immun* **73**(8)**:** 4823-4833

Quayle AJ, Porter EM, Nussbaum AA, Wang YM, Brabec C, Yip KP, Mok SC (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol* **152**(5)**:** 1247-1258

Raj PA, Dentino AR (2002) Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiology Letters* **206**(1)**:** 9-18

Rasmussen CJ (1965) A biological study of the Egtved disease (INuL). *Ann N Y Acad Sci* **126**(1)**:** 427-460

Rocha A, Ruiz S, Tafalla C, Coll JM (2004a) Conformation- and fusion-defective mutations in the hypothetical phospholipid-binding and fusion peptides of viral hemorrhagic septicemia salmonid rhabdovirus protein G. *J Virol* **78**(17)**:** 9115-9122

Rocha A, Ruiz S, Tafalla C, Coll JM (2004b) Characterisation of the syncytia formed by VHS salmonid rhabdovirus G gene transfected cells. *Vet Immunol Immunopathol* **99**(3-4)**:** 143-152

Roche S, Bressanelli S, Rey FA, Gaudin Y (2006) Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. *Science* **313**(5784)**:** 187-191

Roche S, Rey FA, Gaudin Y, Bressanelli S (2007) Structure of the prefusion form of the vesicular stomatitis virus glycoprotein G. *Science* **315**(5813)**:** 843-848

Rodrigues PN, Dixon B, Roelofs J, Rombout JH, Egberts E, Pohajdak B, Stet RJ (1998) Expression and temperature-dependent regulation of the beta2-microglobulin (Cyca-B2m) gene in a cold-blooded vertebrate, the common carp (Cyprinus carpio L.). *Dev Immunol* **5**(4)**:** 263-275

Sakamoto N, Mukae H, Fujii T, Ishii H, Yoshioka S, Kakugawa T, Sugiyama K, Mizuta Y, Kadota J, Nakazato M, Kohno S (2005) Differential effects of alpha- and beta-defensin on cytokine production by cultured human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **288**(3)**:** L508-513

Salonius K, Simard N, Harland R, Ulmer JB (2007) The road to licensure of a DNA vaccine. *Curr Opin Investig Drugs* **8**(8)**:** 635-641

Sanz FA, Coll JM (1992) Detection of hemorrhagic virus of samonid fishes by use of an enzyme-linked immunosorbent assay containing high sodium chloride concentration and two concompetitive monoclonal antibodies against early viral nucleoproteins. *American Journal Veterinary Research* **53:** 897-903

Schlegel R, Sue TT, Willingham MC, Pastan I (1983) Inhibition of VSV binding and infectivity by phosphatidylserine: Is phosphatidylserine a VSV-binding site? *Cell* **32:** 639-646

Schlotfeldt HJ, Ahne W, Vestergard-Jorgensen PE, Glende W (1991) Occurrence of viral haemorrhagic septicaemia in turbot (Scophthalmus maximus)-A natural outbreak. *Bulletin European Association Fish Pathology* **11:** 105-107

Schnell MJ, Conzelmann K-K (1995) Polymerase Activity ofin VitroMutated Rabies Virus L Protein. *Virology* **214**(2)**:** 522-530

Schutte BC, McCray PB, Jr. (2002) ß-Defensins in lung host defense. *Annu Rev Physiol* **64**(1)**:** 709-748

Schutte BC, Mitros JP, Bartlett JA, Walters JD, Jia HP, Welsh MJ, Casavant TL, McCray PB, Jr. (2002) Discovery of five conserved beta -defensin gene clusters using a computational search strategy. *Proc Natl Acad Sci U S A* **99**(4)**:** 2129-2133

Schutze H, Enzmann P, Kuchling R, Mundt E, Niemann H, Mettenleiter T (1995) Complete genomic sequence of the fish rhabdovirus infectious haematopoietic necrosis virus. *J Gen Virol* **76**(10)**:** 2519-2527

Schutze H, Enzmann PJ, Mundt E, Mettenleiter TC (1996) Identification of the non-virion (NV) protein of fish rhabdoviruses viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *J Gen Virol* **77 (Pt 6):** 1259-1263

Schutze H, Mundt E, Mettenleiter TC (1999) Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes* **19**(1)**:** 59-65

Secombes CJ, Wang T, Hong S, Peddie S, Crampe M, Laing KJ, Cunningham C, Zou J (2001) Cytokines and innate immunity of fish. *Developmental & Comparative Immunology* **25**(8-9)**:** 713-723

Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. *Nat Immunol* **6**(6)**:** 551-557

Shum BP, Azumi K, Zhang S, Kehrer SR, Raison RL, Detrich HW, Parham P (1996) Unexpected beta2-microglobulin sequence diversity in individual rainbow trout. *Proc Natl Acad Sci U S A* **93**(7)**:** 2779-2784

Sinha S, Cheshenko N, Lehrer RI, Herold BC (2003) NP-1, a Rabbit {alpha}-Defensin, Prevents the Entry and Intercellular Spread of Herpes Simplex Virus Type 2. *Antimicrob Agents Chemother* **47**(2)**:** 494-500

Skall HF, Olesen NJ, Mellergaard S (2005a) Prevalence of viral haemorrhagic septicaemia virus in Danish marine fishes and its occurrence in new host species. *Dis Aquat Organ* **66**(2)**:** 145-151

Skall HF, Olesen NJ, Mellergaard S (2005b) Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming--a review. *J Fish Dis* **28**(9)**:** 509-529

Snow M, Smail DA (1999) Experimental susceptibility of turbot Scophthalmus maximus to viral haemorrhagic septicaemia virus isolated from cultivated turbot. *Dis Aquat Organ* **38**(3)**:** 163-168

Stuge TB, Wilson, M. R., Zhou, H., Barker, K. S., Bengten, E., Chinchar, G., Miller, N. W., and Clem, L. W (2000) Development and analysis of various clonal alloantigen-dependent cytotoxic cell lines from channel catfish. *Journal of immunology* **164:** 2971-2977

Svinarich DM, Wolf NA, Gomez R, Gonik B, Romero R (1997) Detection of human defensin 5 in reproductive tissues. *Am J Obstet Gynecol* **176**(2)**:** 470-475

Tafalla C, Chico V, Perez L, Coll JM, Estepa A (2007) In vitro and in vivo differential expression of rainbow trout (Oncorhynchus mykiss) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV. *Fish Shellfish Immunol* **23**(1)**:** 210-221

Tang YQ, Yuan J, Osapay G, Osapay K, Tran D, Miller CJ, Ouellette AJ, Selsted ME (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins [see comments]. *Science* **286:** 498-502

Thiery R, de Boisseson C, Jeffroy J, Castric J, de Kinkelin P, Benmansour A (2002) Phylogenetic analysis of viral haemorrhagic septicaemia virus (VHSV) isolates from France (1971-1999). *Dis Aquat Organ* **52**(1)**:** 29-37

Thomma BP, Cammue BP, Thevissen K (2002) Plant defensins. *Planta* **216**(2)**:** 193-202

Thoulouze MI, Bouguyon E, Carpentier C, Bremont M (2004) Essential role of the NV protein of Novirhabdovirus for pathogenicity in rainbow trout. *J Virol* **78**(8)**:** 4098-4107

Tort L, Balasch, J. C: and MacKenzie, S (2004) Fish health challenge after stress. Indicators of immunocompetence. *CONTRIBUTIONS to SCIENCE,* **2(4):** 443-454

Utke K, Bergmann S, Lorenzen N, Kollner B, Ototake M, Fischer U (2007a) Cell-mediated cytotoxicity in rainbow trout, Oncorhynchus mykiss, infected with viral haemorrhagic septicaemia virus. *Fish & Shellfish Immunology* **22**(3)**:** 182-196

Utke K, Kock H, Schuetze H, Bergmann SM, Lorenzen N, Einer-Jensen K, Kollner B, Dalmo RA, Vesely T, Ototake M, Fischer U (2007b) Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. *Dev Comp Immunol*

Valore EV, Ganz T (1992) Posttranslational processing of defensins in immature human myeloid cells. *Blood* **79**(6)**:** 1538-1544

Valore EV, Park CH, Quayle AJ, Wiles KR, McCray PB, Jr., Ganz T (1998) Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J Clin Invest* **101**(8)**:** 1633-1642

Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS (1997) Effect of defensins on interleukin-8 synthesis in airway epithelial cells. *Am J Physiol* **272**(5 Pt 1)**:** L888-896

Vestergaard-Jorgensen PE (1972) Egtved virus: antigenic variation in 76 virus isolates examined in neutralization test and by means of the fluorescent antibody technique. *Symposiums Zoological Society* **30:** 333-339

Wagner RR (1975) Reproduction of Rhabdoviruses. In *Plenum Press.*, pp 1-93. New york: Comprensive.Virology

Wagner RR (1987) The rhabdoviruses. *Plenum Press NY***:** 543

Walker PJ, Kongsuwan K (1999) Deduced structural model for animal rhabdovirus glycoproteins. *J Gen Virol* **80**(Pt 5)**:** 1211-1220.

Wang W, Owen SM, Rudolph DL, Cole AM, Hong T, Waring AJ, Lal RB, Lehrer RI (2004) Activity of alpha- and theta-defensins against primary isolates of HIV-1. *J Immunol* **173**(1)**:** 515-520

Wilde CG, Griffith JE, Marra MN, Snable JL, Scott RW (1989) Purification and characterization of human neutrophil peptide 4, a novel member of the defensin family. *J Biol Chem* **264**(19)**:** 11200-11203

Wilhelm SWaS, C.A. (1999) Viruses and nutrient cycles in the sea. *Bioscience* **49:** 781-788
Wolf F, Quimby MC (1962) Established eurythermic line of fish cells in vitro. *Science* **135:** 1065-1066

Wolf K (1988) In: Fish virus y fish viral diseases. *Cornell University press, Ithaca and London pp 476*

Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* **22:** 181-215

Yang D, Biragyn A, Kwak LW, Oppenheim JJ (2002) Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* **23**(6)**:** 291-296

Yang D, Chen Q, Chertov O, Oppenheim JJ (2000) Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *J Leukoc Biol* **68**(1)**:** 9-14

Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, Oppenheim JJ (2003) Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J Leukoc Biol* **74**(3)**:** 448-455

Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schroder JM, Wang JM, Howard OM, Oppenheim JJ (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**(5439)**:** 525-528

Yasin B, Wang W, Pang M, Cheshenko N, Hong T, Waring AJ, Herold BC, Wagar EA, Lehrer RI (2004) Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J Virol* **78**(10)**:** 5147-5156

Yoshiura Y, Kiryu I, Fujiwara A, Suetake H, Suzuki Y, Nakanishi T, Ototake M (2003) Identification and characterization of Fugu orthologues of mammalian interleukin-12 subunits. *Immunogenetics* **55**(5)**:** 296-306

Yount NY, Bayer AS, Xiong YQ, Yeaman MR (2006) Advances in antimicrobial peptide immunobiology. *Biopolymers* **84**(5)**:** 435-458

Zaharatos GJ, He T, Lopez P, Yu W, Yu J, Zhang L (2004) alpha-defensins released into stimulated CD8+ T-cell supernatants are likely derived from residual granulocytes within the irradiated allogeneic peripheral blood mononuclear cells used as feeders. *J Acquir Immune Defic Syndr* **36**(5)**:** 993-1005

Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* **75**(1)**:** 39-48

Zhang YA, Zou J, Chang CI, Secombes CJ (2004a) Discovery and characterization of two types of liver-expressed antimicrobial peptide 2 (LEAP-2) genes in rainbow trout. *Vet Immunol Immunopathol* **101**(3-4)**:** 259-269

Zhang YB, Li Q, Gui JF (2004b) Differential expression of two Carassius auratus Mx genes in cultured CAB cells induced by grass carp hemorrhage virus and interferon. *Immunogenetics* **56**(1)**:** 68-75

Zou J, Mercier C, Koussounadis A, Secombes C (2006) Discovery of multiple beta-defensin like homologues in teleost fish. *Mol Immunol*

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Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): Inactivation of virus particles and induction of a type I interferon-related response

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Abstract

It is well known that human alpha-defensin-1, also designated as human neutrophil peptide 1 (HNP1), is a potent inhibitor towards several enveloped virus infecting mammals. In this report, we analyzed the mechanism of the antiviral action of this antimicrobial peptide (AMP) on viral haemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus. Against VHSV, synthetic HNP1 possesses two antiviral activities. The inactivation of VHSV particles probably through interfering with VHSV-G protein-dependent fusion and the inhibition of VHSV replication in target cells by up-regulating genes related to the type I interferon (IFN) response, such as Mx. Neither induction of IFN-stimulated genes (ISGs) by HNP1 nor their antiviral activity against fish rhabovirus has been previously reported. Therefore, we can conclude that besides to acting as direct effector, HNP1 acts across species and can elicit one of the strongest antiviral responses mediated by innate immune system. Since the application of vaccine-based immunization strategies is very limited, the used of chemicals is restricted because of their potential harmful impact on the environment and no antimicrobial peptides from fish that exhibit both antiviral and immunoenhancing capabilities have been described so far, HNP1 could be a model molecule for the development of antiviral agents for fish. In addition, these results further confirm that molecules that mediate the innate resistance of animals to virus may prove useful as templates for new antivirals in both human and animal health.

Keywords: HNP1; VHSV; IFN; Mx; AMP; Rhabdovirus; Alpha-defensin

1. Introduction

An increasing number of antiviral agents are presently in various stages of development and testing, and an increasing number have recently been licensed for use in humans and animals. However, most of the available antiviral drugs often lead to the development of viral resistance coupled with the problem of side effects, recurrence and viral latency. In this regard, antiviral drug development focusing on the regulation of innate defense system is an attractive approach.

The innate response is the first line of defense against infectious agents. Upon a first encounter with a virus, the innate immunity is triggered immediately in the organism. This early immune response is characterized by the production of different cytokines, as well as other immune intermediators and antiviral factors, such as antimicrobial peptides (AMPs) that control viral replication and provide time for the generation of a more-effective host adaptive immunity response. Defensins (Ganz et al., 1985; Selsted et al., 1985), a family of cysteine-rich cationic antimicrobial peptides, are probably the AMPs that show the broadest range of antiviral activity being active against both enveloped and non-enveloped virus (reviewed in reference Klotman and Chang, 2006). In addition to their direct antiviral effects, defensins also modulate the host immune response and provide a link between the innate (early) and the adaptive (late) mammal immune responses (Lillard et al., 1999; Selsted and Ouellette, 2005; Tani et al., 2000; Yang et al., 2002). This dual role of defensins as direct effectors and inducers of immune responses qualify defensins as potential antiviral drugs.

Defensins are polypeptides of fewer than 100 amino acids (Ganz, 2003) with β-pleated sheet structures stabilized by intramolecular disulphide bonds (Ganz, 2003; Lehrer and Ganz,

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2002). They are classified into α-, β- and θ-defensins, which differ in the distribution of the disulphide bonds between their six conserved cysteine residues (reviewed in references Klotman and Chang, 2006; Selsted and Ouellette, 2005; Yang et al., 2002). While disulphide bonds are not required for some of their functions such as the antibacterial activities of human α-defensin 1 (HNP1), human β-defensin-3 (HBD3) or the mouse Paneth-cell-derived α-defensin, cryptdin-4 (Klotman and Chang, 2006; Maemoto et al., 2004; Mandal and Nagaraj, 2002; Wu et al., 2003), they might be required for some others such as binding to HSV-1 and HIV virions (Daher et al., 1986; W. Wang et al., 2004).

Defensins and/or defensin-like peptides have been found in mammals (Ganz, 1999, 2003; Ganz et al., 1985; Klotman and Chang, 2006; Selsted and Ouellette, 2005), insects (Bulet and Stocklin, 2005; Lehrer and Ganz, 2002; Otvos, 2000), plants (Castro and Fontes, 2005; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005; Lehrer and Ganz, 2002; Selsted and Ouellette, 2005) and birds (Martin et al., 1995; Sugiarto and Yu, 2004). Unexpectedly, no defensin-like peptides have been isolated from fish so far but genomic sequences show that β-defensins are most likely expressed. Thus, homologue sequences to β-defensins from higher vertebrates and one to human β-defensin-26 have been recently identified in non-salmonid fish (Zou et al., 2007) and rainbow trout (Falco et al., unpublished results), respectively, by bioinformatic analysis of EST and genome databases, demonstrating that the β-defensin family of AMPs is present in the fish genomes. Several other AMPs families have been reported in fish, including misgurin, pleurocidin, paradaxins, parasin I, hepcidin, piscidins/moronecidin, oncorhyncins, LEAP-2, perforin and cathelicidin (Cole et al., 1997; Fernandes et al., 2004; Lauth et al., 2002; Noga and Silphaduang, 2003; Oren and Shai, 1996; Park et al., 1997, 1998; Shike et al., 2002; Zhang et al., 2004; Hwang et al., 2004; C.I. Chang et al., 2005). To date, antiviral activity against fish virus has only been studied for piscidins (Chinchar et al., 2004) and the activity of non-piscine AMPs against fish viruses has been only reported for cecropins (Chiou et al., 2002). In addition, no AMPs from fish with both antiviral and immunoenhancing capabilities have been described.

Because of the economic and social impact of viral infections in aquaculture and since no efficient therapeutic agents against fish viral infections have yet been developed, antiviral agents, such as defensins, could be of great interest. Therefore, we have evaluated the ability of synthetic HNP1 to inhibit the infectivity of viral haemorrhagic septicaemia rhabdovirus (VHSV), one of the most devastating viruses for worldwide aquaculture (Lorenzen and LaPatra, 2005; Olesen and Korsholm, 1997). HNP1 was chosen because it has been shown to be effective against both non-enveloped (Buck et al., 2006) and enveloped viruses (reviewed in reference Klotman and Chang, 2006) including the rhabdovirus causing vesicular stomatitis in mammals (Daher et al., 1986), and the mechanism underlying the antiviral activity of HNPs against certain enveloped virus is partially known (Chang et al., 2003; T.L. Chang et al., 2005; Mackewicz et al., 2003; Sinha et al., 2003).

The results obtained in this work showed that HNP1 causes a dose-dependent inhibition of VHSV infectivity *in vitro* in the absence of cellular toxicity. Preliminary characterization of its mechanism of inhibition indicated that HNP1 exhibited anti-VHSV activity at least on two levels: directly by inactivating virus particles and indirectly by inducing cellular antiviral responses in the host fish cells. In addition, HNP1 showed immunomodulatory activity since *ex vivo* treatment of trout head kidney leucocytes with HNP1 increased the transcriptional expression level of IFN-stimulated genes (ISGs) and other immune related transcripts. Work is in progress to evaluate the possible *in vivo* activity of HNP1 by using protein- or gene-transfer based methodologies to explore the potential use of these AMPs for the development of novel fish therapeutic agents and/or vaccines.

2. Materials and methods

2.1. Fish cell lines and viral haemorrhagic septicaemia virus (VHSV)

The fish cell lines epithelioma papulosum cyprini (EPC) (Fijan et al., 1983), purchased from the European collection of cell cultures (ECACC No. 93120820), and RTG-2 (rainbow trout gonad) (Wolf and Quimby, 1962), purchased from the American Type Culture Collection (ATCC CCL 55), were used.

EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen Corporation, UK) cell culture medium containing 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO, USA), 1 mM pyruvate (Gibco, Invitrogen Corporation, UK), 2 mM L-glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone. Likewise, RTG-2 were maintained at 20 °C in a 5% CO_2 atmosphere with MEM (with Earle's salts) cell culture medium (Gibco) containing 10% fetal calf serum (Sigma), 2 mM glutamine (Gibco) and 50 μg/ml neomycin sulphonate (Sigma).

Viral haemorrhagic septicaemia virus (VHSV 07.71) isolated in France from rainbow trout, *Oncorhynchus mykiss* (LeBerre et al., 1977) was propagated in EPC cells at 14 °C as previously reported (Basurco and Coll, 1989). Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* for 20 min and stored in aliquots at −70 °C. Viruses from clarified supernatants were concentrated to 10^{11} foci forming units (f.f.u.) per ml by ultracentrifugation at 100,000 × *g* for 45 min (Basurco and Coll, 1989).

2.2. Synthetic HNP1

Synthetic human alpha-defensin-1 (ACYCRIPACIAGERRYGTCIYQGRLWAFCC-NH_2) was purchased from Peptides International (Louisville, KY, USA). The purity of peptide was >98%. HNP1 was reconstituted to a final concentration of 1 μg/μl in sterile distilled water and stored until used in suitable aliquots at −20 °C. Several batches have to be tested for optimal activity, most probably due to differences in their disulphide bound patterns (native, fully active HNP1 has disulphide bonds between cysteins 2–30, 4–19 and 9–29).

2.3. Viral infectivity assays

To assay for VHSV infectivity, a previously developed immunostaining focus assay (focus forming units, f.f.u.) was used (Lorenzo et al., 1996; Mas et al., 2002, 2006; Perez et al., 2002; Micol et al., 2005). To test the influence of pre-incubation of VHSV with HNP1, different concentrations of HNP1 (up to 20 μg/ml) was incubated with 10^3 ffu from replication-competent stocks of concentrated VHSV (10^{10} ffu/ml) for 12 h at 14 °C in 25 μl serum-free cell culture medium supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin. After incubation, VHSV–HNP1 mixtures were added to the RTG-2 or EPC cell monolayers, grown in 96-well plates, in a final volume of 100 μl per well. The infected cell monolayers were then either not washed, or washed when indicated, and further incubated for 24 h at 14 °C. Alternatively, EPC and/or RTG-2 cell monolayers were either infected with VHSV (m.o.i. of 10^{-3}) in the presence of different concentrations of HNP1 or pre-incubated with HNP1 for 12 h at 28 and 20 °C, respectively, washed extensively and then infected with VHSV (m.o.i. of 10^{-3}). In both cases, infected cells were incubated for 24 h at 14 °C. The cell monolayers were then fixed for 10 min in cold methanol and air-dried. Monoclonal antibody (MAb) 2C9 directed towards the N protein of VHSV diluted 1000-fold in dilution buffer (0.24 mM merthiolate, 5 g/l Tween 20, 50/l mg of phenol red in PBS, pH 6.8) were added to the wells (100 μl/well) and incubated for 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled rabbit anti-IgG mouse antibody (Ab) (Nordic, Tilburg, The Netherlands) were added per well, and incubation was continued for 30 min. After three washes by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma) in PBS containing H_2O_2 were added (Lorenzo et al., 1996; Sanz and Coll, 1992) and the reaction allowed to proceed until brown foci were detected with an inverted microscope (Nikon Eclipse TE2000-U, Nikon instruments Inc., NY, USA). Once washed with water and air dried, brown foci of DAB stained cells (VHSV-infected cell foci) were counted with an inverted microscope with a 10× ocular eye grid (Lorenzo et al., 1996). The results were expressed as the percentage of infectivity and calculated by the formula: (number of VHSV-infected cell foci in the presence of HNP1/total number of VHSV-infected cell foci in the absence of HNP1) × 100.

The mRNA corresponding to the N and G proteins of VHSV in EPC cells either infected with VHSV (m.o.i. of 10^{-3}) in the presence of 20 μg/ml of HNP1 or pre-incubated with 20 μg/ml of HNP1 for 12 h at 28 °C, washed and then infected with VHSV (m.o.i. of 10^{-3}) were also quantified 9 h post-infection by quantitative RT-PCR using specific primers for the VHSV-N and G genes (Chico et al., 2006).

2.4. Determination of VHSV binding to solid-phase HNP1 by enzyme-linked immunosorbent assay (ELISA)

DTT-treated HNP1 was obtained by incubating HNP1 (20 μg/ml) with 50 mM DTT for 30 min at 37 °C. Polystyrene plates (Dynatech, Plochingen, Germany) were coated with 0, 0.5, 1 or 2 μg of untreated HNP1 or with 2 μg of dithiothreitol (DTT)-treated HNP1 per well in 100 μl of distilled water, incubated overnight at 37 °C to dryness, and kept sealed with blue silica gel at 4 °C until used. After blocking with 3% dry milk in dilution buffer (0.24 mM merthiolate, 0.1% Tween 20, 0.005% phenol red in PBS) for 1 h at room temperature and washing, the plates were incubated for 3 h at 14 °C with concentrated VHSV (10^7 ffu/well) in 100 μl of distilled water or 100 μl/well of PBS. The unbound viral particles were then removed by washing the plates four times by immersion in distilled water. Polyclonal antibody (PoAb) anti-HNP1 (Alpha Diagnostic, San Antonio, USA) and MAb I10 (Fernandez-Alonso et al., 1998) reactive against VHSV-G protein diluted 500- and 200-fold, respectively, in dilution buffer (100 μl/well) was added to the wells and incubated for 90 min at room temperature. After washing with distilled water, 100 μl/well of a peroxidase-labeled goat anti-mouse IgG Ab (Sigma) diluted 300-fold in dilution buffer were added per well and the incubation was continued for 45 min. For color development, the plates were washed three times with distilled water and 100 μl of substrate buffer (150 mM sodium citrate, 3 mM H_2O_2 and 1 mg/l *o*-phenylenediamine, pH 4.8) were pipetted per well. The reaction was stopped after 30 min with 100 μl per well of 4N H_2SO_4. Absorbance readings at 492 nm to estimate enzymatic activity and 620 nm for estimation of each individual well background readings were measured using an ELISA plate reader (Anthos, LabTec Instruments).

2.5. Generation of a permanently transformed EPC cell line expressing green fluorescent protein (EPC-eGFP)

An EPC-eGFP cell line was obtained as previously described (Brocal et al., 2006) with minor modifications. Briefly, EPC cell monolayers in six-well plates were co-transfected with 1.5 μg of pMCV1.4-eGFP plus 0.5 μg of pAE6-*pac* (puromycin resistance gene) plasmid constructions (Brocal et al., 2006). The pMCV 1.4-eGFP plasmid was obtained by subcloning the eGFP cDNA sequence from the pGFP plasmid (Clontech, CA, USA) into pMCV 1.4 vector (Ready-Vector, Madrid, Spain) (Rocha et al., 2004) following standard procedures. After transfection, puromycin resistant cells were selected by adding 20 μg/ml of puromycin (Sigma) to the cell culture media at 6 days. Resulting puromycin-resistant cells were seeded in 96-well plates at a density from 1 to 50 cells/well (limiting dilution) and grown in cell culture medium conditioned by the growth of non-transfected EPC cells to favor growth of isolated cells. Twenty-four hours later, the wells were screened for the presence of single cells. Two weeks later, single colonies were transferred to wells of 48-well plates and grown in conditioned medium. Cell lines expressing eGFP were selected among the puromycin-resistant clones using an inverted fluorescence microscope (Nikon). Three EPC-eGFP cell lines were obtained and one of them selected for further work. The selected EPC-eGFP cell line was grown in 96-well plates and gradually transferred into cell culture flasks. The EPC-eGFP cell line has been maintained by continuous culture (about one subculture per week) in the absence of puromycin as described above for non-transformed EPC cell monolayers during more than 3 years.

2.6. Viral binding assays

To determine if HNP1 inhibited the binding of VHSV to cells, EPC-eGFP cell monolayers, grown on six-well plates, were incubated with VHSV (m.o.i. 0.3) in the presence or absence of 20 μg/ml of HNP1 or with VHSV pre-treated with HNP1 (12 h at 14 °C in serum-free cell culture medium) for 2 h at 4 °C. Cells were washed three times with PBS to remove unbound virus, and cell-bound virus was then detected by probing Western blots of cell lysates with the anti-VHSV N protein MAb 2C9 and an anti-GFP MAb (Santa Cruz Biotechnology, CA, USA) to control for protein loading. Briefly, infected cells were frozen and thawed and supernatants from cell lysates clarified by centrifugation (1000 × *g* for 10 min). SDS-polyacrylamide gels at 12% were loaded with 20 μl of samples in buffer containing β-mercaptoethanol. The proteins in the gel were transferred during 3 h at 125 mM in 2.5 mM Tris, 9 mM glycine, 20% methanol to nitro-cellulose membranes (BioRad, Richmond, VI, USA). The membranes were blocked with 2% dry milk, 0.05% Tween-20 and 0.3% rabbit serum in PBS and incubated for 2 h at room temperature with the above mentioned antibodies. Blots were then incubated with peroxidase-conjugated rabbit anti-IgG mouse antibody (SIGMA) diluted 1/500 in 2% milk-containing PBS for 45 min. Finally, the peroxidase activity was detected using the ECL chemiluminescence reagents (Amersham Biosciences, UK) and revealed by exposure to X-ray films (Amersham).

2.7. VHSV G protein-mediated syncytia formation in VHSV-infected cells in the presence of HNP1

EPC-eGFP cell monolayers, grown on 96-well plates, were infected with VHSV at a m.o.i. of 10^{-3} to 10^{-2}. Twenty-four hours post-infection, cells were washed and treated with HNP1 (20 μg/ml) in serum-free medium for 45 min at 14 °C or untreated. After cell washing, fusion was triggered by incubating cells with fusion medium (Mas et al., 2002) at pH 6 for 30 min at 14 °C. Monolayers were then washed and incubated with fusion medium at pH 7.5 for 2 h at room temperature. The cell monolayers were fixed with 4% paraformaldehyde in PBS (15 min at room temperature) and then incubated with the MAb anti-VHSV G protein I10 diluted 200-fold in PBS for 2 h at room temperature. The indirect staining was carried out by using rabbit anti-mouse antibody conjugate to rhodamine (TRITC, Sigma). To visualize nuclei, cells were incubated with 0.1 mg/ml of the DNA stain Hoechst (Sigma) for 10 min. Cells were viewed and photographed with an inverted fluorescence microscope (Nikon) provided with a digital camera (Nikon DS-1QM). To analyze the fusion in RTG-2 cells, cells were fixed with cold methanol, dried and stained with Giemsa (5 mg/ml in PBS). To measure the extent of fusion, the number of nuclei in syncytia of three or more nuclei per syncytia was counted among 10,000 nuclei per well as previously described (Estepa and Coll, 1997; Estepa et al., 2001).

2.8. Treatment of RTG-2 and head kidney leucocytes with HNP1

Confluent monolayers of RTG-2 cells in 24-well tissue culture plates were treated with PBS, 20 μg/ml of HNP1, 20 μg/ml of synthetic Pleurocidin (Ple) from winter flounder (Cole et al., 1997; Brocal et al., 2006) or 30 μg/ml of Polyribocytidylic acid (poly I:C) (Pharmacia, Piscataway, NJ, USA). At 24 h post-induction, cells in control and induced wells were harvested. In all cases, after incubation period, the medium was removed, cells desattached with Ca^{2+} and Mg^{2+}-free PBS and total RNA extracted.

Head kidney leukocytes were isolated following the method previously described (Graham et al., 1988). Briefly, fish were sacrificed by overexposure to MS-222 and the anterior kidney removed aseptically and passed through a 100 μm nylon mesh using RPMI-1640 Dutch modified culture medium supplemented with 10% fetal calf serum (Sigma), 1 mM pyruvate (Gibco), 2 mM glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone. The viable cell concentration was determined by Trypan blue exclusion. Cells were resuspended in RPMI-1640 with 10% FCS and 2% trout serum, dispensed into 24-well plates at a concentration of 1×10^6 cells/ml, and then incubated with PBS, 20 μg/ml of HNP1 or 30 μg/ml of poly I:C. After 24 h, total RNA was extracted from the cells as described below.

2.9. RNA isolation and cDNA synthesis

The "Total RNA Isolation System" (Promega) was used for cellular RNA extraction following manufacturer's instructions. Isolated RNAs were treated with DNase (RQ1 RNAase-Free Dnase, Promega), resuspended diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C until used. Two micrograms of RNA were used to obtain cDNA using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen). Briefly, RNA was incubated with 1 μl of random hexamers (50 μM) (Roche) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of M-MLV reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min and the resulting cDNA stored at −20 °C.

2.10. Detection of Mx1, Mx2, Mx3, Vig-1, TLR3, IL1β and iNOS cDNA by PCR

All amplification reactions contained 200 μM of each deoxynucleotide triphosphate, 1 unit of Taq polymerase (Roche), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 μM of each primer and 1 μl of cDNA in a final volume of 25 μl. PCR amplifications with primers for glyceraldehyde 3-phosphate dehydrogenase (GADPH) were performed with all samples as internal reference amplification for RT-PCR, since GADPH is constitutively expressed in all organs. Primers used

Table 1
Genes, sequence of primers and size of amplicons used in this study

Gene	Name	Sequence 5′–3′	Size (bp)	Reference
Mx1 protein	Mx1	**F**: ATGCCACCCTACAGGAGATGAT **R**: TAACTTCTATTACATTTACTATGCAA	421	Tafalla et al. (2007)
Mx2 protein	Mx2	**F**: ATGCCACCCTACAGGAGATGAT **R**: GGAAGCATAGTAACTTTATTATAAC	400	Tafalla et al. (2007)
Mx3 protein	Mx3	**F**: ATGCCACCCTACAGGAGATGAT **R**: CCACAGTGTACATTTAGTTG	381	McLauchlan et al. (2003)
VHSV-induced gene 1	Vig-1	**F**: CAGTTCAGTGGCTTTGACGA **R**: ACAAACGCCTCAAGGTATGG	232	Boudinot et al. (1999)
Toll-like receptor 3	TLR3	**F**: TGACAGAGCTTAACCTGGCT **R**: AAGAACTTCCAGCATGGACA	538	Rodriguez et al. (2005)
Interleukin 1 beta	IL1β	**F**: AGGGAGGCAGCAGCTACCACAA **R**: GGGGGCTGCCTTCTGACACAT	353	Wang et al. (2002)
Inducible nitric oxide synthase	iNOS	**F**: CATACGCCCCCAACAAACCAGTGC **R**: CCTCGCCTTCTCATCTCCAGTGTC	746	Lindenstrom et al. (2004)
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	**F**: ATGTCAGACCTCTGTGTTGG **R**: TCCTCGATGCCGAAGTTGTCG	514	T. Wang et al. (2004)

F, Forward primer; R, reverse primer.

for mRNA amplification and the sizes of the different PCR products are shown in Table 1. All PCRs were carried out in a Perkin-Elmer 2400 cycler and all samples were amplified twice to verify the results. Amplification conditions consisted of a denaturing step (5 min at 94 °C) followed by different specific cycling conditions and a final extension of 7 min at 72 °C. These conditions were established for each gene following protocols described previously by the references shown in Table 1. The PCR products in 8 μl were visualised on a 1.6% agarose gel stained with ethidium bromide. Samples that were to be compared were run in the same agarose gel. A 100 bp ladder was used as a size marker. The optical density (OD) of the amplification bands was estimated using the Scion image software. The mRNA expression for each gene was determined relative to the expression of the GADPH gene in the same sample using the formula: OD of mRNA band/OD of corresponding GADPH band.

2.11. Detection of the mRNA of the N protein of VHSV by quantitative RT-PCR

Primers and the FAM-labeled (TaqMan®) probe for the N gene of VHSV were described in a previous report (Chico et al., 2006). Quantitative PCR assays were performed using an ABI PRISM® 7300 Sequence Detector System. Reactions were carried out in a final volume of 25 μl, containing 300 nM of each primer, 100 nM of the probe, 2 μl of cDNA and 1× Absolute Q-PCR ROX Mix (ABGene). The polymerase chain reaction conditions consisted of one cycle of 2 min at 50 °C and 15 min at 95 °C followed by 40 cycles of 15 s

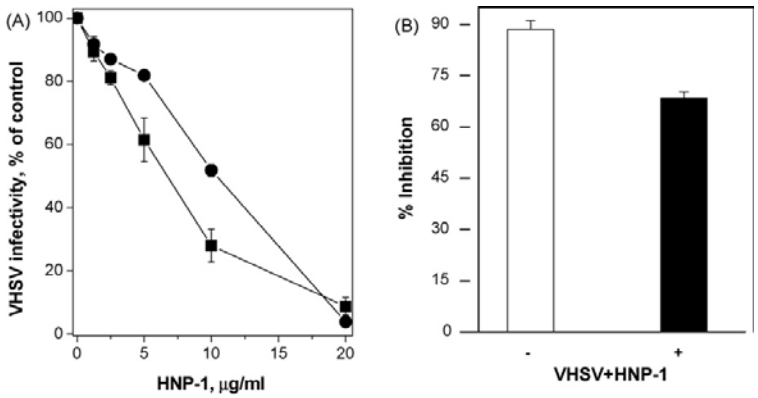

Fig. 1. Pre-incubation of VHSV with HNP1 in the absence of serum inhibits VHSV infectivity. 10^3 ffu of VHSV were mixed with increasing concentrations of HNP1 in 25 μl of serum-free cell culture medium and incubated at 14 °C for 12 h. The VHSV–HNP1 mixtures were then diluted five-fold to a final volume of 100 μl by adding 75 μl of cell culture medium containing 2% FCS. Then the mixtures were added to EPC (■) or RTG-2 (●) cell monolayers, grown in 96-well plates. Two hours later, the infected cell monolayers were either not washed (A and inset B−, white bar) or washed (inset B+, black bar) and further incubated at 14 °C during 24 h. The VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. Data are mean ± standard deviations (S.D.) from three different experiments, each experiment performed in triplicate.

at 90 °C and 1 min at 60 °C. Endogenous control included for quantitation was the 18S ribosomal RNA gene as determined with the TaqMan® Ribosomal RNA Control Reagents Kit (Applied Biosystems) following the manufacturer's guidelines.

3. Results

3.1. Pre-incubation of VHSV with HNP1 in the absence of serum inhibits VHSV infectivity

The ability of HNP1 to inactivate VHSV was studied by preincubating increasing concentrations of HNP1 with 10^3 ffu of cell-free VHSV in 25 μl of serum-free cell culture medium. The VHSV–HNP1 mixtures were then diluted with 75 μl of cell culture medium containing 2% of FCS (five-fold dilution) and added to and incubated with the fish cell monolayers until analysis of VHSV infectivity (24 h post-infection). Serum-free medium was used for those incubations because the inhibitory effect of HNP1 on other virions has been reported to be reduced by serum proteins (T.L. Chang et al., 2005; Daher et al., 1986; Mackewicz et al., 2003). Fig. 1A shows that HNP1 inhibited VHSV-infected cell foci formation in a dose-dependent manner in both EPC and RTG-2 cell lines, two fish cell lines of different origin, indicating that VHSV inhibition by HNP1 was independent on the cell line. Maxima inhibition (>90% of the ffu in both cell lines) was observed when 20 μg/ml of HNP1 were preincubated with VHSV.

On the other hand, no cytotoxicity was observed when the EPC cell monolayers were treated with HNP1 at different concentrations up to 20 μg/ml (data not shown), indicating that the effects of HNP1 on VHSV infectivity were not due to non-specific cytotoxicity.

3.2. Infection of EPC cell monolayers with VHSV in the presence of serum and HNP1 also inhibits VHSV infectivity without altering the VHSV binding to EPC cell monolayers

The previously commented inhibitory assays were performed by pre-incubating VHSV with HNP1, adding the VHSV–HNP1 mixture to the EPC cell monolayers and maintaining their presence throughout all the time of incubation until analysis. However, when the excess of HNP1 and unbound VHSV were removed from the cell culture medium 2 h post-infection and then incubation proceeded with fresh medium devoid of HNP1, the antiviral effect of HNP1 was ∼30% reduced (Fig. 1B). To investigate any potential antiviral effects caused by the presence of HNP1 during the infection time, EPC cells were infected in the presence of HNP1 with non-treated VHSV in cell cultured medium containing 2% of serum. The results showed that HNP1 when added from the beginning of the infection ($t = 0$) also inhibited VHSV infectivity in EPC cells (Fig. 2A) with a very similar inhibitory profile to that shown when VHSV and HNP1 were pre-incubated in the absence of serum before infection (Fig. 1A). Thus, VHSV infectivity was reduced to 86, 80, 57 and 19% by the presence of 1.25, 5, 10 and 20 μg/ml of HNP1, respectively (Fig. 2A). This result suggested that in the presence of serum some HNP1 antiviral mechanism other than that exerted directly on VHSV particles could be operating.

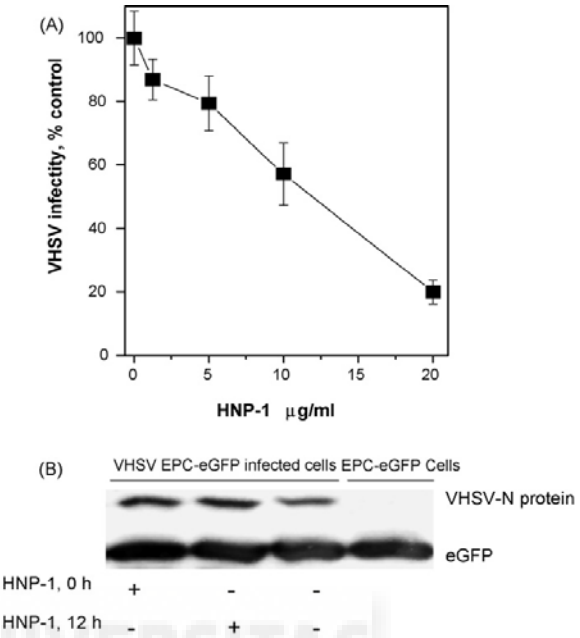

Fig. 2. Inhibition of VHSV infectivity by HNP1 (A) and effect of HNP1 on binding of VHSV to EPC cell monolayers (B). (A) EPC cell monolayers grown in 96-well plates, were infected with VHSV in the presence of different concentrations of HNP1 in cell culture media containing 2% of FCS. Twenty-four hours later, VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. Data are mean ± S.D. from three different experiments, each performed in triplicate. (B) EPC-eGFP cell monolayers (permanently expressing eGFP), grown in six-well plates, were incubated in 2% FCS containing cell culture medium during 2 h at 4 °C with VHSV in the absence of HNP1 (HNP1, 0 h, −), in the presence of 20 μg/ml of HNP1 (HNP1, 0 h, +) or with pre-incubated VHSV + HNP1 as in Fig. 1 (HNP1, 12 h, +). After washing unbound virus, cell lysates were prepared and separated by SDS-PAGE, and cell-bound virus was visualized by Western blotting with anti-N 2C9 or anti-eGFP MAbs. The inmunostained gel is representative of three independent experiments.

The inhibition of VHSV infectivity by the presence of HNP1 at the infection time could not be explained by changes in the binding of VHSV to the EPC cell monolayers because incubation of VHSV with EPC-eGFP cells at 4 °C with or without 20 μg/ml of HNP1 resulted in similar amounts of EPC cell-associated VHSV N protein (VHSV binding assay) (Fig. 2B). Similar results were found for VHSV pre-incubated with 20 μg/ml of HNP1 (Fig. 2B).

3.3. Pre-incubation of EPC cell monolayers with HNP1 in the presence of serum prior infection also inhibits VHSV infection

Cell-mediated antiviral effects induced by pre-incubation of cells with HNP1 before or during viral infection have been previously reported (Klotman and Chang, 2006). To investigate

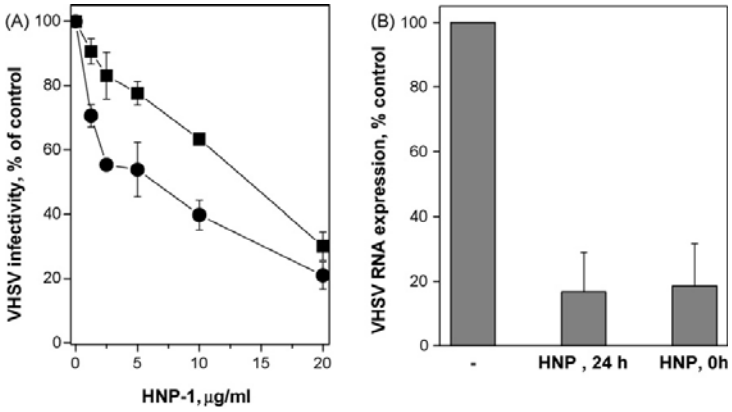

Fig. 3. Inhibition of VHSV infectivity (A) and VHSV RNA synthesis (B) by pre-incubation of cell monolayers with HNP1. EPC (■) and RTG-2 (●) cell monolayers were pre-incubated with HNP1 at the indicated concentrations in cell culture medium containing 10% FCS for 24 h. Cell monolayers were then washed, infected with VHSV in cell culture media containing 2% of FCS and incubated during 24 h at 14 °C. VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. Data are mean ± S.D. from three different experiments, each performed in triplicate. (B) EPC cell monolayers were incubated with 20 μg/ml HNP1 in cell culture media containing FCS during 24 h (24 h) before infection with VHSV or only during the time of VHSV addition (0 h). Viral RNA levels were determined at 9 h post-infection by TaqMan® quantitative RT-PCR. The average value of the non-treated VHSV-infected samples was 100%. The values are given as mean ± S.D. of duplicates.

whether cellular anti-VHSV defense mechanisms are induced by HNP1 in fish cells, monolayers of EPC and RTG-2 cells were treated with HNP1 for 24 h in the presence of 10% FCS followed by washing off HNP1 before infection with VHSV. Fig. 3A shows that also in this case, VHSV infectivity was similarly reduced in a dose-dependent manner.

The maximal inhibition of VHSV infectivity of 70–80% was observed in both EPC and RTG-2 cell monolayers incubated (Fig. 2A) or pre-incubated (Fig. 3A) with 20 μg/ml of HNP1. Similar results of 80–90% reduction were obtained by estimating VHSV infectivity by the mRNA levels of their proteins N (Fig. 3B) and G (data not shown).

Taken together, all these results indicate that in the presence of serum, HNP1 acts on fish VHSV target cells by inducing protection against the VHSV infection and that this effect is independent on the fish cell line and persisted after washing out the HNP1.

3.4. Interaction between HNP1 and VHSV in the absence of serum

If HNP1 inhibits VHSV infectivity in the absence of serum by inactivating VHSV virions, interactions of HNP1 with VHSV particles should be detectable. To investigate this possibility, VHSV binding to solid phase HNP1 was estimated by using specific MAbs to the G protein of VHSV. First, HNP1 was shown to be present in the solid phase since it could be detected by using a polyclonal antibody (PoAb) anti-HNP1 (Fig. 4A). The binding of DTT-treated HNP1 to solid phase was similar to that of the untreated HNP1 (not shown). Fig. 4A shows an HNP1 concentration- and disulphide-dependent recognition of VHSV bound to solid-phase HNP1 by a non-conformational anti VHSV-G protein MAb, indicating that a conformation-dependent HNP1 interacts with VHSV. The elimination of the HNP1 recognition of VHSV by reduction with DTT (Fig. 4A) suggests that the disulphide bonds are important for the recognition of VHSV by HNP1.

3.5. HNP1 inhibits VHSV G protein-mediated syncytia formation in VHSV-infected cell monolayers

The interaction of HNP1 with VHSV could be due to binding to the G protein or to the phospholipids of the VHSV membrane, the only types of molecules exposed on the VHSV surface. Because in the absence of serum, HNP1 can inactivate enveloped mammal virus particles by interacting with their surface glycoproteins (Klotman and Chang, 2006; Sinha et al., 2003; W. Wang et al., 2004; Yasin et al., 2004), we further studied the possible interaction of HNP1 with the VHSV-G protein by using a VHSV G protein-dependent fusion assay. VHSV-infected fish cells express VHSV G protein at the cell membrane of infected fish cells and those infected cells can be induced to fuse by lowering the cell culture media to pH 5–6. Cell-to-cell fusion of infected cells results in the formation of syncytia (multinucleated cells). Therefore, to examine whether HNP1 modifies the VHSV-G protein fusion properties, we assayed the possible interference of HNP1 in a syncytium-forming assay. This assay has been described before (Estepa et al., 2001; Mas et al., 2004), except in this case, we used the permanently transformed EPC cell line EPC-eGFP, to best detect the nuclei in syncytia. No syncytia of more than two to three nuclei per syncytia were detected in VHSV-infected EPC-eGFP cell monolayers incubated with HNP1 (Fig. 4D) despite the presence of protein G in the membrane of infected cells (Fig. 4D2). In contrast, syncytia were abundant in VHSV infected and non-treated cells (∼19 and 35% of nuclei in syncytia at m.o.i. 0.001 and 0.01, respectively) (Fig. 4B and C). On the other hand, the incubation of both uninfected EPC-eGFP cell monolayers (data not shown)

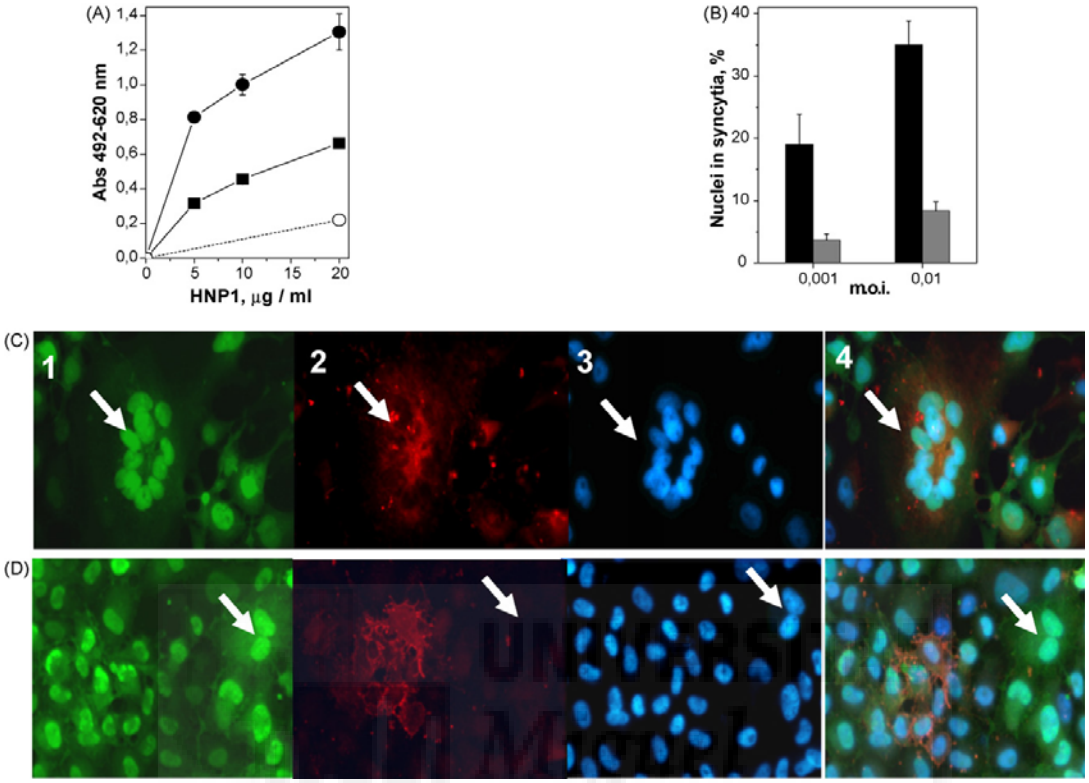

Fig. 4. Binding of VHSV to solid-phase HNP1 (A) and inhibition of syncytia formation in VHSV-infected EPC cell monolayers by HNP1 (B–D). (A) Different amounts of HNP1 or DTT-treated HNP1 were dried onto solid-phase 96-well plates and HNP1 was then detected by using a PoAb anti HNP1 (■). Concentrated VHSV was then added to each well and incubated during 3 h at 14 °C. After washing MAb I10 against VHSV-G protein was used to detect DTT-HNP1- (◯) and HNP1-bound VHSV (●). Data are means ± S.D. from two experiments, each performed in triplicates. (B) EPC-eGFP cell monolayers infected with VHSV during 24 h were washed and then incubated or not with HNP1 during 45 min. After washing the cell monolayers, cell-to-cell fusion was triggered by incubating with fusion medium at pH 6 during 30 min. Cell monolayers were then fixed, fluorescent microphotographies taken and number of nuclei in syncitya counted. Results are expressed as the percentage of nuclei in syncytia by the formula: number of nuclei in syncytia/total number of nuclei × 100. Data are the means ± S.D. from two different experiments, each performed in triplicates. Black bars, not incubated with HNP1. White bars, incubated with HNP1. (C and D) Fluorescent microphotographies of the VHSV infected EPC-eGFP cell monolayers at m.o.i. 0.001. (C) Not incubated with HNP1. (D) Incubated with HNP1. (1) GFP fluorescence (green fluorescence), (2) stained with MAb I10 anti-G protein of VHSV and anti Igs-TRITC (red fluorescence), (3) stained with the Hoechst DNA stain (blue fluorescence), (4) merged fluorescence of fields 1, 2 and 3. Arrows, syncytia.

and VHSV-infected EPC-eGFP cell monolayers with HNP1 (Fig. 4D) induced the unspecific formation of small syncytia (3–4%) of two to three nuclei per syncytia (Fig. 4B).

3.6. HNP1 induces mRNA expression of the Mx3 gene in RTG-2 cell monolayers

The inhibition of VHSV infectivity when using cell monolayers pre-incubated with HNP1 before the VHSV infection suggested that some cellular antiviral defense mechanisms might have been induced by HNP1 in fish cells. To investigate whether a type I IFN response could be induced by HNP1, RTG-2 cell monolayers were incubated during 24 h with HNP1 or poly I:C, a well-known type I IFN-inducer. As negative control, cell monolayers were not incubated or incubated with Pleurocidin (Ple), an AMP from flat fish that does not affect the expression levels of genes associated with the IFN response (Chiou et al., 2006). The TLR3 and the IFN-inducible Mx3 genes were selected to represent the IFN response genes. The Mx3 gene was chosen as marker for IFN responses among the three different rainbow trout Mx genes, because Mx3 was the Mx isoform predominantly expressed in RTG-2 cells in response to different IFN inducers (Tafalla et al., 2007). Both TLR3 and Mx3 genes were up-regulated in RTG-2 cell monolayers treated with HNP1 or poly I:C although induction was always higher in response to poly I:C (Fig. 5A and B). No changes in either TLR3 nor Mx3 gene expression levels were observed in response to Ple (data not shown).

3.7. HNP1 induces mRNA expression of immune response-related genes in trout head kidney leucocytes

To investigate the possible effect of HNP1 on fish immune responses *in vitro*, the mRNA expression profile of a set of repre-

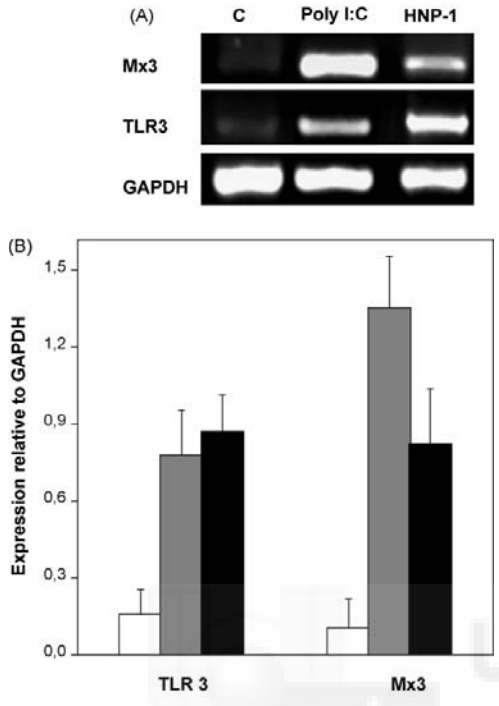

Fig. 5. Expression of transcripts from TLR3 and Mx3 genes in RTG-2 cells in response to HNP1 and poly I:C. Monolayers of RTG-2 cells were incubated with HNP1 or poly I:C. After 24 h of incubation at 20 °C, total RNA was extracted and the expression of transcripts from TLR3 and IFN-inducible Mx3 genes were then estimated by RT-PCR. The mRNA expression relative to GADPH was calculated by the formula: OD of mRNA band/OD of the corresponding GADPH band. (A) Photography of an agarose gel of Mx3, TLR3 and GAPDH mRNA amplified by RT-PCR. The gel is representative of three experiments. (B) Data are mean ± S.D. from two experiments, each performed in duplicate. White bars, untreated RTG-2 cells. Gray bars, HNP1-treated RTG-2 cells. Black bars, poly I:C-treated RTG-2 cells.

sentative immune response-related genes was analyzed in trout head kidney leucocytes. Fig. 6 shows, that the levels of Mx1, Mx2, Mx3, Vig-1, TLR3, IL1β and iNOS transcripts, as assayed by RT-PCR, were increased in head kidney leucocytes incubated with HNP1. A similar effect was shown for samples incubated with poly I:C with the exception of the iNOS gene transcripts.

4. Discussion

This work shows that synthetic human HNP1 is active in fish since it inhibits VHSV replication in fish cells and has immunoregulatory activity on fish leucocytes.

It has been previously described that the mechanisms underlying the antiviral actions of mammal defensins are multiple and complex and include direct effects on the virion as well as effects on the target cell and on innate and adaptive immunity (Klotman and Chang, 2006). In agreement with those findings HNP1 showed at least two mechanisms of anti-VHSV activity. Thus, HNP1 inhibits VHSV replication directly by interacting with the VHSV particles (Figs. 1 and 4) and indirectly by affecting the target cells (Figs. 2 and 3).

In the absence of serum, HNP1 inactivated VHSV before cell infection as it had been shown for type 1 and type 2 herpes simplex viruses (HSV-1 and -2, respectively) (Daher et al., 1986) and HIV-1 (Chang et al., 2003; T.L. Chang et al., 2005) as well as for vesicular stomatitis virus (VSV) (Daher et al., 1986), the prototype virus of rhabdoviridae family. In the presence of serum and at non-cytotoxic concentrations, as shown by the HNP1 solid phase binding assay (Fig. 4A), there was binding between VHSV and HNP1 and this binding is abrogated by treatment of HNP1 with DTT (Fig. 4A), suggesting that disulphide bonds were required for the interaction of this HNP1 with the surface of VHSV particles. Similarly, the direct effect of the HNP1 or θ-defensins on HSV-1 and HIV virions was abolished when their disulphide bonds were disrupted by treatment with the reducing agents DTT and iodoacetamide (Daher et al., 1986; W. Wang et al., 2004). HNP1 inhibited cell-to-cell fusion mediated by the low pH conformation of VHSV-G protein expressed at the surface of VHSV-infected EPC cells (Fig. 4). Consequently, inactivation of VHSV particles by HNP1 may involve interactions with VHSV-G protein rather than with the VHSV envelope. Recent studies have demonstrated that defensins, included HNP1, can inactivate enveloped virus by interacting with *N*-linked or *O*-linked glycans of viral surface glycoproteins in a lectin-dependant manner (Gallo et al., 2006; Hazrati et al., 2006; Klotman and Chang, 2006; Leikina et al., 2005; W. Wang et al., 2004; Yasin et al., 2004). This kind of binding alters the ability of these glycoproteins to bind to their receptors at the target cells or to fuse with the host cellular membranes. Whether the interaction of HNP1 with the G glycoprotein of VHSV occurs by a similar mechanism or by a different one remains to be determined. The relevant interest will be to determine whether HNP1 interacts with the VHSV-G glycoprotein regions implicated in the membrane fusion process since defensins also inhibit HIV-1 by preventing 6-helix bundle formation (Gallo et al., 2006) and a similar structure has been recently reported in the VSV G glycoprotein (Roche et al., 2006). In addition, HNP1 also promoted some cell-to-cell fusion among uninfected EPC cell monolayers as shown by induction of small syncytia of two to three nuclei per syncytia in those monolayers (Fig. 4D, arrows), showing that HNP1 also interacts with the cell membranes of fish cells.

Our data also suggest that HNP1 acted also intracellulary since the inhibition of VHSV infectivity was also obtained when VHSV was not treated with HNP1 but the fish cell monolayers were either infected in the presence of HNP1 (Fig. 2A) or pretreated with HNP1 before the VHSV infection (Fig. 3A and B). Regarding the mechanisms underlying the intracellular activity of HNP1, It has been shown that HNP1 in the presence of serum inhibited HIV-1 infectivity after HIV-1 entry into cells by interfering with cell-signalling pathways required for HIV-1 replication (T.L. Chang et al., 2005). Although a similar signalling pathway-related mechanism might also be implicated in the inhibition of VHSV infectivity by HNP1, other IFN-related mechanisms were operating in fish cells as shown by the up-regulation of Mx3, a well known marker of IFN-induction in

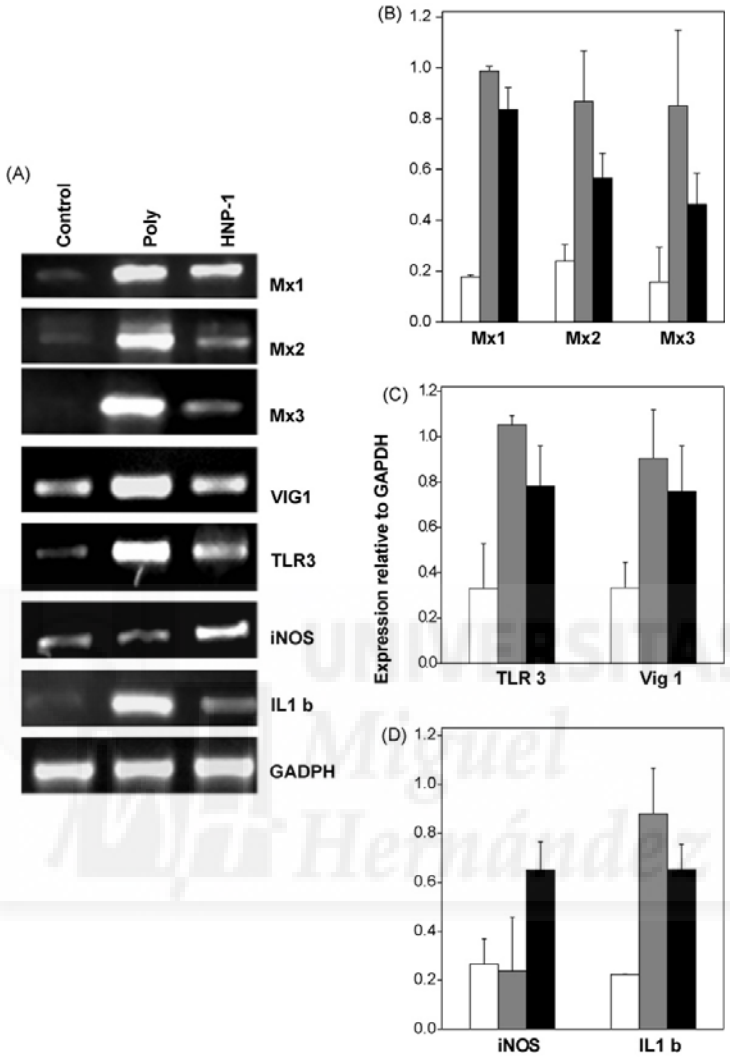

Fig. 6. Expression of transcripts from type I IFN-related genes Mx1, Mx2, Mx3 (A and D), Vig-1, TLR3 (A and C) and IL1β and iNOS (A and D) genes in head kidney leucocytes in response to HNP1. After incubating head kidney leucocytes with HNP1 during 24 h, total RNA was extracted and Mx1, Mx2, Mx3, TLR3, Vig-1, IL1β and iNOS mRNA were analyzed by RT-PCR and agarose gel electrophoresis. The mRNA expression relative to GADPH was calculated by the formula: OD of mRNA band/OD of the corresponding GADPH band. Data are means ± S.D. from three experiments, each in duplicate. White bars, untreated head kidney leucocytes. Gray bars, HNP1-treated head kidney leucocytes. Black bars, poly I:C-treated head kidney leucocytes. (A) Photography of a representative agarose gel ($n = 3$) of mRNAs amplified by RT-PCR. (B–D) Quantification of A by densitometry.

fish, in RTG-2 cells incubated with HNP1. In addition, similar up-regulated levels of TLR3 to those induced by poly I:C (a ligand of TLR3), were observed in response to HNP1, raising the question: How HNP1 activates TLR3? To date, the only evidence that HNPs are TLR ligands is the fact that murine β-defensin-2 interacts with TLR4 in dendritic cells (Froy, 2005; Yang et al., 1999). It could be possible however that for fish cells, the interaction is between HNP1 and TLR3. Learning how HNP1 induces Mx3 and TLR3 gene expression requires further study. Possibilities include the binding of HNP1 to a cellular receptor/s, receptor-mediated or independent endocytosis, transport across channels, binding to and endocytosis together with serum glycoproteins, such as transferrins (Hazrati et al., 2006), etc. In fact, accumulation of HNP1 within the cytoplasm of cells that do not synthesize HNP1, including CD4-T cells (Mackewicz et al., 2003; Zhang et al., 2002), smooth muscle cells (Nassar et al., 2002), epithelial cells and human cervical cells (CaSki cell line) (Hazrati et al., 2006) it has been previously reported although their uptake remains still unclear.

Because different effects of HNP1 have been described depending on the cells used (primary cells or transformed cell lines) (Chang et al., 2003; T.L. Chang et al., 2005; Klotman

and Chang, 2006), trout head kidney leucocytes were also used to define the HNP1 ability to modulate fish immune response. Four IFN-related genes (Mx1, Mx2, Mx3 and vig-1) were significantly modulated in head kidney leucocytes incubated with HNP1, thus confirming that HNP1 might trigger an antiviral response dependent of IFN induction. Moreover, IL1β and iNOs genes were also up-regulated indicating the immunomodulatory role of HNP1 on the leucocytes-regulated immune response in fish. In mammals the induction of proinflammatory cytokines (Froy, 2005; Lehrer and Ganz, 2002; Lillard et al., 1999; Yang et al., 2002) and IFN-γ but not IFN-α/β by HNPs has been previously reported (T.L. Chang et al., 2005; Klotman and Chang, 2006; Selsted and Ouellette, 2005).

Therefore, further studies of the antiviral activity of the heterologous HNP1 in fish and/or of HNP1-like homologous fish peptides could help the development of new orally- (medicated food) or bath immersion-administrated therapeutants for VHSV prevention as well as advance our understanding of how AMPs work to block virus replication. In addition, the inactivation of VHSV particles by HNP1 further confirms the previously proposed common mechanism that might account for a broad range of activity of the innate immune response against viruses that use a common pathway of membrane fusion for entering host cells (Klotman and Chang, 2006). Taken together, all these results suggest that HNP1, HNP1-like peptides and other innate immune system-related molecules may prove useful as templates for novel antivirals of broad range of activity in both human and animal health.

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References

Basurco, B., Coll, J.M., 1989. Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. Bull. Eur. Assoc. Fish Pathol. 9, 92–95.

Boudinot, P., Massin, P., Blanco, M., Riffault, S., Benmansour, A., 1999. vig-1, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family. J. Virol. 73 (3), 1846–1852.

Brocal, I., Falco, A., Mas, V., Rocha, A., Perez, L., Coll, J.M., Estepa, A., 2006. Stable expression of bioactive recombinant pleurocidin in a fish cell line. Appl. Microbiol. Biotechnol. 72, 1217–1228.

Buck, C.B., Day, P.M., Thompson, C.D., Lubkowski, J., Lu, W., Lowy, D.R., Schiller, J.T., 2006. Human alpha-defensins block papillomavirus infection. Proc. Natl. Acad. Sci. U.S.A. 103 (5), 1516–1521.

Bulet, P., Stocklin, R., 2005. Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept. Lett. 12 (1), 3–11.

Chang, C.I., Pleguezuelos, O., Zhang, Y.A., Zou, J., Secombes, C.J., 2005. Identification of a novel cathelicidin gene in the rainbow trout *Oncorhynchus mykiss*. Infect. Immun. 73 (8), 5053–5064.

Castro, M.S., Fontes, W., 2005. Plant defense and antimicrobial peptides. Protein Pept. Lett. 12 (1), 13–18.

Chang, T.L., Francois, F., Mosoian, A., Klotman, M.E., 2003. CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition. J. Virol. 77 (12), 6777–6784.

Chico, V., Gomez, N., Estepa, A., Perez, L., 2006. Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR. J. Virol. Methods 132 (1–2), 154–159.

Chinchar, V.G., Bryan, L., Silphadaung, U., Noga, E., Wade, D., Rollins-Smith, L., 2004. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. Virology 323 (2), 268–275.

Chiou, P.P., Lin, C.M., Perez, L., Chen, T.T., 2002. Effect of cecropin B and a synthetic analogue on propagation of fish viruses in vitro. Mar. Biotechnol. (NY) 4 (3), 294–302.

Chiou, P., Khoo, J., Bols, N.C., Douglas, S., Chen, T.T., 2006. Effects of linear cationic alpha-helical antimicrobial peptides on immune-relevant genes in trout macrophages. Dev. Comp. Immunol. 30 (9), 797–806.

Cole, A.M., Weis, P., Diamond, G., 1997. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J. Biol. Chem. 272 (18), 12008–12013.

Daher, K.A., Selsted, M.E., Lehrer, R.I., 1986. Direct inactivation of viruses by human granulocyte defensins. J. Virol. 60 (3), 1068–1074.

Estepa, A., Coll, J.M., 1997. Temperature and pH requirements for viral haemorrhagic septicemia virus induced cell fusion. Dis. Aquat. Organ. 28, 185–189.

Estepa, A.M., Rocha, A.I., Mas, V., Perez, L., Encinar, J.A., Nunez, E., Fernandez, A., Gonzalez Ros, J.M., Gavilanes, F., Coll, J.M., 2001. A protein G fragment from the salmonid viral hemorrhagic septicemia rhabdovirus induces cell-to-cell fusion and membrane phosphatidylserine translocation at low pH. J. Biol. Chem. 276 (49), 46268–46275.

Fernandes, J.M.O., Molle, G., Kemp, G.D., Smith, V.J., 2004. Isolation and characterisation of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 28 (2), 127–138.

Fernandez-Alonso, M., Lorenzo, G., Perez, L., Bullido, R., Estepa, A., Lorenzen, N., Coll, J.M., 1998. Mapping of linear antibody epitopes of the glycoprotein of VHSV, a salmonid rhabdovirus. Dis. Aquat. Organ. 34, 167–176.

Fijan, N., Sulimanovic, D., Bearzotti, M., Mizinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F., de Kinkelin, P., 1983. Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Ann. Virol. 134, 207–220.

Froy, O., 2005. Regulation of mammalian defensin expression by Toll-like receptor-dependent and independent signalling pathways. Cell. Microbiol. 7 (10), 1387–1397.

Gallo, S.A., Wang, W., Rawat, S.S., Jung, G., Waring, A.J., Cole, A.M., Lu, H., Yan, X., Daly, N.L., Craik, D.J., Jiang, S., Lehrer, R.I., Blumenthal, R., 2006. Theta-defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. J. Biol. Chem. 281 (27), 18787–18792.

Ganz, T., 1999. Defensins and host defense. Science 286 (5439), 420–421.

Ganz, T., 2003. Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. 3 (9), 710–720.

Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F., Lehrer, R.I., 1985. Defensins. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. 76 (4), 1427–1435.

Garcia-Olmedo, F., Molina, A., Alamillo, J.M., Rodriguez-Palenzuela, P., 1998. Plant defense peptides. Biopolymers 47 (6), 479–491.

Graham, S., Jeffries, A.H., Secombes, C.J., 1988. A novel assay to detect macrophages bactericidal activity in fish. Factors influencing the killing of *Aeromonas salmonicida*. J. Fish Dis. 11, 389–396.

Hazrati, E., Galen, B., Lu, W., Wang, W., Ouyang, Y., Keller, M.J., Lehrer, R.I., Herold, B.C., 2006. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. J. Immunol. 177 (12), 8658–8666.

Hwang, G., Muller, F., Rahman, M.A., Williams, D.W., Murdock, P.J., Pasi, K.J., Goldspink, G., Farahmand, H., Maclean, N., 2004. Fish as bioreactors: transgene expression of human coagulation factor VII in fish embryos. Mar. Biotechnol. 6, 485–492.

Klotman, M.E., Chang, T.L., 2006. Defensins in innate antiviral immunity. Nat. Rev. Immunol. 6 (6), 447–456.

Lauth, X., Shike, H., Burns, J.C., Westerman, M.E., Ostland, V.E., Carlberg, J.M., Van Olst, J.C., Nizet, V., Taylor, S.W., Shimizu, C., Bulet, P., 2002. Discovery and characterization of two isoforms of moronecidin, a novel

antimicrobial peptide from hybrid striped bass. J. Biol. Chem. 277 (7), 5030–5039.
Lay, F.T., Anderson, M.A., 2005. Defensins—components of the innate immune system in plants. Curr. Protein Pept. Sci. 6 (1), 85–101.
LeBerre, M., De Kinkelin, P., Metzger, A., 1977. Identification sérologique des rhabdovirus des salmonidés. Bull. Off. Int. Epizooties 87, 391–393.
Lehrer, R.I., Ganz, T., 2002. Defensins of vertebrate animals. Cur. Op. Immunol. 14 (1), 96–102.
Leikina, E., Delanoe-Ayari, H., Melikov, K., Cho, M.S., Chen, A., Waring, A.J., Wang, W., Xie, Y., Loo, J.A., Lehrer, R.I., Chernomordik, L.V., 2005. Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. Nat. Immunol. 6 (10), 995–1001.
Lillard Jr., J.W., Boyaka, P.N., Chertov, O., Oppenheim, J.J., McGhee, J.R., 1999. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. Proc. Natl. Acad. Sci. U.S.A. 96 (2), 651–656.
Lindenstrom, T., Secombes, C.J., Buchmann, K., 2004. Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. Vet. Immunol. Immunopathol. 97 (3–4), 137–148.
Lorenzen, N., LaPatra, S.E., 2005. DNA vaccines for aquacultured fish. Rev. Sci. Tech. 24 (1), 201–213.
Lorenzo, G., Estepa, A., Coll, J.M., 1996. Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicemia with anti-nucleoprotein monoclonal antibody. J. Virol. Methods 58, 1–6.
Mackewicz, C.E., Yuan, J., Tran, P., Diaz, L., Mack, E., Selsted, M.E., Levy, J.A., 2003. Alpha-defensins can have anti-HIV activity but are not CD8 cell anti-HIV factors. Aids 17 (14), F23–F32.
Maemoto, A., Qu, X., Rosengren, K.J., Tanabe, H., Henschen-Edman, A., Craik, D.J., Ouellette, A.J., 2004. Functional analysis of the alpha-defensin disulfide array in mouse cryptdin-4. J. Biol. Chem. 279 (42), 44188–44196.
Mandal, M., Nagaraj, R., 2002. Antibacterial activities and conformations of synthetic alpha-defensin HNP-1 and analogs with one, two and three disulfide bridges. J. Pept. Res. 59 (3), 95–104.
Martin, E., Ganz, T., Lehrer, R.I., 1995. Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukoc. Biol. 58 (2), 128–136.
Mas, V., Perez, L., Encinar, J.A., Pastor, M.T., Rocha, A., Perez-Paya, E., Ferrer-Montiel, A., Gonzalez Ros, J.M., Estepa, A., Coll, J.M., 2002. Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library. J. Gen. Virol. 83 (Pt 11), 2671–2681.
Mas, V., Rocha, A., Perez, L., Coll, J.M., Estepa, A., 2004. Reversible inhibition of spreading of in vitro infection and imbalance of viral protein accumulation at low pH in viral haemorrhagic septicaemia rhabdovirus (VHSV), a salmonid rhabdovirus. J. Virol. 78 (4), 1936–1944.
Mas, V., Falco, A., Brocal, I., Perez, L., Coll, J.M., Estepa, A., 2006. Identification of selective inhibitors of VHSV from biased combinatorial libraries of *N*,*N*′-disubstituted 2,5-piperazinediones. Antiviral Res..
McLauchlan, P.E., Collet, B., Ingerslev, E., Secombes, C.J., Lorenzen, N., Ellis, A.E., 2003. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression. Fish Shellfish Immunol. 15 (1), 39–50.
Micol, V., Caturla, N., Perez-Fons, L., Mas, V., Perez, L., Estepa, A., 2005. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). Antiviral Res. 66 (2–3), 129–136.
Nassar, T., Akkawi, S., Bar-Shavit, R., Haj-Yehia, A., Bdeir, K., Al-Mehdi, A.B., Tarshis, M., Higazi, A.A., 2002. Human alpha-defensin regulates smooth muscle cell contraction: a role for low-density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. Blood 100 (12), 4026–4032.
Noga, E.J., Silphaduang, U., 2003. Piscidins: a novel family of peptide antibiotics from fish. Drug News Perspect. 16 (2), 87–92.
Olesen, N.J., Korsholm, H., 1997. Control measures for viral diseases in aquaculture: eradication of VHS and IHN. Bull. Eur. Assoc. Fish Pathol. 17, 229–233.
Oren, Z., Shai, Y., 1996. A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from Moses sole fish *Pardachirus marmoratus*. Eur. J. Biochem. 237 (1), 303–310.
Otvos Jr., L., 2000. Antibacterial peptides isolated from insects. J. Pept. Sci. 6 (10), 497–511.
Park, C.B., Lee, J.H., Park, I.Y., Kim, M.S., Kim, S.C., 1997. A novel antimicrobial peptide from the loach, *Misgurnus anguillicaudatus*. FEBS Lett. 411 (2–3), 173–178.
Park, I.Y., Park, C.B., Kim, M.S., Kim, S.C., 1998. Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. FEBS Lett. 437 (3), 258–262.
Perez, L., Mas, V., Coll, J., Estepa, A., 2002. Enhanced detection of viral hemorrhagic septicemia virus (a salmonid rhabdovirus) by pretreatment of the virus with a combinatorial library-selected peptide. J. Virol. Methods 106 (1), 17–23.
Rocha, A., Ruiz, S., Coll, J.M., 2004. Improvement of transfection efficiency of epithelioma papulosum cyprini carp cells by modification of cell cycle and use of an optimal promoter. Mar. Biotechnol. (NY) 6 (5), 401–410.
Roche, S., Bressanelli, S., Rey, F.A., Gaudin, Y., 2006. Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science 313 (5784), 187–191.
Rodriguez, M.F., Wiens, G.D., Purcell, M.K., Palti, Y., 2005. Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics 57 (7), 510–519.
Sanz, F., Coll, J.M., 1992. Detection of viral haemorrhagic septicemia virus by direct immunoperoxidase with selected anti-nucleoprotein monoclonal antibody. Bull. Eur. Ass. Fish Pathol. 12, 116–119.
Selsted, M.E., Ouellette, A.J., 2005. Mammalian defensins in the antimicrobial immune response. Nat. Immunol. 6 (6), 551–557.
Selsted, M.E., Harwig, S.S., Ganz, T., Schilling, J.W., Lehrer, R.I., 1985. Primary structures of three human neutrophil defensins. J. Clin. Invest. 76 (4), 1436–1439.
Shike, H., Lauth, X., Westerman, M.E., Ostland, V.E., Carlberg, J.M., Van Olst, J.C., Shimizu, C., Bulet, P., Burns, J.C., 2002. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur. J. Biochem. 269 (8), 2232–2237.
Sinha, S., Cheshenko, N., Lehrer, R.I., Herold, B.C., 2003. NP-1, a rabbit {alpha}-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. Antimicrob. Agents Chemother. 47 (2), 494–500.
Sugiarto, H., Yu, P.L., 2004. Avian antimicrobial peptides: the defense role of beta-defensins. Biochem. Biophys. Res. Commun. 323 (3), 721–727.
Chang, T.L., Vargas Jr., J., DelPortillo, A., Klotman, M.E., 2005. Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity. J. Clin. Invest. 115 (3), 765–773.
Tafalla, C., Chico, V., Perez, L., Coll, J.M., Estepa, A., 2007. In vitro and in vivo differential expression of rainbow trout (*O. mykiss*) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV. Fish Shellfish Immunol. 23, 210–221.
Tani, K., Murphy, W.J., Chertov, O., Salcedo, R., Koh, C.Y., Utsunomiya, I., Funakoshi, S., Asai, O., Herrmann, S.H., Wang, J.M., Kwak, L.W., Oppenheim, J.J., 2000. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. Int. Immunol. 12 (5), 691–700.
Wang, T., Johnson, N., Zou, J., Bols, N., Secombes, C.J., 2004. Sequencing and expression of the second allele of the interleukin-1beta1 gene in rainbow trout (*Oncorhynchus mykiss*): identification of a novel SINE in the third intron. Fish Shellfish Immunol. 16 (3), 335–358.
Wang, W., Owen, S.M., Rudolph, D.L., Cole, A.M., Hong, T., Waring, A.J., Lal, R.B., Lehrer, R.I., 2004. Activity of alpha- and theta-defensins against primary isolates of HIV-1. J. Immunol. 173 (1), 515–520.
Wang, T., Zou, J., Cunningham, C., Secombes, C.J., 2002. Cloning and functional characterisation of the interleukin-1 beta 1 promoter of rainbow trout (*Oncorhynchus mykiss*). Biochim. Biophys. Acta 1575 (1–3), 108–116.
Wolf, F., Quimby, M.C., 1962. Established eurythermic line of fish cells in vitro. Science 135, 1065–1066.
Wu, Z., Hoover, D.M., Yang, D., Boulegue, C., Santamaria, F., Oppenheim, J.J., Lubkowski, J., Lu, W., 2003. Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. Proc. Natl. Acad. Sci. U.S.A. 100 (15), 8880–8885.
Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schroder, J.M., Wang, J.M., Howard, O.M., Oppenheim, J.J., 1999. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286 (5439), 525–528.

Yang, D., Biragyn, A., Kwak, L.W., Oppenheim, J.J., 2002. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol. 23 (6), 291–296.

Yasin, B., Wang, W., Pang, M., Cheshenko, N., Hong, T., Waring, A.J., Herold, B.C., Wagar, E.A., Lehrer, R.I., 2004. Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J. Virol. 78 (10), 5147–5156.

Zhang, L., Yu, W., He, T., Yu, J., Caffrey, R.E., Dalmasso, E.A., Fu, S., Pham, T., Mei, J., Ho, J.J., Zhang, W., Lopez, P., Ho, D.D., 2002. Contribution of human {alpha}-defensin-1, -2 and -3 to the anti-HIV-1 activity of CD8 antiviral factor. Science, 1076185.

Zhang, Y.A., Zou, J., Chang, C.I., Secombes, C.J., 2004. Discovery and characterization of two types of liver-expressed antimicrobial peptide 2 (LEAP-2) genes in rainbow trout. Vet. Immunol. Immunopathol. 101 (3–4), 259–269.

Zou, J., Mercier, C., Koussounadis, A., Secombes, C., 2007. Discovery of multiple beta-defensin like homologues in teleost fish. Mol. Immunol. 44, 638–647.

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In vivo modulation of the rainbow trout (*Oncorhynchus mykiss*) immune response by the human alpha defensin 1, HNP1

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Abstract Recent studies have demonstrated that the synthetic human defensin-α1, also designated as human neutrophil peptide 1 (HNP1), not only has in vitro antiviral activity against viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus, but can also modulate some immune activities of rainbow trout (*Oncorhynchus mykiss*) head kidney leucocytes. However, none of these HNP1 properties have been analysed in vivo so far. Thus, in the current work, we have studied the in vivo immunomodulatory capacity of HNP1 on the rainbow trout immune system as a first approach to evaluate the possible use of this family of antimicrobial peptides (AMPs) to increase fish resistance by enhancing non-specific defence mechanisms. The intramuscular injection of synthetic HNP1 induced the transcript expression of genes encoding both pro-inflammatory cytokines (IL-1β, TNF-α1 and specially IL-8) and CC chemokines (CK5B, CK6 and CK7A) as well as of the genes related to type I interferon (IFN) production (Mx1, Mx2, Mx3 and IFN regulatory factor 3, IRF-3) in different trout tissues (muscle, head kidney and blood). Furthermore, the chemotactic capacity of HNP1 towards trout leucocytes has been clearly revealed. All together, these results demonstrate that in vivo HNP1 is active across species and can modulate fish immune responses. Therefore, in a moment when most pathogens have developed resistance to commonly used antibiotics, natural antimicrobial peptides with interspecific activity, such as HNP1, might prove to be useful model molecules for the development of novel therapeutic agents that exhibit both microbicidal and immunoenhancing capabilities.

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Introduction

Despite advances in prevention, diagnosis, and treatment, infectious diseases continue to challenge the aquaculture industry. Due to the limitations of vaccine-based immunization strategies and the increasing resistance of microbes to existing antibiotics, research related to innate host defence mechanisms that are not dependant on specific recognition of individual antigens offers a promising field to search for new antibiotics or immunostimulants. In this context, defensins, are now considered as model molecules for the development of novel therapeutic agents that exhibit both microbicidal and immunoenhancing capabilities.

Defensins are cationic antimicrobial peptides (AMPs) with β-sheet structures stabilised by three intramolecular disulphide bonds [1,2]. Mammalian defensins are classified into α, β, or θ depending on the distribution of the disulphide bonds between their six conserved cysteine residues. Human α-defensins, also designated as human neutrophil peptides (HNP1, HNP2, HNP3 and HNP4), are synthesised as prepropeptides, and therefore contain an amino-terminal signal sequence, an anionic propiece and a carboxy-terminal mature peptide of approximately 30 amino acid [2]. Alpha-defensins are mainly produced by neutrophils, as part of their granule content, but they can also be produced by other cell types such as NK cells [3]. These molecules not only exhibit antimicrobial properties, but are also capable of modulating the immune response; thus they constitute very good candidates for therapy or for use as adjuvants in vaccination.

Due to their high homology, HNP1-3 are usually studied as a group although differences in their microbicidal [4] and immunoregulatory activities have been reported [5]. HNPs can influence various mechanisms of the innate immune response; thus they have been shown to modulate the production of chemokines, such as interleukin 8 (IL-8) [6,7], apart from being chemoattractant for some cell types themselves [5]. HNPs also regulate NK-mediated cytotoxicity, although the mechanism of action is not entirely clear yet [3]. Furthermore, HNPs can also modulate the adaptive immune response and have been shown to enhance specific antibody and cellular responses [8—10], acting as effective adjuvants. The mechanism through which HNPs exert their adjuvant activity has not been fully elucidated yet in mammals, and many hypothesis such as direct modulation of lymphocyte responses or modulation of antigen presenting cell function, through enhanced chemotaxis, have been postulated [11]. In fact, there is even some controversy dealing with the hypothesis that the main role of HNPs in vivo is the immunomodulation since their microbicidal effects is abrogated in the presence of serum and albumin [12].

Using a database mining approach, genes encoding β-defensin-like peptides have been recently discovered in three non-salmonid fish species (zebrafish, puffer fish, and tetraodon) [13] and rainbow trout (GenBank accession nos. AM286737, AM282656, AM282655 and AM282657), but biological activity has been only shown for the trout β-defensin-like peptide [14]. To date, although there is no evidence of α-defensins in fish, their presence can not be excluded until more expressed sequence databases and genome sequences become available. Moreover, the in vitro inhibition of viral hemorrhagic septicemia rhabdovirus (VHSV) infectivity by HNP1 as well as the induction of transcript expression of genes related to interferon (IFN) production and other immune-related genes (interleukin 1β, IL-1β and inducible nitric oxide synthase, iNOS) after ex vivo treatment of rainbow trout head kidney leukocytes with HNP1 has been recently demonstrated [15], suggesting the presence of α-defensins in fish.

While awaiting for the discovery of fish α-defensins and following the in vitro results previously found in fish with HNP1, in this work, we have evaluated the effect of HNP1 on the levels of expression of genes relevant to the early inflammatory response (IL-1β; IL-8 and tumour necrosis factor α1, TNF-α1), chemokines belonging to the CC family such as CK5B, CK6 and CK7A, genes related to IFN production (Mx1, Mx2, Mx3 and interferon regulatory factor 3, IRF-3), to macrophage activation (iNOS and IFN-γ) or to antigen presentation (major histocompatibility complex, MHC-I and MHC-II) in rainbow trout. In order to establish if there is a direct effect of defensins on fish leukocyte chemotaxis, we have also studied the capacity of HNP1 to attract trout leukocytes.

These results can be useful towards the understanding of how antimicrobial peptides work in fish, and might help elucidate whether α-defensin homologues are likely to be present in fish. Moreover, due to the effectiveness of DNA vaccination in fish [16], and the possibility of incorporating molecular adjuvants in these plasmids in the form of gene sequences within the vaccine plasmid, it is not outrageous to further investigate the effects of the incorporation of sequences that code for these human defensins which not only are immunostimulatory, but also exert antiviral effects against VHSV [15].

Materials and methods

Fish

Rainbow trout (*Oncorhynchus mykiss*) of approximately 8—10 cm obtained from Lillogen (Leon, Spain) were maintained in 50 L tanks at the Miguel Hernandez University (Elche, Spain) laboratory at 14 °C with a re-circulating water system using water from the fish farm. Fish were fed daily until satiated with a commercial diet (Trow, Leon, Spain). Prior to the experiments, fish were acclimatised to laboratory conditions for 2 weeks.

Peptides

Synthetic human alpha-Defensin-1 (HNP1) (ACYCRIPACIAGERRYGTCIYQGRLWAFCC-NH_2, disulfide bonds: 2—30, 4—19 and 9—29) was purchased from Peptides International (Louisville, KY, USA). The purity of the peptide was >98%. The mature sequence of Limanda (*Limanda limanda*) pleurocidin (LmPle) (GWKKWFKKATHVGKHVGKAALDAYL) [17] was used as a control to verify that the effects of HNP1 on the trout immune response were specific of HNP1 and not only due to the injection of a foreign peptide. Synthetic LmPle was obtained from Diverdrugs (Diverdrugs

S.A., Barcelona, Spain). The purity of peptide was >95%. Both HNP1 and pleurocidin were reconstituted to a final concentration of 1 $\mu g\ \mu l^{-1}$ in sterile distilled water and stored until used in suitable aliquots at −20 °C.

Injection of HNP1 and LmPle into rainbow trout muscle

To determine the effect of HNP1 on the rainbow trout immune system, fish were divided into 3 groups (12 fish each). One group was intramuscularly injected with the human defensin HNP1 (1 μg in 100 μl of phosphate buffered saline, PBS, per fish) another one was injected with LmPle (1 μg in 100 μl of phosphate buffered saline, PBS, per fish) and the last one was mock-injected with the same volume of PBS. At days 1, 3, and 7 post-injection four trout from each group were sacrificed by overexposure to MS-222, muscle and head kidney removed and blood extracted from the caudal vein.

cDNA synthesis

Total RNA of the different tissues was extracted using Trizol (Invitrogen, UK). Individual organs were homogenized in 1 ml of Trizol in an ice bath, and mixed with 200 μl of chloroform. The suspension was then centrifuged at 12,000 × *g* for 15 min. The clear upper phase was aspirated and placed in a clean tube. Five hundred μl of isopropanol were then added, and the samples were again centrifuged at 12,000 × *g* for 10 min. The RNA pellet was washed with 75% ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C.

Two μg of RNA were used to obtain cDNA using the Superscript II reverse transcriptase (Invitrogen, UK). Briefly, RNA was incubated with 1 μl of oligo (dT)12–18 (0.5 $\mu g\ ml^{-1}$) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of Superscript II reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA was diluted in a 1:5 proportion with DEPC-treated water and stored at −20 °C.

PCR of immune genes

All amplification reactions were performed using 0.5 μl dNTP mix (10 mM each), 0.2 μl Taq polymerase (5 units μl^{-1}, Invitrogen, UK), 2.5 μl Taq 10× buffer, 0.75 μl $MgCl_2$ 50 mM, 0.5 μl of each primer (50 μM) and 1 μl of cDNA in a final volume of 25 μl. First, a PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with all samples as a positive control for RT-PCR, since GAPDH is constitutively expressed in all organs. These PCR products also allowed the verification that equivalent amounts of cDNA were present in the different samples and therefore amplifications of the different immune genes were comparable among samples. A negative control in which no cDNA was added was included in all PCR reactions.

Primers used for gene amplification, and the sizes of the different PCR products are shown in Table 1. All PCRs were carried out in a Perkin Elmer 2720 cycler and amplification conditions always consisted of a denaturing step of 94 °C for 5 min followed by the different specific cycling conditions shown in Table 1 followed by a final extension of 7 min at 72 °C. For each gene, after optimising the conditions following protocols described previously and referenced in Table 1, at least two PCRs with different number of cycles were performed in order to determine at which point of the amplification differences were evident among samples. Once the optimal number of cycles (Table 1) was determined, all samples were amplified twice to verify the results. The PCR products (8 μl) were visualized on a 2% agarose gel stained with ethidium bromide. Samples that were to be compared were always run in the same agarose gel. A 100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Image Gauge v. 4.0 software (Fujifilm, Barcelona, Spain). Semi-quantitative analysis of mRNA transcription for each gene was performed relative to the GAPDH expression of the same sample using the formula: intensity of target gene band/intensity of its corresponding GAPDH band. Data were then analysed using Student's *t*-test comparing values obtained in mock-injected fish and fish injected with HNP1. Differences were considered statistically significant at $p < 0.05$.

Blood leukocyte isolation

Blood leukocytes were isolated following the method previously described [18]. Briefly, blood obtained from the tail vein was diluted 5 times with Leibovitz medium (L-15, Gibco, Invitrogen, UK) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), heparin (10 units ml^{-1}) and 2% foetal calf serum (FCS, Gibco). The resulting cell suspension was placed onto 51% Percoll density gradients. The gradients were centrifuged at 500 × *g* for 30 min at 4 °C. The interface cells were collected and washed twice at 500 × *g* for 5 min in L-15 containing 0.1% FCS. The viable cell concentration was determined by Trypan blue exclusion. Cells were resuspended in L-15 with 5% FCS at a concentration of 5×10^5 cells ml^{-1}.

Chemotaxis experiments

The capacity of HNP1 to induce specific migration in trout blood leukocytes was studied using 96-well chemotaxis chambers (Neuroprobe, Gaithersburg, MD, USA) in which the different concentrations of HNP1 (0.1, 1 and 2 $\mu g\ ml^{-1}$) were diluted in L-15 medium to make a final volume of 30 μl. Controls consisted in L-15 medium alone. Blood leukocytes (30 μl of a suspension containing 5×10^6 cells ml^{-1}) were dispensed in the upper chamber, separated by a 3 μm polycarbonate membrane. After 60 min of incubation at 20 °C, the number of cells that had migrated to the bottom wells was estimated using CellTiter 96 (Promega, Madison, WI, USA) according to manufacturer's instructions. This assay is based on the bioreduction of an MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopehyl)-2H-tetrazolium] into a coloured formazan product soluble in culture medium which can be then estimated by its absorbance at 490 nm.

Table 1 Primer sequences, sizes of PCR products and amplification conditions for the different genes studied

Gene	Primers	Size of PCR product (bp)	N° of cycles	Cycling conditions	Reference
GAPDH	F: 5′ ATGTCAGACCTCTGTGTTGG 3′ R: 5′ TCCTCGATGCCGAAGTTGTCG 3′	514 bp	32	94 °C 30 s 58 °C 30 s 72 °C 1 min	[25]
IL-1β	F: 5′ AGGGAGGCAGCAGCTACCACAA 3′ R: 5′ GGGGGCTGCCTTCTGACACAT 3′	353 bp	35	94 °C 30 s 60 °C 30 72 °C 30 s	[26]
IL-8	F: 5′ GAATGTCAGCCAGCCTTGTC 3′ R: 5′ TCCAGACAAATCTCCTGACCG 3′	226 bp	35	94 °C 30 s 60 °C 30 s 72 °C 30 s	[20]
TNF-α1	F: 5′ TTCGGGCAAATATTCAGTCG 3′ R: 5′ GCCGTCATCCTTTCTCCACT 3′	433 bp	10	94 °C 1 min 60 °C 1 min 72 °C 20 s	[27]
			25	94 °C 1 min 60 °C 1 min 72 °C 20 s + 1 s per cycle	
CK5B	F: 5′ TTTGCTGATCGTCAGATACCC 3′ R: 5′ GGACCATGACTGCTCTCTCTG 3′	315 bp	37	94 °C 20 s 55 °C 20 s 72 °C 30 s	[23]
CK6	F: 5′ CGAATCTGCTCTGACACTTCC 3′ R: 5′ TGGTGAGTTGTTGACCATTGA 3′	219 bp	37	94 °C 20 s 55 °C 20 s 72 °C 30 s	[23]
CK7A	F: 5′ TCTGCAGGTGTCATTAAGTTGG 3′ R: 5′ TCTTTGTGGTGAAAATCAGTGC 3′	139 bp	37	94 °C 20 s 55 °C 20 s 72 °C 30 s	[23]
Mx1	F: 5′ ATGCCACCCTACAGGAGATGAT 3′ R: 5′ TAACTTCTATTACATTTACTATGCAA 3′	421 bp	37	94 °C 30 s 52 °C 30 s 72 °C 30 s	[24]
Mx2	F: 5′ ATGCCACCCTACAGGAGATGAT 3′ R: 5′ GGAAGCATAGTAACTTTATTATAAC 3′	400 bp	37	94 °C 30 s 52 °C 30 s 72 °C 30 s	[24]
Mx3	F: 5′ ATGCCACCCTACAGGAGATGAT 3′ R: 5′ CCACAGTGTACATTTAGTTG 3′	381 bp	37	94 °C 30 s 52 °C 30 s 72 °C 30 s	[28]
IRF-3	F: 5′ GTCCCTCTTTAGCACAAGTC 3′ R: 5′ GGTGGAGCAGTTCACAAATG 3′	690 bp	35	94 °C 20 s 60 °C 20 s 72 °C 20 s	CB515644
iNOS	F: 5′ CATACGCCCCCAACAAACCAGTGC 3′ R: 5′ CCTCGCCTTCTCATCTCCAGTGTC 3′	746 bp	40	94 °C 1 min 62 °C 1 min 72 °C 2 min	[27]
IFN-γ	F: 5′ GTGAGCAGAGGGTGTTGATG 3′ R: 5′ GATGGTAATGAACTCGGACAG 3′	251 bp	40	94 °C 20 s 60 °C 20 s 72 °C 20 s	[29]
MHC-I	F: 5′ CAGTGTCTCTGCTCCAGAAGG 3′ R: 5′ TCAGAACCTCGATGAAGTCCTT 3′	263 bp	28—32	94 °C 30 s 55 °C 30 s 72 °C 30 s	[30]
MHC-II	F: 5′ ATGTCGATGCCAATTGCCTTCTA 3′ R: 5′ TGTCTTGTCCAGTATGGCGCT 3′	336 bp	28	94 °C 30 s 57 °C 30 s 72 °C 30 s	[27]

Results

Effect of HNP1 on the expression of pro-inflammatory cytokines

The effect of HNP1 on the expression of different pro-inflammatory cytokines was studied in order to evaluate the potential use of defensins as adjuvants in fish (Fig. 1). In the head kidney, HNP1 produced no effect on IL-1β and TNF-α1 expression, while it significantly increased IL-8 at day 1 post-injection. In the blood, however, a moderate increase of IL-1β was observed at day 3. For TNF-α1, we observed a significant decrease of the constitutive levels of expression at day 7. In the muscle, there was a strong induction of all three cytokines.

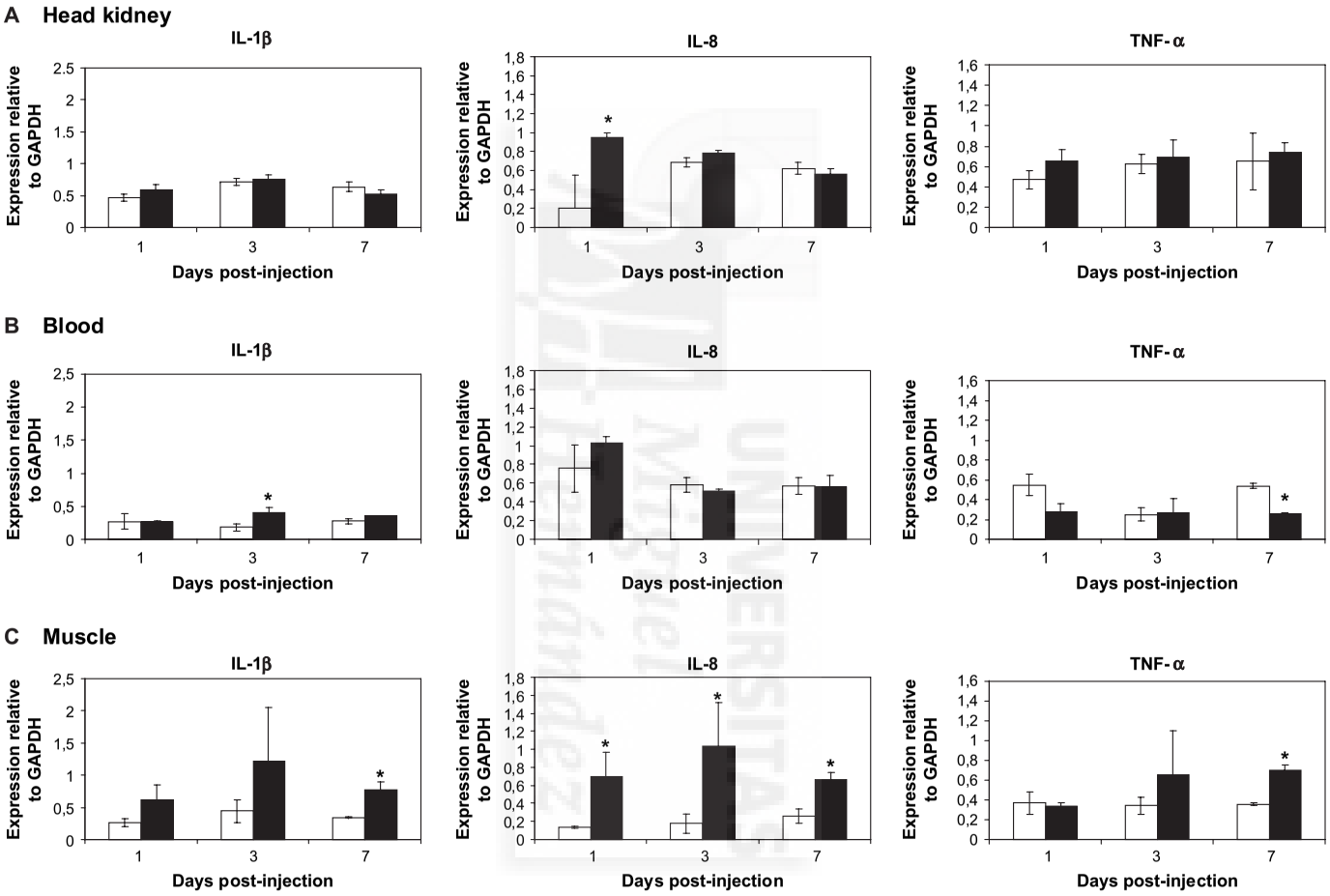

Figure 1 Effect of HNP1 on the expression of pro-inflammatory cytokines: IL-1β, IL-8 and TNF-α1. Levels of expression of the different cytokines were assayed by semi-quantitative RT-PCRs in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly different from that observed in mock-injected controls ($p < 0.05$).

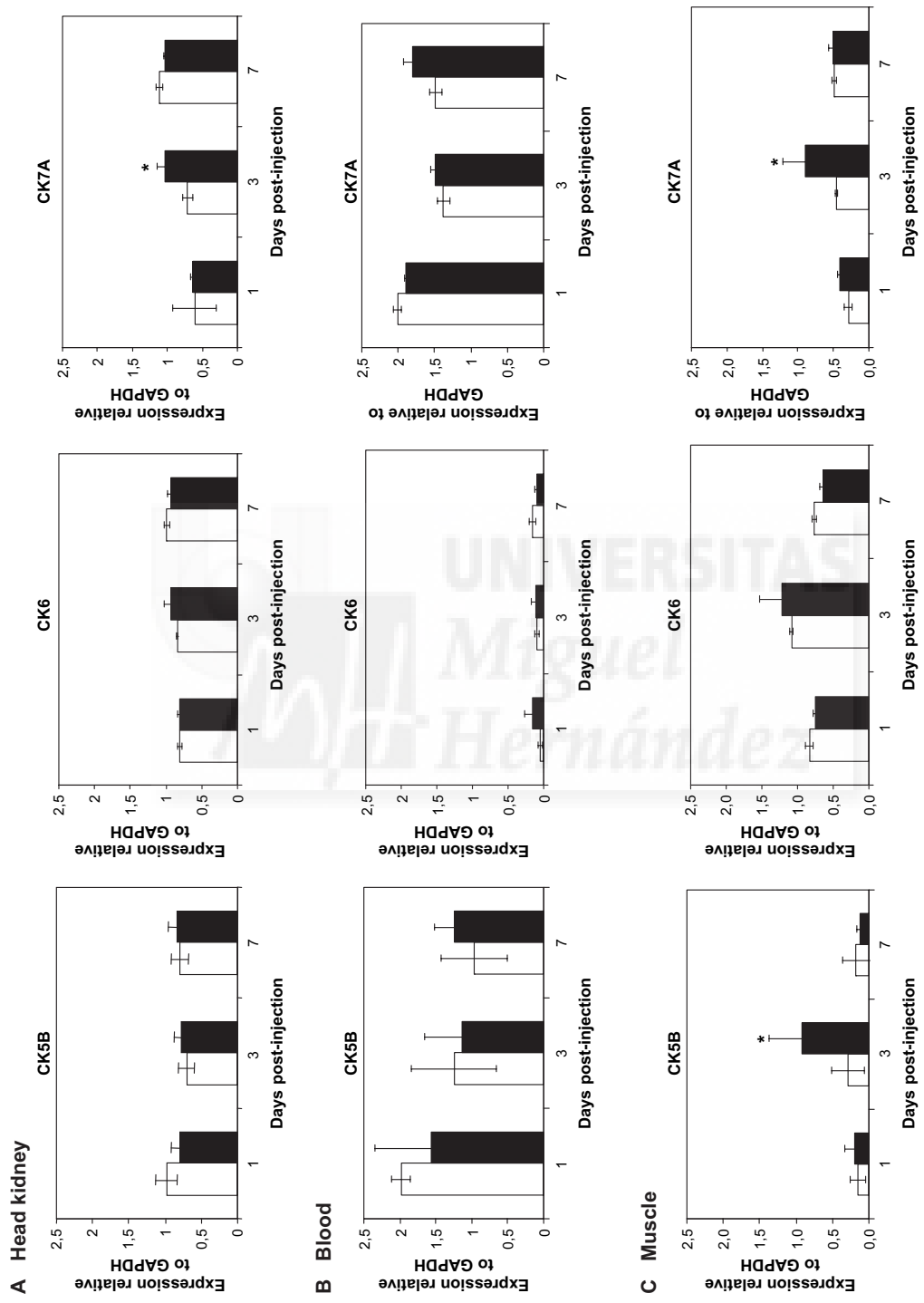

Figure 2 Effect of HNP1 on the expression of the CC chemokines CK5B, CK6 and CK7A. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

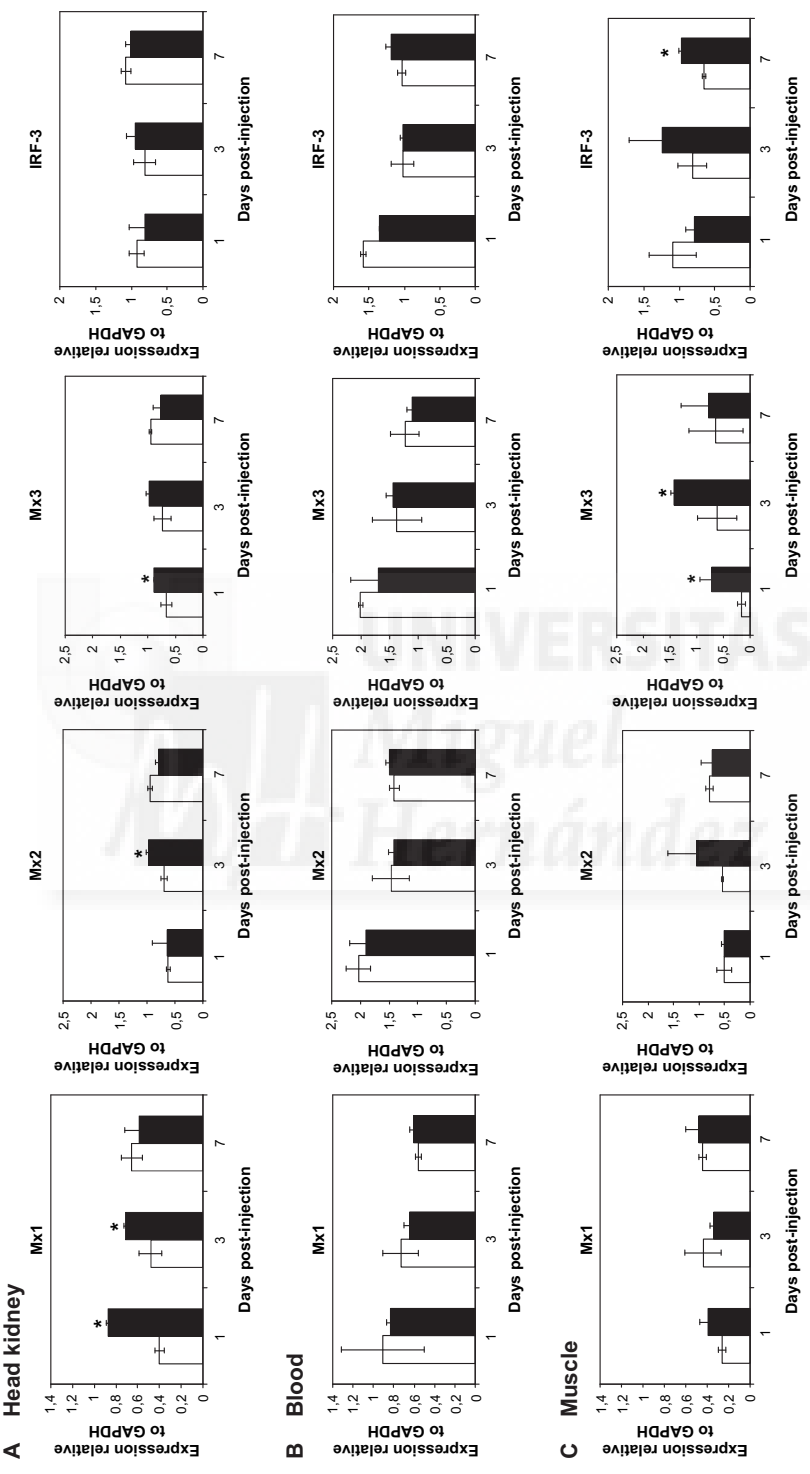

Figure 3 Effect of HNP1 on the expression of genes related to IFN production: *Mx1*, *Mx2*, *Mx3* and IRF-3. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

Effect of HNP1 on the expression of CC chemokines

We also studied the effect of HNP1 administration on the expression of chemokines belonging to the CC family such as CK5B, CK6 and CK7 (Fig. 2). In the head kidney, only the levels of expression of CK7A were increased in treated animals. The levels of expression found in blood were not altered by HNP1, whereas in the muscle, a strong induction of both CK5B and CK7A transcription was observed at day 3.

Effect of HNP1 on the expression of molecules related to the IFN system

The effects of HNP1 on the expression of IFN-induced genes was evaluated through the analysis of the transcript expression of the three trout Mx isoforms and IRF-3 (Fig. 3). In the head kidney, the three Mx isoforms were significantly induced in response to HNP1, while no effect on IRF-3 expression was observed. In the blood, neither of the IFN-related genes studied was altered. In the muscle, Mx3 was the only isoform induced. IRF-3 was also significantly induced in this tissue after 7 days post-injection.

Effect of HNP1 on the expression of molecules related to macrophage functions

The injection of HNP1 did not have a significant effect on the expression of genes related to macrophage activation such as iNOS or IFN-γ in the head kidney nor in blood (Fig. 4). However, a significant up-regulation of iNOS was observed in the muscle at day 3 post-injection.

Effect of HNP1 on the expression of MHC genes

Concerning the effect of HNP1 injection on the expression of MHC genes, only MHC-I was significantly up-regulated when compared to the controls and only in muscle and only on day 7 (Fig. 5).

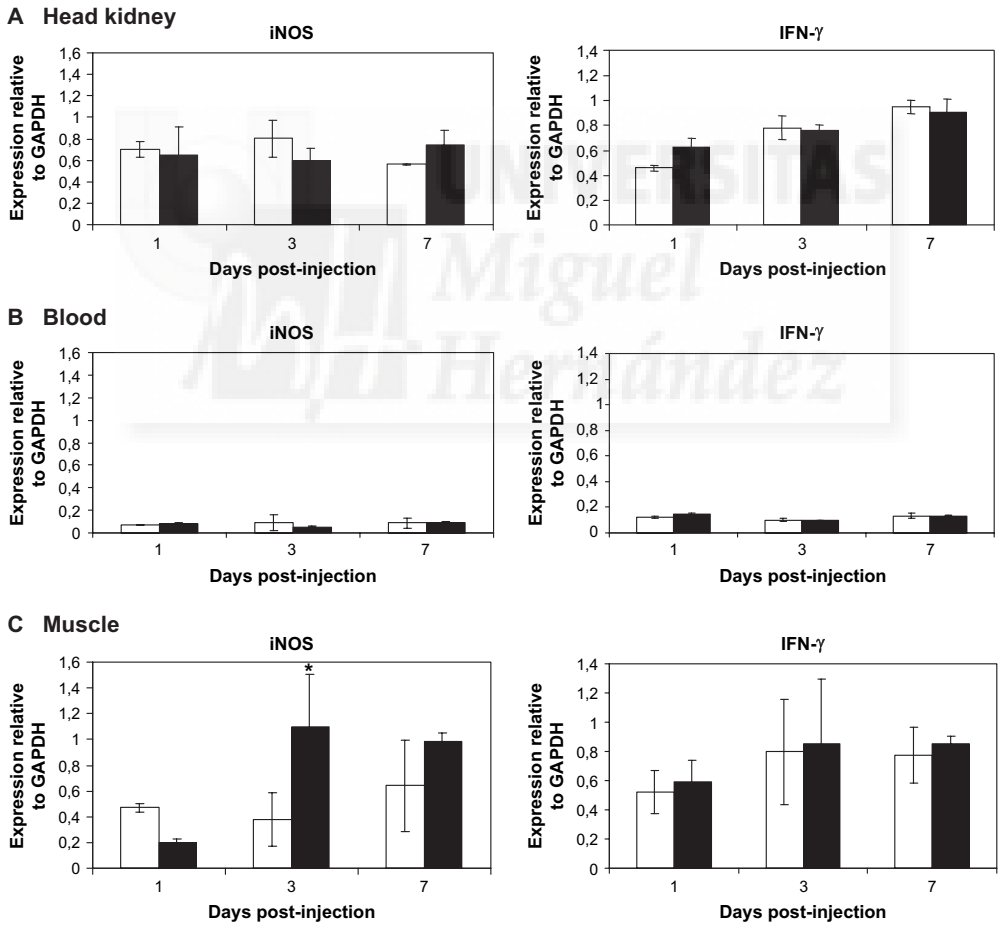

Figure 4 Effect of HNP1 on the expression of genes implicated in macrophage activation: iNOS and IFN-γ. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression $\pm$ SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

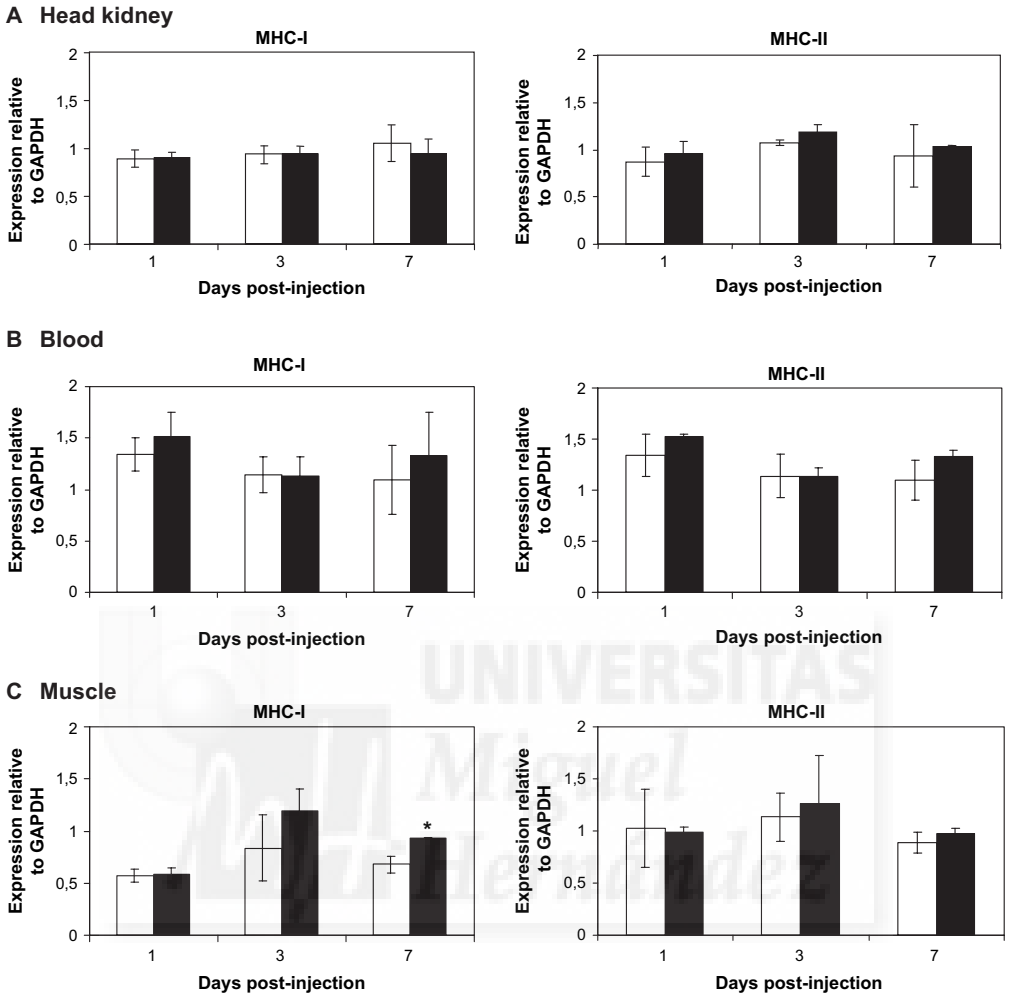

Figure 5 Effect of HNP1 on the expression of MHC genes. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (white bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

Chemoattractant capacity of HNP1

To elucidate a possible direct effect of defensins in fish leukocyte chemotaxis, we studied if HNP1 could specifically attract trout blood leukocytes. First, a high range of HNP1 concentrations was assayed in order to establish the optimal HNP1 doses. Once we determined that the optimal HNP1 doses for this assay ranged from 0.1 and 2 μg ml^{-1} HNP1, we performed the assay in leukocytes belonging to three different trout (Fig. 6). We found a significant migration of leukocytes when compared to controls towards 0.1 and 1 μg ml^{-1} HNP1.

Discussion

In the current study, using human HNP1, we have evaluated for the first time in fish the immunomodulatory capacity of a defensin in vivo. We have demonstrated that HNP1 is active across species in vivo and has diverse immunomodulatory properties in fish, in addition to its established in vitro antiviral activity against VHSV [15].

HNP1 was able to modulate the expression of many genes related to the innate immune response (genes encoding pro-inflammatory cytokines, chemokines, IFN-stimulated genes, etc.) in rainbow trout, not only in the muscle (site of injection) but for some genes also in the blood and head kidney. Since the presence of HNP1 is not expected in the head kidney after intramuscular injection, the effects of HNP1 seen on this organ should be part of a systemic response to this α-defensin. Because the biological effects of AMPs, similarly to their expression and secretion, are often induced by inflammatory stimuli and are influenced by the physiological setting, including the concentration of the peptide,

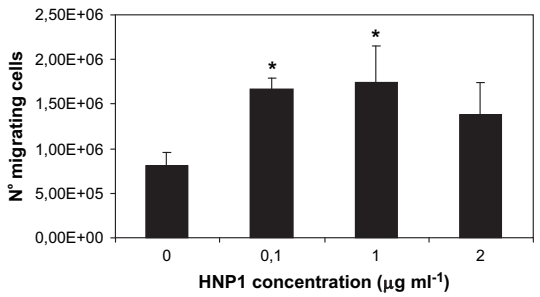

Figure 6 Migration of trout blood leukocytes towards HNP1. Chemotactic activity towards HPN1 was assayed in 96-well chemotaxis chambers. The different dilutions of HPN1 (30 μl) were placed in the lower chambers, while 30 μl of the cell suspensions (5×10^6 cells ml^{-1}) were placed in the upper wells. After 60 min of incubation at 20 °C, the number of cells that had migrated to the bottom chambers were counted using CellTiter 96 (Promega). After the addition of 5 μl of CellTiter96 per well, the plate was incubated at 37 °C for 2 h. After that time, the optical density at 492 nm was determined. The number of cells was estimated using a standard curve with known cell concentrations. Results are presented as the mean number of cells that had migrated ± SD ($N = 3$).

the cellular environment and soluble components of the extracellular milieu, we also analysed the in vivo effect of LmPle, a cationic AMP of 25 amino acid member of a larger family of AMPs present in flatfish, on the expression of these immune genes. Of all genes studied, LmPle only produced a significant up-regulation of the levels of expression of pro-inflammatory cytokines (data not shown), thus, we can conclude that most of the effects of HNP1 are an exclusive response to the HNP1.

Regarding the expression of pro-inflammatory cytokines (IL-1β, TNF-α1 and IL-8) HNP1 strongly increased all three cytokines in the muscle, and also in the blood in the case of IL-1β, and in the head kidney in the case of IL-8. In mammals the effect of HNPs on IL-1β expression is controversial [7], and it seems to be dependant on the cell type. In our work, we observed an up regulation of IL-1β in response to HNP1, however, we also observed a significant increase of IL-1β in response to LmPle (data not shown) which confirms previous results showing induction of IL-1β by Ple in RTS11 cells (trout macrophages) [19]. Thus, it seems that the effect that HNP1 produces on IL-1β and TNF-α1 genes, are mostly part of an inflammatory response to a foreign peptide. The levels of expression of IL8 induced by HNP1 were, however, significantly stronger, in agreement with what had been previously reported in mammals [6,7]. Although IL-8 is characteristic of the early immune response and it belongs to the CXC family of chemokines [20], this cytokine can be classified within the pro-inflammatory cytokines as well as within chemokines. This ''dual character'' of IL8 could be underlying the differential effect of HNP1 on the IL-8 induction related to that observed for IL-1β and TNF-α1. There is a close relation between antimicrobial peptides and chemokines, and although controversial, it has been proposed that some antimicrobial peptides evolved from chemokines [21], since some chemokines have some antimicrobial activity [22], and many antimicrobial peptides have chemoattractant capacity [5]. To investigate whether any relation between HNP1 and chemokines can observed in fish, we also analysed the effect of HNP1 on the expression of other chemokines belonging to the CC family: CK5B, CK6 and CK7A. In rainbow trout, two forms (A and B) are found for CK5 and CK7 [23] but their biological significance is still unknown. For this study, we chose only one of the isoforms for each of these genes. HNP1 was also capable of increasing the levels of expression of two of the three CC chemokines studied, CK5B and CK7A, mostly in the muscle, although for CK7A, some effect was also visible in the head kidney. CK5B and CK7A, are homologues of the mammalian RANTES (regulated on activation, normal T cells expressed and secreted) and MCP (monocyte chemotactic protein), respectively. It seems that this effect on CC chemokines is specific for HNP1, since other peptides such as LmPle or even VHSV (Jana Montero, personal communication) failed to induce their expression. In this context, we thought it was important to study whether HNP1 by itself was chemotactic for fish leucocytes, as in mammals. We found that HNP1 significantly attracted trout blood leukocytes. Again, this demonstrates that antimicrobial peptides play a major role in chemotaxis, in part indirectly, by the activation of other chemokines, and directly by being chemotactic themselves.

Since the in vitro inhibition of VHSV by HNP1 is, at least in part, mediated by a type I IFN-antiviral response [15], we evaluated the effect of HNP1 injection on the expression of IFN-related genes such as the different Mx isoforms found in rainbow trout and IRF-3. In the head kidney, all three Mx isoforms were induced, while only Mx3 was significantly induced in the muscle. Preferential induction of the Mx3 isoform in muscle cells regardless of the IFN inductor used has been recently reported [24]. Previous studies had showed that other antimicrobial peptides such as cecropin and Ple were not able to increase the levels of expression of Mx genes in RTS11 cells [19], thus again, the capacity of HNP1 to modulate the expression of genes related to the IFN system, seems exclusive to HNP1.

Concerning genes related to the macrophage response, only iNOS was significantly up-regulated in the muscle. Studies performed in head kidney leucocytes in vitro also demonstrated an increased iNOS expression in response to HNP1 [14]. More work should be done to determine if the NO released plays a role in the microbicidal activity of defensins. As occurred in response to cecropin and Ple [19], MHC-II gene transcription was not altered by HNP1, but a modest induction of MHC-I genes, probably with an unknown biological significance, was observed in the muscle.

In conclusion, we have demonstrated for the first time in fish that human α-defensins such as HNP1, are able to modulate the cytokine response in vivo, having the most relevant effects on genes related to IFN production and chemokines, since other effects seen for example on pro-inflammatory genes and MHC are most probably due to non-specific responses to a foreign peptide. Moreover, the chemoattractant capacity of a defensin has been established for the first time in fish. Regardless of their possible biological significance, the immunostimulant effect of HNP1 on fish immune response is clearly of interest from an immunotherapeutic and vaccinology perspective.

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References

[1] Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, Bainton DF, et al. Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest 1985;76:1427—35.
[2] Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI. Primary structures of three human neutrophil defensins. J Clin Invest 1985;76:1436—9.
[3] Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 2000;96:3086—93.
[4] Lehrer RI, Ganz T, Szklarek D, Selsted ME. Modulation of the in vitro candidacidal activity of human neutrophil defensins by target cell metabolism and divalent cations. J Clin Invest 1988;81:1829—35.
[5] Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, et al. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. J Biol Chem 1996;271:2935—40.
[6] Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. Am J Physiol 1997;272:L888—96.
[7] Sakamoto N, Mukae H, Fujii T, Ishii H, Yoshioka S, Kakugawa T, et al. Differential effects of alpha- and beta-defensin on cytokine production by cultured human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 2005;288:L508—13.
[8] Lillard Jr JW, Boyaka PN, Chertov O, Oppenheim JJ, McGhee JR. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. Proc Natl Acad Sci U S A 1999;96:651—6.
[9] Tani K, Murphy WJ, Chertov O, Salcedo R, Koh CY, Utsunomiya I, et al. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. Int Immunol 2000;12:691—700.
[10] Brogden KA, Heidari M, Sacco RE, Palmquist D, Guthmiller JM, Johnson GK, et al. Defensin-induced adaptive immunity in mice and its potential in preventing periodontal disease. Oral Microbiol Immunol 2003;18:95—9.
[11] Bowdish DM, Davidson DJ, Hancock RE. Immunomodulatory properties of defensins and cathelicidins. Curr Top Microbiol Immunol 2006;306:27—66.
[12] Daher KA, Selsted ME, Lehrer RI. Direct inactivation of viruses by human granulocyte defensins. J Virol 1986;60:1068—74.
[13] Zou J, Mercier C, Koussounadis A, Secombes C. Discovery of multiple beta-defensin like homologues in teleost fish. Mol Immunol 2006:80.
[14] Falco A, Chico V, Marroqui L, Perez L, Coll J, Estepa A. Expression and antiviral activity of a beta-defensin like peptide identified in the rainbow trout (*Oncorhynchus mykiss*) EST sequences. Mol Immunol 2007. doi:10.1016/j.molimm.2007.06.358.
[15] Falco A, Mas V, Tafalla C, Perez L, Coll J, Estepa A. Human alpha-defensin 1 inhibits in vitro infection of viral haemorrhagic septicaemia rhabdovirus (VHSV) by inactivating virus particles and inducing a type I interferon antiviral response in fish cells. Antiviral Res 2007;76:111—23.
[16] Lorenzen N. Use of plasmid DNA for induction of protective immunity. Bull EAFP 2004;24:11—5.
[17] Brocal I, Falco A, Mas V, Rocha A, Perez L, Coll JM, et al. Stable expression of bioactive recombinant pleurocidin in a fish cell line. Appl Microbiol Biotechnol 2006;72:1217—28.
[18] Chung S, Secombes CJ. Activation of rainbow trout macrophages. J Fish Biol 1987;31:51—6.
[19] Peter Chiou P, Khoo J, Bols NC, Douglas S, Chen TT. Effects of linear cationic alpha-helical antimicrobial peptides on immune-relevant genes in trout macrophages. Dev Comp Immunol 2006;30:797—806.
[20] Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T, et al. Identification and analysis of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss*. Dev Comp Immunol 2002;26:433—44.
[21] Durr M, Peschel A. Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. Infect Immun 2002;70:6515—7.
[22] Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, et al. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. J Leukoc Biol 2003;74:448—55.
[23] Laing KJ, Secombes CJ. Trout CC chemokines: comparison of their sequences and expression patterns. Mol Immunol 2004;41:793—808.
[24] Tafalla C, Chico V, Pérez L, Coll J, Estepa A. In vitro and in vivo differential expression of rainbow trout (*Oncorhynchus mykiss*) Mx isoforms in response to viral hemorrhagic septicemia virus (VHSV) G gene, Poly I:C and VHSV. Fish Shellfish Immunol 2007;23:210—21.
[25] Wang T, Zou J, Cunningham C, Secombes CJ. Cloning and functional characterisation of the interleukin-1 beta 1 promoter of rainbow trout (*Oncorhynchus mykiss*). Biochim Biophys Acta 2002;1575:108—16.
[26] Zou J, Peddie S, Scapigliati G, Zhang Y, Bols NC, Ellis AE, et al. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. Dev Comp Immunol 2003;27:813—22.
[27] Lindenstrom T, Secombes CJ, Buchmann K. Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. Vet Immunol Immunopathol 2004;97:137—48.
[28] McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, Ellis AE. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression. Fish Shellfish Immunol 2003;15:39—50.
[29] Zou J, Carrington A, Collet B, Dijkstra JM, Yoshiura Y, Bols N, et al. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish. J Immunol 2005;175:2484—94.
[30] Hansen JD, La Patra S. Induction of the rainbow trout MHC class I pathway during acute IHNV infection. Immunogenetics 2002;54:654—61.

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Expression and antiviral activity of a β-defensin-like peptide identified in the rainbow trout (*Oncorhynchus mykiss*) EST sequences

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Abstract

The *in silico* identification of a β-defensin-like peptide sequence (omBD-1) in the rainbow trout (*Oncorhynchuss mykiss*) database of salmonid EST is reported here. We have studied the transcript expression of this β-defensin-like sequence in different organs and expressed the recombinant peptide in a fish cell line. Finally, we have demonstrated the *in vitro* antiviral activity of the recombinant trout β-defensin-like peptide against viral haemorrhagic septicaemia rhabdovirus (VHSV), one of the most devastating viruses for worldwide aquaculture. Thus, the resistance to VHSV infection of EPC cells transfected with pMCV 1.4-omBD-1 has been shown. Since EPC cells transfected with omBD-1 produced acid and heat stable antiviral activity and up regulation of Mx, a type I IFN-mediated mechanism of antiviral action is suggested. To our knowledge, this is the first report showing biological activity of a β-defensin-like peptide from any fish.

Keywords: Antimicrobial peptide; Defensin; VHSV; IFN; Mx

1. Introduction

Antimicrobial peptides (AMPs) are ancient components of the innate immune system and have been isolated from organisms spanning the phylogenetic spectrum. Defensins constitute a family of evolutionarily related vertebrate AMPs with antimicrobial, antiviral and immunomodulatory properties that have a characteristic β-sheet-rich fold and a framework of six disulphide-linked cysteines (Ganz, 2003; Selsted et al., 1985). The vertebrate defensins are classified into α-, β- and θ-defensins, which differ in the distribution of the disulphide bonds between their six conserved cysteine residues (Klotman and Chang, 2006; Selsted and Ouellette, 2005; Yang et al., 2002). Both α- and β-defensins have been identified in almost every vertebrate species searched but not θ-defensins (Pazgier et al., 2006; Tang et al., 1999). Defensin and defensin-like peptides have also been identified in invertebrates (Castro and Fontes, 2005; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005; Lehrer and Ganz, 2002; Selsted and Ouellette, 2005) and plants (Castro and Fontes, 2005; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005; Lehrer and Ganz, 2002; Selsted and Ouellette, 2005).

In fish, homologue sequences to β-defensins from higher vertebrates have been recently identified only in three species, zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*) and tetraodron (*Tetraodon nigroviridis*) (Zou et al., 2007). No antimicrobial, antiviral or immunomodulatory properties of these fish β-defensins-like peptides have been described so far. The presence of α-defensins in fish has not been reported yet.

Because of the economic and social impact of both bacterial and viral infections in aquaculture and since no efficient therapeutic agents against fish pathogens have yet been developed, defensin identification and characterization in commercially important fish species could be of great interest. In light of this, the identification of a β-defensin-like sequence from rainbow trout (*Oncorhynchuss mykiss*), a continental fish specie of economical importance in aquaculture is reported here. Moreover, we have studied the expression of this trout β-defensin-like sequence (omBD-1) in different trout organs, and expressed the recombinant peptide in a fish cell line. Finally, we have also assessed the biological activity of the recombinant omBD-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV),

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one of the most devastating viruses for worldwide aquaculture (Lorenzen and LaPatra, 2005; Olesen and Korsholm, 1997). All the results obtained suggest that further studies of this novel trout β-defensin-like peptide as well as its place in the fish innate immunity *in vivo* could lead to development of new strategies and agents to prevent and/or treat fish viral infections.

2. Materials and methods

2.1. Search of β-defensin-like sequences in salmonids EST databases

The individual nucleotide coding sequences of each of the 36 human β-defensins sequences (http://www.expasy.org/cgi-bin/sprot-search-de?human%20beta%20defensin) shown in Table 1 were used to screen the database of expressed sequence tags (ESTs) from salmonid (http://grasp.mbb.sfu.ca). BLAST analysis was made with the BLAST software from the EST BLAST server on the Genomic Research on Atlantic Salmon Project home page (http://grasp.mbb.sfu.ca). It retrieved one related sequence of 779 bp (GeneBank accession number, BX073898) from rainbow trout (*O. mykiss*). Analysis of the translated protein sequence was made by the translate tool from ExPASy proteomic server (http://www.expasy.org/tools/dna.html).

2.2. Sequences analysis, phylogenetics analysis and protein modelling

The existence of a signal peptide was predicted by the SignalP 3.0 program (Bendtsen et al., 2004). The molecular weight, the isoelectric point (p*I*) and the net charge of the peptide were calculated using the ProtParam tool (http://www.expasy.org/tools). Multiple sequence alignments between omBD-1 and the known β-defensin-like of several fish and human β-defensin-26 (hBD-26) sequences (Table 2) (UniProtKB/Swiss-Prot database) were generated with the Clustal W program (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994), and identity percentages (IP) among sequences were determined with the LALING program (http://www.ch.embnet.org/software/LALIGN_form.html). A phylogenetic tree based on the deduced aminoacid sequences was performed by using the Neighbour–Joining (NJ) algorithm. Finally, omBD-1 amino acid sequence was modelled using the Swiss-PdbViewer program (Guex and Peitsch, 1997). Mouse β-defensin 8 (PDB file code: IE4R) was selected as template

Table 1
Human β-defensin (hBD) mature peptide sequences used to screen the salmonid EST database

hBD	UniProtKB/Swiss-Prot entry
hBD-1	P60022
hBD-2	O15263
hBD-3	P81534
hBD-4	Q8WTQ1
hBD-5	Q8NG35
hBD-6	Q8N104
hBD-7	Q8IZN7
hBD-8	Q8NET1
hBD-9	Q30KR1
hBD-10	Q30KR0
hBD-11	Q30KQ9
hBD-12	Q30KQ8
hBD-13	Q30KQ7
hBD-14	Q30KQ6
hBD-15	Q30KQ5
hBD-16	Q30KQ4
hBD-17[a]	Q30KQ3
hBD-18	Q96PH6
hBD-19	Q8N690
hBD-20	Q8N689
hBD-21	Q5J5C9
hBD-23	Q8N688
hBD-24	Q8NES8
hBD-25	Q8N687
hBD-26	Q9BYW3
hBD-27	Q9H1M4
hBD-28	Q7Z7B8
hBD-29	Q9H1M3
hBD-30	Q30KQ2
hBD-31	P59861
hBD-32	Q7Z7B7
hBD-33	Q30KQ1
hBD-34	Q4QY38
hBD-35	Q30KP9
hBD-36	Q30KP8

[a] Fragment, not complete sequence.

Table 2
β-Defensin (BD) mature peptide sequences used to compare with omBD-1

Name	Accession number	Specie	aa	Net charge	Mw (KDa)	pI
omBD-1	918595	***O**ncorhynchus* ***m**ykiss* (Trout)	41	+2	4.38	8.35
zfBD-1	AM181358	*Danio rerio* (**Z**ebra fish)	43	+1	4.52	7.82
zfBD-2	AM181359	*Danio rerio* (**Z**ebra fish)	43	+3	5.11	8.68
zfDB-3	AM181360	*Danio rerio* (**Z**ebra fish)	43	+7	5.29	9.50
fuBD-1	BN000875	*Takifugu rubripes* (**Fu**gu)	42	+2	4.54	8.35
tnBD-1	BN000873	***T**etraodon* ***n**igroviridis* (Green spotted pufferfish)	42	+2	4.50	8.35
tnBD-2	BN000874	***T**etraodon* ***n**igroviridis* (Green spotted pufferfish)	45	+2	5.41	8.34
ogBD-1	AY129305	*Epinephelus coioides* (**O**range spotted grouper)	43	+4	5.17	8.92
hBD-26	AF525928	*Homo sapiens* (**H**uman)	43	+4	4.95	8.84

Letters in bold in species names were used to identify the BD names. Fish β-defensin mature peptide sequences were obtained from Zou et al. (2007) and hBD-26 mature peptide sequence (NWYVKKCLND VGICKKKCKPEEMHVKNGWAMCGKQRDCCVP ADRRANYPVFCVQ TKTTRISTVTATTATTTLMMTT ASMSSMAPTPVSPTG) correspond to UniProtKB/Swiss-Prot entry Q9BYW3.

structure because of its higher sequence similarity among all the available β-defensin PDB files.

2.3. Fish

Rainbow trout of approximately 8–10 cm obtained from Lillogen (Leon, Spain) were maintained at the University Miguel Hernández (UMH) aquarium at 12–14 °C and fed daily with a commercial diet (Trouw, Leon, Spain). Prior to experiments, fish were acclimatised to laboratory conditions during at least 2 weeks.

2.4. Expression studies

The expression of omBD-1 in different trout tissues was analyzed by RT-PCR in 12 healthy trout by using specific primers deduced from the trout EST encoding the omBD-1 (omBD-1 forward 5′-ATGGTCACTTTGGTGCTCCTGG-3′ and reverse 5′-TTAGAAATGAGAAACACAGCACAAG-3′). Fish were sacrificed by overexposure to tricaine methanesulfonate, MS222 (Sigma) and head kidney, spleen, liver and muscle dissected for RNA extraction. The "Total RNA Isolation System" kit (Promega) was used for RNA extraction from the different rainbow trout organs following manufacturer's instructions. Isolated RNAs were resuspended in diethylpyrocarbonate (DEPC)-treated water, treated with DNase (RQ1 RNAase-Free Dnase, Promega) and stored at −80 °C until used. Two micrograms of RNA were used to obtain cDNA by using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen). Briefly, RNA was incubated with 1 μl of random hexamers (50 μM) (Roche) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of M-MLV reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min and the resulting cDNA stored at −20 °C.

All PCR amplification reactions were performed by using 0.5 μl dNTP mix (10 mM each), 0.125 μl Taq DNA polymerase (Roche, Barcelona, Spain), 2.5 μl Taq 10× buffer, 1 μl of each primer (20 μM) and 2.5 μl of cDNA in a final volume of 25 μl. A parallel PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Wang et al., 2004) was performed with all samples as a house-keeping gene to standardise the RT-PCR results using the conditions previously described (Tafalla et al., 2007; Wang et al., 2004). PCRs were carried out in a GeneAmp® PCR System 2700 cycler (Applied Biosystems). The amplification conditions consisted in a denaturing step of 94 °C for 5 min followed by 25–30 cycles of 94 °C 30 s, 52 °C 30 s and 72 °C 30 s followed by a final extension step of 72 °C 7 min. PCR products (8 μl) were visualised on a 1.5% agarose gel stained with ethidium bromide. A 100 bp ladder was used as a size marker.

2.5. Cloning and sequencing of omBD-1

For cloning purposes, all PCR products amplified from trout head kideny and muscle were resolved on a 1% agarose gel; bands excised from the gel, extracted by using Gene-Clean (Bio 101, La Jolla, CA, USA) and then cloned into the PCR II-Topo vector (Invitrogen, CA, USA). The sequence of the inserts was determined by Sistemas Genómicos S.L. (Valencia, Spain) using specific primers for the PCR II-Topo vector. After sequencing, one of the PCR product obtained from muscle (183 bp) was excised from the PCR II-Topo with the restriction enzymes KpnI and XhoI and subcloned into the KpnI/XhoI site of the expression vector pMCV 1.4 (Ready-Vector, Madrid, Spain) following standard procedures. The pMCV 1.4 vector contains the human cytomegalovirus immediate-early-promoter (CMV).

2.6. Cell cultures and virus

The fish cell line EPC (*epithelioma papulosum cyprinid*) (Fijan et al., 1983), purchased from the European collection of cell cultures (ECACC n°. 93120820) was used in this work. EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium containing 10% fetal calf serum (Sigma Chem. Co., St. Louis, MO, USA), 1 mM pyruvate (Gibco), 2 mM glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone.

Viral haemorrhagic septicaemia virus (VHSV 07.71) isolated in France from rainbow trout, *O. mykiss* (LeBerre et al., 1977) was propagated in EPC cells at 14 °C as previously reported (Basurco and Coll, 1989). Supernatants from VHSV 07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* for 20 min and kept in aliquots at −70 °C. Clarified supernatants were used for the experiments.

2.7. Transfection assays and analysis of the expression of omBD-1 transcripts by RT-PCR in EPC transfected cells

For cell transfection, EPC cell monolayers, grown in culture flasks of 75 cm^2, were detached using trypsine (Sigma), washed, resuspended in culture medium supplemented with 10% of FCS and dispensed into 96-well plates at a concentration of 3×10^4 cells per well in a final volume of 100 μl. Next day, different concentrations (from 0.25 to 2 μg/ml) of either pMCV 1.4-omBD-1 or pMCV 1.4 complexed with 0,3 μl of FuGene 6 (Roche) were incubated for 15 min in 25 μl of RPMI-1640 containing 2 mM Cl_2Ca and then added to each well in 100 μl of culture medium with 10% of FCS (Brocal et al., 2006; Lopez et al., 2001). As an additional control, EPC cells were transfected with FuGene 6 alone following the same procedure. The plates were further incubated at 28 °C for 48 h. The expression of transcripts from the recombinant omBD-1 in transfected cells was then assessed by RT-PCR. RNA extraction from transfected cells, synthesis of cDNA and PCR amplification of the omBD-1 sequence were performed as described above (Section 2.3) but carp β-actin and their primers (Y.B. Zhang et al., 2004). PCR products (8 μl) were visualised on a 1% agarose gel stained with ethidium bromide. Samples to be compared were run in the same agarose gel. The intensity of the amplified bands was estimated using the Imagen Scion Program (www.scionorg.com). Analysis of mRNA transcription of the

recombinant omBD-1 was performed and expressed as relative to the β-actin gene transcription (expression relative to β-actin). PCR products amplified from transfected EPC cells were sequenced.

2.8. Preparation of conditioned medium (CM) from EPC cell cultures

In order to prepare conditioned medium (CM) from transfected cells, EPC cells were transfected with 1 μg/ml of pMCV 1.4-omBD-1, pMCV 1.4 or FuGene alone as indicated above (Section 2.6) for 24 h. After extensive washes with PBS, 100 μl of fresh medium supplemented with 10% FCS were added to each well. Following incubation at 28 °C for 24 h, the supernatants collected from transfected cells were clarified by centrifugation at 800 × *g* for 10 min. Clarified supernatants were then treated with 1N HCl, which reduced the pH to 2. After overnight incubation at 4 °C, the samples were heated to 50 °C for 60 min and the pH was restored to 7.6 by addition of 1N NaOH.

2.9. Viral infectivity assays

To assay for VHSV infectivity, a previously developed immunostaining focus assay (focus forming units, f.f.u.) was used (Lorenzo et al., 1996; Mas et al., 2002, 2006; Micol et al., 2005; Perez et al., 2002). Briefly, EPC cells, grown in 96-well plates, either non-transfected, transfected or treated during 24 h with CM (diluted from 1 to 1/500) were washed extensively with PBS and then infected with VHSV (multiplicity of infection (m.o.i.) of 2×10^{-3}) in a final volume of 100 μl/well of culture medium supplemented with 2% FCS. Infected EPC cells were incubated for 24 h at 14 °C. The EPC cell monolayers were then fixed for 10 min in cold methanol and air-dried. Monoclonal antibody (MAb) 2C9 directed towards the N protein of VHSV diluted 1000-fold in dilution buffer (0.24 mM merthiolate, 5 g/l Tween 20, 50 per l mg of phenol red in PBS, pH 6.8) were added to the wells (100 μl/well) and incubated for 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled rabbit anti-IgG mouse antibody (Ab) (Nordic, Tilburg, The Netherlands) were added per well, and incubation was continued for 30 min. After three washings by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma) in PBS containing H_2O_2 were added (Lorenzo et al., 1996; Sanz and Coll, 1992) and the reaction allowed to proceed until brown foci were detected with an inverted microscope (Nikon Eclipse TE2000-U, Nikon Instruments Inc., NY, USA). Once washed with water and air dried, brown foci of DAB stained cells (VHSV-infected cell foci) were counted with an inverted microscope with a 10× ocular eye grid (Lorenzo et al., 1996).

2.10. Detection of the mRNA of carp Mx1 gene in transfected EPC cells by RT-PCR

The expression of the IFN-inducible Mx1 gene of carp was analyzed by RT-PCR in EPC cells transfected with different amounts of pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 alone as indicated in Section 2.6. As positive control of Mx expression, EPC cells, grown in 96-well plates, were treated with 30 μg/ml of polyribocytidylic acid (poly I:C) (Pharmacia, Piscataway, NJ, USA) a well-known type I IFN-inducer. After an incubation of 48 h at 28 °C cells were harvested. In all cases, after the incubation period, the medium was removed, cells dettached with Ca^{2+} and Mg^{2+}-free PBS, total RNA extracted and cDNA synthesized as indicated in Section 2.3. The primers previously designed (Y.B. Zhang et al., 2004) to amplify a 349 bp fragment in the Mx1 sequence of carp were used. The amplification conditions consisted in a denaturing step of 94 °C for 2 min followed by 30 cycles of 94 °C 1 min, 60 °C 1 min and 72 °C 1.30 min followed by a final extension step of 72 °C 5 min. PCR products (8 μl) were visualised on a 1.5% agarose gel stained with ethidium bromide. A 100 bp ladder was used as a size marker. Analysis of mRNA transcription of the carp Mx was performed and expressed as relative to the β-actin gene transcription (expression relative to β-actin). Furthermore, PCR products were sequenced.

3. Results

3.1. Sequence analysis

The search in the database of salmonid EST with one of the 36 human β-defensin nucleotide sequences used in this study (Table 1), the β-defensin-26 (hBD-26), scored a rainbow trout (*O. mykiss*) EST of 715 bp. Further analysis of the corresponding translated amino acid sequence from this EST showed that it contained a β-defensin-like peptide of 60 amino acids (Fig. 1A) that was named *O. mykiss* β-defensin 1 (omBD-1). The N terminus of omBD-1 had the features consistent with a signal peptide as defined by the SignalP 3.0 program analysis with a putative cleavage site located after position 19 (vertical arrow; Fig. 1A). After cleavage, a resulting propeptide of 41 aa (underlined; Fig. 1A), which is present in positions equivalent to those described in the β-defensins consensus sequence six conserved cysteine residues (C-$X_{4–8}$-C-$X_{3–5}$-C-$X_{9–13}$-C-$X_{4–7}$-C-C), showed 85.7% identity with the previously described β-defensin1-like sequences from other fish (zebrafish, Fugu and tetraodon) (Zou et al., 2007) and 34.9% with the hBD-26 from humans (UniProtKB/Swiss-Prot accession number Q9BYW3). As all of them, omBD-1 has a net cationic charge of +2, an isoelectric point of ∼8 and a molecular weight of 4–5 kDa (Table 2). In addition, the alignment of omBD-1 with hBD-26 and the other fish β-defensins-like (Fig. 1B) indicated the existence of other conserved amino acid residues at positions 10 (aromatic residue), 11 (serine/threonine), 17 (glycine), 20 and 21 (positively charged residues), 28 (glutamic acid) and 42 (aromatic residue). The modelling of the amino acid sequence of omBD-1 (data not shown) showed a similar peptide fold to those described for mammalian and the other fish β-defensins.

The phylogenetic tree constructed with β-defensin-like sequences from fish and hBD-26 (Fig. 2) showed that the omBD-

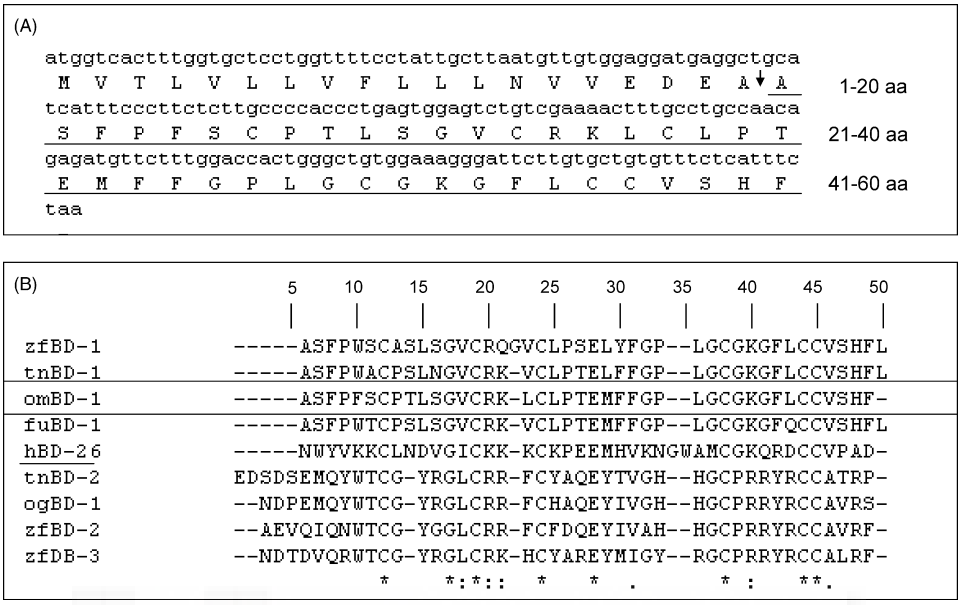

Fig. 1. Sequence (nucleotide and amino acid) of omBD-1 (A) and multiple sequence alignment of omBD-1 with β-defensin-like peptides from zebrafish, Fugu and tetraodon and hBD-26 from humans (B). (A) The nucleotide sequence is indicated in lower case letters. The deduced amino acid sequence (amino acids are numbered) is shown below the nucleotide sequence. The predicted mature peptide is underlined. The arrow shows the putative cleaveage site for signal peptidase. (B) Multiple alignment was performed using the CLUSTAL W program. Fish β-defensin mature peptide sequences were obtained from Zou et al. (2007) and the hBD-26 mature peptide sequence correspond to UniProtKB/Swiss-Prot entry Q9BYW3. (*) Indicates identity, whilst (.) or (:) indicate similarity. The positions of the alignment are numbered. h, Human; om, *Oncorhynchus mykiss*; fu, *Takifugu rubripes*; tn, *Tetraodon nigroviridis*; zf, *Danio rerio*; og, orange spotted grouper; BD, β-defensin.

1 sequence branched together with tetraodon, Fugu and zebrafish BD-1 sequences, whilst tetraodon, Fugu and zebrafish BD-2 sequences and zebrafish BD-3 sequence branched in other group. By contrast, hBD-26 clusters alone showing that is more distantly related.

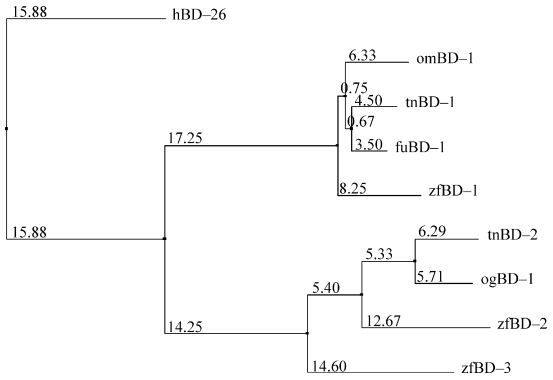

Fig. 2. Phylogenetic analysis of the mature peptide sequence of omBD-1 with known fish β-defensins-like and hBD-26 sequences. The sequences were aligned using CLUSTAL W program and the tree was generated using the Neighbour–Joining method. Fish β-defensin mature peptide sequences were chosen from Zou et al. (2007). hBD-26 mature peptide sequence correspond to UniProtKB/Swiss-Prot entry: Q9BYW3. h, Human; om, *Oncorhynchus mykiss*; fu, *Takifugu rubripes*; tn, *Tetraodon nigroviridis*; zf, *Danio rerio*; og, orange spotted grouper; BD, β-defensin.

3.2. Tissue distribution of omBD-1 mRNA revealed by RT-PCR

RT-PCR was employed by using total RNA extracted from different trout tissues (liver, head kidney, spleen and muscle) to investigate the tissue distribution of omBD-1 in healthy rainbow trout. Furthermore, the constitutive expression of omBD-1 was also assessed in the RTG-2 cell line derived from trout. In all samples analyzed a GAPDH product was amplified in parallel to confirm the good quality of the cDNA used and to serve as an internal control. Constitutive expression of omBD-1 transcripts was mostly detectable in the muscle but low levels of expression were also detected in the head kidney of all individual fingerling trout (Fig. 3). No expression was found of omBD-1 in the RTG-2 cell line (not shown).

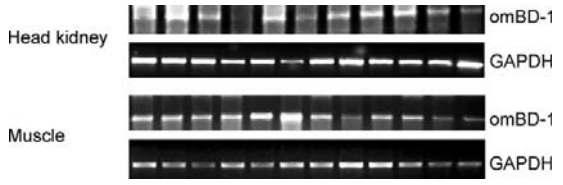

Fig. 3. RT-PCR analysis of omBD-1 mRNA expression in muscle and head kidney of healthy rainbow trout. Total RNA was extracted from these tissues from 11 fingerling rainbow trout, cDNA obtained and PCR amplifications performed as indicated in Section 2. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

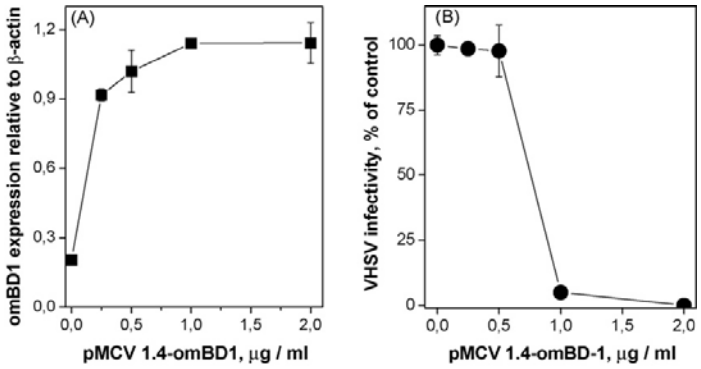

Fig. 4. Expression of omBD-1 mRNA (A) and inhibition of VHSV infectivity in EPC cells transfected with omBD-1 (B). (A) Monolayers of EPC cells were transfected with pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 alone. After 48 h of incubation at 28 °C, total RNA was extracted and the expression of transcripts from omBD-1 was then estimated by RT-PCR. The mRNA expression relative to β-actin was calculated by the formula: intensity of omBD-1 band/intensity of the corresponding β-actin band. Data are mean ± S.D. from two experiments, each performed in triplicate. (B) EPC cells were transfected with pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6. After 24 h, transfected cell monolayers were extensively washed, infected with VHSV in cell culture media containing 2% of FCS and incubated during 24 h at 14 °C. VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. The results were expressed as the percentage of infectivity and calculated by the formula: number of VHSV-infected cell foci in cells transfected with pMCV 1.4-omBD-1/number of VHSV-infected cell foci in cells transfected with FuGene 6 × 100. Data are the mean ± S.D. from two experiments, each performed in triplicate.

3.3. Resistance of EPC cells expressing omBD-1 to VHSV infection

Since several reports showed that β-defensins possess antiviral activity against enveloped virus (Chattopadhyay et al., 2006; Hazrati et al., 2006; Klotman and Chang, 2006; Leikina et al., 2005; Sun et al., 2005), the antiviral properties of omBD-1 against a fish enveloped virus such as VHSV were studied. To carry out the antiviral activity studies of omBD-1, EPC cells were transiently transfected with different amounts of pMCV 1.4-omBD-1. RT-PCR analysis of these cells revealed that this expression vector efficiently expressed omBD-1 mRNA in EPC cells (Fig. 4A). Twenty-four hours after transfection, the EPC cells transiently expressing omBD-1 were infected with VHSV and 24 h later VHSV infectivity was determined. The results showed a 80–90% reduced VHSV infectivity in EPC cells transfected with concentrations higher than 0.50 μg/ml of pMCV 1.4-omBD-1 (Fig. 4B). The control EPC cells (EPC cells transfected with pMCV 1.4 or FuGene 6) efficiently propagated the virus since approximately 130 VHSV-infected cell foci per well were observed (data no shown). These data revealed that EPC cells expressing omBD-1 had reduced susceptibility to VHSV.

3.4. Resistance of EPC cells treated with conditioned medium from EPC cells expressing omBD-1 to VHSV infection

The inhibition of VHSV infectivity in EPC cells transfected with pMCV 1.4-omBD-1 suggested that some cellular antiviral defense mechanisms might have been induced by the expression of omBD-1 in these cells. To investigate the possibility that the expression of omBD-1 can induce secretion of soluble factors with antiviral activity, such as type I IFN, CM medium from non-transfected EPC (CM) and from EPC cells transfected with either pMCV 1.4-omBD-1 (omBD-1-CM), pMCV 1.4 (V-CM) or FuGene 6 alone (F-CM) were prepared. Fresh EPC cells were then treated with these different CM during 24 h and after extensively washing infected with VHSV in the absence of CM. As expected, the treatment of EPC cells with CM from EPC cells transfected with pMCV 1.4-omBD-1 conferred protection against VHSV infection (Fig. 5) but not to those treated with CM from non-transfected EPC cells or from EPC cells transfected with either pMCV 1.4 (not shown) or FuGene 6. Since heat and acid treatments of omBD-1-CM did not eliminate its antiviral activity against VHSV the presence of type I IFN in supernatants from EPC cells transfected with pMCV 1.4-omBD-1 is suggested.

3.5. Transfection of EPC cells with omBD-1 induces expression of the Mx1 gene

If the antiviral activity against VHSV observed in EPC transfected with pMCV 1.4-omDB was mediated by type I IFN, the up regulation of IFN-stimulated genes (ISGs) should be detectable in these cells. To investigate this possibility, EPC cells were transfected with pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 or incubated with poly I:C (a well-known type I IFN-inducer) and the expression of the IFN-induced gene Mx1 was then assessed by RT-PCR. The IFN-inducible Mx1 gene of carp was selected as a marker for IFN responses because direct induction of this gene by carp IFN has been demonstrated (Y.B. Zhang et al., 2004). Carp Mx1 gene was up-regulated in both poly I:C treated (not shown) and omBD-1 transfected EPC cells (Fig. 6) but not in EPC cells transfected only with pMCV 1.4 or FuGene 6. The level of expression of carp Mx1 transcripts was proportional up to eight-fold to the amount of pMCV 1.4-omBD-1 transfected showing a plateau-like at DNA concentrations ≥0.5 μg/ml (Fig. 6B).

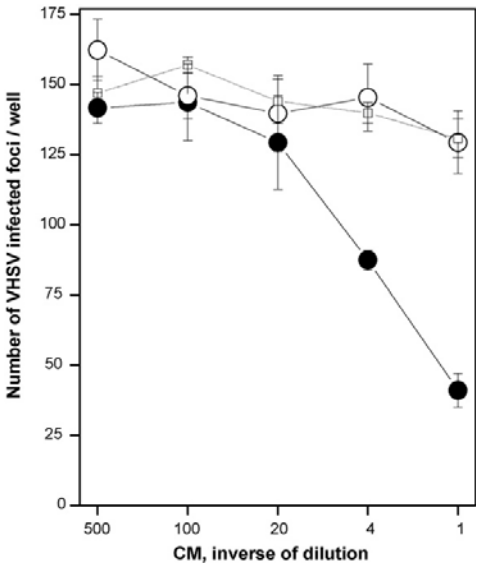

Fig. 5. Inhibition of VHSV infectivity in EPC cells treated with conditioned medium prepared from cells transfected with pMCV 1.4-omBD-1. Fresh EPC cells were incubated during 24 h with CM prepared from EPC cells non-transfected (CM) or transfected with pMCV 1.4-omBD-1 (omBD-1-CM), pMCV 1.4 (V-CM) or FuGene 6 (F-CM). Treated EPC cells were then infected with VHSV in cell culture medium containing 2% of FCS and incubated during 24 h at 14 °C. VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. The results were expressed as the number of VHSV infected cell foci per well. Data are the mean ± S.D. from two experiments, each performed in triplicate. EPC cells treated prior to the infection with CM (□), F-CM (◯) or omBD-1-CM (●).

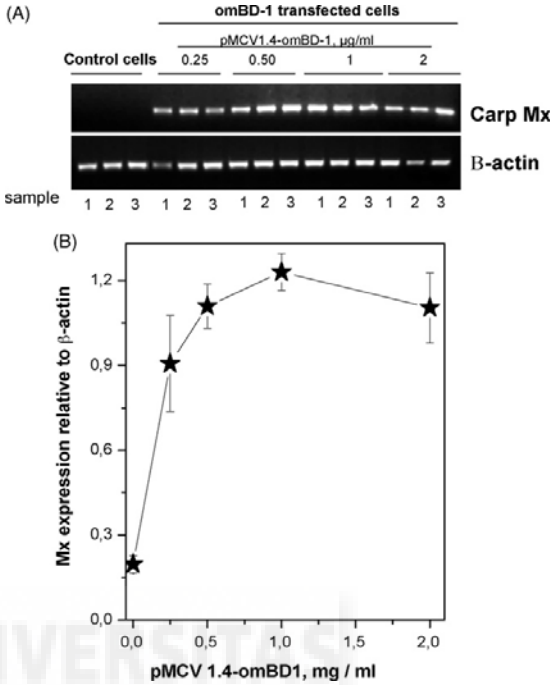

Fig. 6. Expression of carp Mx1 transcripts in EPC cells transfected with pMCV 1.4-omBD-1. Monolayers of EPC cells were transfected with of pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 alone. After 48 h of incubation at 28 °C, total RNA was extracted and the expression of transcripts from the IFN-inducible carp Mx1 gene was then estimated by RT-PCR. The mRNA expression relative to β-actin was calculated by the formula: intensity of Mx1 band/intensity of the corresponding β-actin band. (A) Photography of an agarose gel of Mx1 and β-actin band mRNA amplified by RT-PCR. The gel is representative of two experiments. (B) Data are the mean ± S.D. from two experiments, each performed in triplicate.

4. Discussion

In the present study, we describe the identification of a β-defensin-like sequence in the present EST sequences from trout and demonstrated their *in vivo* expression in different tissues of rainbow trout. In addition, we have cloned the β-defensin-like peptide into an eukaryotic expression vector and shown that fish cells transfected with omBD-1 were protected against infection with VHSV, a fish rhabdovirus. Although homologue sequences to β-defensins from higher vertebrates have been previously described for three non-salmonid species (zebrafish, Fugu and tetraodon) (Zou et al., 2007), their possible biological activities have not yet been described.

Based on the homology comparison and phylogenetic analysis, the multiple defensin-like genes discovered recently in zebrafish, pufferfish and tetraodon have been classified into two subgroups, the BD-1 group and another group containing BD-2 and 3 (Zou et al., 2007). A phylogenetic tree (Fig. 2) constructed with omBD-1 and zebrafish, Fugu and tetraodon β-defensin-like peptides as well as hBD-26 peptides included omBD-1 in the BD-1 group of fish β-defensins, close to fugu and tetraodon BD-1 but in a different branch than zebrafish BD-1. On the contrary, fish β-defensins-like and hBD-26 grouped in a distant branch in this tree.

The omBD-1 expression analyzed by RT-PCR was constitutive in muscle and head kidney (Fig. 3). In contrast, zebrafish β-defensin-like homologues were constitutively expressed in every organ analyzed (Zou et al., 2007). The differential tissue expression of omBD-1 could suggest a tissue-specific regulation of β-defensin in fish such as it has been described for β-defensins from other vertebrates (Pazgier et al., 2006). In mammals, where defensins are more diversified than in other animal groups, β-defensins are predominantly expressed in epithelial cell tissues (Ganz, 2003; Klotman and Chang, 2006; Yang et al., 2004) whilst α-defensins are expressed in immune cells. Since there is no evidence of fish α-defensins existence so far, it might be not surprising that fish β-defensins could be expressed in a broader range of tissues/organs including immune system related organs such as the head kidney. However, the present ESTs might not include yet every trout possible expressed sequence. On the other hand, the lack of omBD-1 expression in the trout liver may be related to the presence of other antimicrobial peptides produced there, such as hepcidins (Douglas et al., 2003) and LEAP-2 (Y.A. Zhang et al., 2004), similarly to what was previously proposed to explain the absence

of expression of fish cathelicidins in fish liver (Chang et al., 2006).

Although the antiviral activity of β-defensins is well known long time ago, the importance of β-defensins against viruses has only recently coming to light. Thus, it has been reported against several viruses, including HIV-1 (Quinones-Mateu et al., 2003; Sun et al., 2005), adenovirus (Klotman and Chang, 2006), influenza virus (Leikina et al., 2005), parainfluenza virus 3 (PIV-3) (Grubor et al., 2004), respiratory syncytial virus (RSV) (Meyerholz et al., 2007), vaccinia virus (VV) (Howell et al., 2007) herpes simplex virus (Hazrati et al., 2006) and Chandipura virus (Chattopadhyay et al., 2006).

To investigate whether the omBD-1 also exhibited antiviral activity, omBD-1 was transiently expressed in EPC cells (a carp derived cell line) under the control of the cytomegalovirus immediate-early (CMV) promoter and then the transfected EPC cells challenged with the viral haemorrhagic septicaemia rhabdovirus (VHSV), one of the most devastating viruses for worldwide aquaculture. The *in vitro* expression system CMV promoter/EPC cells was chosen because: (i) the CMV promoter-enhancer has been found previously to work very efficiently in fish cells (Anderson et al., 1996; Hansen et al., 1991; Trobridge et al., 1997) and (ii) EPC cells are highly susceptible to VHSV (can yield up to 10^9 ffu/ml; Marroqui et al., 2007; Mas et al., 2004) and can be readily transfected (Castric et al., 1992; Lopez et al., 2001; Moav et al., 1992; Rocha et al., 2004) showing higher transfection rates than trout cell lines. In fact, one of the most important limitations of fish cell lines is their relatively low transfection efficiencies compared to those of mammalian cell lines (Altmann et al., 2003). As an example, the RTG-2 cell line, a fibroblastic cell line originated from rainbow trout and theoretically suitable to perform the present study, has transfection efficiencies ranging from 5 to 7% (Tafalla et al., 2007). The results of the transfection–infection assays demonstrated that omBD-1 was expressed in EPC cells (Fig. 4A) and induced an antiviral activity against VHSV (Fig. 4B). Since EPC cells transfected with omBD-1 produced acid and heat-stable antiviral activity (Fig. 5) and also showed up regulation of carp Mx1 gene (Fig. 6), a type I IFN-related antiviral response could be operating in the fish cells transfected with omBD-1. However, further studies are needed to clarify the mechanism underlying the antiviral activity of omBD-1.

Acknowledgements

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References

Altmann, S.M., Mellon, M.T., Distel, D.L., Kim, C.H., 2003. Molecular and functional analysis of an interferon gene from the zebrafish *Danio rerio*. J. Virol. 77, 1992–2002.

Anderson, E.D., Mourich, D.V., Fahrenkrug, S.C., LaPatra, S.C., Shepherd, J., Leong, J.C., 1996. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol. Mar. Biol. Biotechnol. 5, 114–122.

Basurco, B., Coll, J.M., 1989. Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. Bull. Eur. Assoc. Fish Pathol. 9, 92–95.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Brocal, I., Falco, A., Mas, V., Rocha, A., Perez, L., Coll, J.M., Estepa, A., 2006. Stable expression of bioactive recombinant pleurocidin in a fish cell line. Appl. Microbiol. Biotechnol. 72, 1217–1228.

Castric, J., Jeffroy, J., Bearzotti, M., DeKinkelin, P., 1992. Isolation of viral haemorrhagic septicemia virus (VHSV) from wild elvers *Anguilla anguilla*. Bull. Eur. Assoc. Fish Pathol. 12, 21–23.

Castro, M.S., Fontes, W., 2005. Plant defense and antimicrobial peptides. Protein Pept. Lett. 12, 13–18.

Chang, C.I., Zhang, Y.A., Zou, J., Nie, P., Secombes, C.J., 2006. Two cathelicidin genes are present in both rainbow trout (*Oncorhynchus mykiss*) and atlantic salmon (*Salmo salar*). Antimicrob. Agents Chemother. 50, 185–195.

Chattopadhyay, S., Sinha, N.K., Banerjee, S., Roy, D., Chattopadhyay, D., Roy, S., 2006. Small cationic protein from a marine turtle has beta-defensin-like fold and antibacterial and antiviral activity. Proteins 64, 524–531.

Douglas, S.E., Gallant, J.W., Liebscher, R.S., Dacanay, A., Tsoi, S.C., 2003. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev. Comp. Immunol. 27, 589–601.

Fijan, N., Sulimanovic, D., Bearzotti, M., Mizinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F., de Kinkelin, P., 1983. Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Ann. Virol. 134, 207–220.

Ganz, T., 2003. Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. 3, 710–720.

Garcia-Olmedo, F., Molina, A., Alamillo, J.M., Rodriguez-Palenzuela, P., 1998. Plant defense peptides. Biopolymers 47, 479–491.

Grubor, B., Gallup, J.M., Meyerholz, D.K., Crouch, E.C., Evans, R.B., Brogden, K.A., Lehmkuhl, H.D., Ackermann, M.R., 2004. Enhanced surfactant protein and defensin mRNA levels and reduced viral replication during parainfluenza virus type 3 pneumonia in neonatal lambs. Clin. Diagn. Lab. Immunol. 11, 599–607.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.

Hansen, E., Fernandes, K., Goldspink, G., Buterworth, P., Umeda, P.K., Chang, K.-C., 1991. Strong expression of foreign genes following direct injection into fish muscle. FEBS Lett. 290, 73–76.

Hazrati, E., Galen, B., Lu, W., Wang, W., Ouyang, Y., Keller, M.J., Lehrer, R.I., Herold, B.C., 2006. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. J. Immunol. 177, 8658–8666.

Howell, M.D., Streib, J.E., Leung, D.Y.M., 2007. Antiviral activity of human [beta]-defensin 3 against vaccinia virus. J. Allergy Clin. Immunol. 119, 1022–1025.

Klotman, M.E., Chang, T.L., 2006. Defensins in innate antiviral immunity. Nat. Rev. Immunol. 6, 447–456.

Lay, F.T., Anderson, M.A., 2005. Defensins—components of the innate immune system in plants. Curr. Protein Pept. Sci. 6, 85–101.

LeBerre, M., De Kinkelin, P., Metzger, A., 1977. Identification sérologique des rhabdovirus des salmonidés. Bull. Office Int. Epizooties 87, 391–393.

Lehrer, R.I., Ganz, T., 2002. Defensins of vertebrate animals. Curr. Opin. Immunol. 14, 96–102.

Leikina, E., Delanoe-Ayari, H., Melikov, K., Cho, M.S., Chen, A., Waring, A.J., Wang, W., Xie, Y., Loo, J.A., Lehrer, R.I., Chernomordik, L.V., 2005. Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. Nat. Immunol. 6, 995–1001.

Lopez, A., Fernandez-Alonso, M., Rocha, A., Estepa, A., Coll, J.M., 2001. Transfection of epitheloma papulosum cyprini (EPC) carp cells. Biotech. Lett. 23, 481–487.

Lorenzen, N., LaPatra, S.E., 2005. DNA vaccines for aquacultured fish. Rev. Sci. Tech. 24, 201–213.

Lorenzo, G., Estepa, A., Coll, J.M., 1996. Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicemia with anti-nucleoprotein monoclonal antibody. J. Virol. Methods 58, 1–6.

Marroqui, L., Estepa, A., Perez, L., 2007. Assessment of the inhibitory effect of ribavirin on the rainbow trout rhabdovirus VHSV by real-time reverse-transcription PCR. Vet. Microbiol. 122, 52–60.

Mas, V., Perez, L., Encinar, J.A., Pastor, M.T., Rocha, A., Perez-Paya, E., Ferrer-Montiel, A., Gonzalez Ros, J.M., Estepa, A., Coll, J.M., 2002. Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library. J. Gen. Virol. 83, 2671–2681.

Mas, V., Rocha, A., Perez, L., Coll, J.M., Estepa, A., 2004. Reversible inhibition of spreading of in vitro infection and imbalance of viral protein accumulation at low pH in viral haemorrhagic septicaemia rhabdovirus (VHSV), a salmonid rhabdovirus. J. Virol. 78 (4), 1936–1944.

Mas, V., Falco, A., Brocal, I., Perez, L., Coll, J.M., Estepa, A., 2006. Identification of selective inhibitors of VHSV from biased combinatorial libraries of *N*,*N*′-disubstituted 2,5-piperazinediones. Antiviral Res. 72, 107–115.

Meyerholz, D.K., Gallup, J.M., Lazic, T., De Macedo, M.M.A., Lehmkuhl, H.D., Ackermann, M.R., 2007. Pretreatment with recombinant human vascular endothelial growth factor reduces virus replication and inflammation in a perinatal lamb model of respiratory syncytial virus infection. Viral Immunol. 20, 188–196.

Micol, V., Caturla, N., Perez-Fons, L., Mas, V., Perez, L., Estepa, A., 2005. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). Antiviral Res. 66, 129–136.

Moav, B., Liu, Z., Groll, Y., Hackett, P.B., 1992. Selection of promoters for gene transfer into fish. Mol. Mar. Biol. Biotechnol. 1, 338–345.

Olesen, N.J., Korsholm, H., 1997. Control measures for viral diseases in aquaculture: eradication of VHS and IHN. Bull. Eur. Assoc. Fish Pathol. 17, 229–233.

Pazgier, M., Hoover, D., Yang, D., Lu, W., Lubkowski, J., 2006. Human β-defensins. Cell. Mol. Life Sci. (CMLS) 63, 1294–1313.

Perez, L., Mas, V., Coll, J., Estepa, A., 2002. Enhanced detection of viral hemorrhagic septicemia virus (a salmonid rhabdovirus) by pretreatment of the virus with a combinatorial library-selected peptide. J. Virol. Methods 106, 17–23.

Quinones-Mateu, M.E., Lederman, M.M., Feng, Z., Chakraborty, B., Weber, J., Rangel, H.R., Marotta, M.L., Mirza, M., Jiang, B., Kiser, P., Medvik, K., Sieg, S.F., Weinberg, A., 2003. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. Aids 17, F39–F48.

Rocha, A., Ruiz, S., Coll, J.M., 2004. Improvement of transfection efficiency of epithelioma papulosum cyprini carp cells by modification of cell cycle and use of an optimal promoter. Mar. Biotechnol. (NY) 6, 401–410.

Sanz, F., Coll, J.M., 1992. Detection of viral haemorrhagic septicemia virus by direct immunoperoxidase with selected anti-nucleoprotein monoclonal antibody. Bull. Eur. Assoc. Fish Pathol. 12, 116–119.

Selsted, M.E., Harwig, S.S., Ganz, T., Schilling, J.W., Lehrer, R.I., 1985. Primary structures of three human neutrophil defensins. J. Clin. Invest. 76, 1436–1439.

Selsted, M.E., Ouellette, A.J., 2005. Mammalian defensins in the antimicrobial immune response. Nat. Immunol. 6, 551–557.

Sun, L., Finnegan, C.M., Kish-Catalone, T., Blumenthal, R., Garzino-Demo, P., La Terra Maggiore, G.M., Berrone, S., Kleinman, C., Wu, Z., Abdelwahab, S., Lu, W., Garzino-Demo, A., 2005. Human {beta}-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection. J. Virol. 79, 14318–14329.

Tafalla, C., Chico, V., Perez, L., Coll, J.M., Estepa, A., 2007. In vitro and in vivo differential expression of rainbow trout (*Oncorhynchus mykiss*) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV. Fish Shellfish Immunol. 23, 210–221.

Tang, Y.Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C.J., Ouellette, A.J., Selsted, M.E., 1999. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science 286, 498–502 (See comments).

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Trobridge, G.D., Chiou, P.P., Leong, J.C., 1997. Cloning of the rainbow trout (*Oncorhynchus mykiss*) Mx2 and Mx3 cDNAs and characterization of trout Mx protein expression in salmon cells. J. Virol. 71, 5304–5311.

Wang, T., Johnson, N., Zou, J., Bols, N., Secombes, C.J., 2004. Sequencing and expression of the second allele of the interleukin-1beta1 gene in rainbow trout (*Oncorhynchus mykiss*): identification of a novel SINE in the third intron. Fish Shellfish Immunol. 16, 335–358.

Yang, D., Biragyn, A., Hoover, D.M., Lubkowski, J., Oppenheim, J.J., 2004. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu. Rev. Immunol. 22, 181–215.

Yang, D., Biragyn, A., Kwak, L.W., Oppenheim, J.J., 2002. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol. 23, 291–296.

Zhang, Y.A., Zou, J., Chang, C.I., Secombes, C.J., 2004. Discovery and characterization of two types of liver-expressed antimicrobial peptide 2 (LEAP-2) genes in rainbow trout. Vet. Immunol. Immunopathol. 101, 259–269.

Zhang, Y.B., Li, Q., Gui, J.F., 2004. Differential expression of two *Carassius auratus* Mx genes in cultured CAB cells induced by grass carp hemorrhage virus and interferon. Immunogenetics 56, 68–75.

Zou, J., Mercier, C., Koussounadis, A., Secombes, C., 2007. Discovery of multiple beta-defensin-like homologues in teleost fish. Mol. Immunol. 44, 638–647.