



## Full length article

## Inhibition of SERPINE1 reduces rhabdoviral infections in zebrafish

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## ABSTRACT

While exploring the molecular mechanisms behind the fin hemorrhages that follow zebrafish (*Danio rerio*) early infection with viral haemorrhagic septicemia virus (VHSV), we discovered that most *serpin* (serine protease inhibitor) gene transcripts were upregulated, except those of *serpine1*. Surprisingly, only SERPINE1-derived 14-mer peptide and low molecular weight drugs targeting SERPINE1 (i.e. tannic acid, EGCG, tiplaxtinin) inhibited *in vitro* infections not only of VHSV, but also of other fish rhabdoviruses such as infectious hematopoietic necrosis virus (IHNV) and spring viremia carp virus (SVCV). While the mechanisms that inhibited rhabdoviral infections remain speculative, these and other results suggested that SERPINE1-derived peptide specifically targeted viral infectivity rather than virions. Practical applications might be developed from these studies since preliminary evidences showed that tannic acid could be used to reduce VHSV-caused mortalities. These studies are an example of how the identification of host genes targeted by viral infections using microarrays might facilitate the identification of novel prevention drugs in aquaculture and illuminate viral infection mechanisms.

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## 1. Introduction

In many fish, external (mouth, fin, epithelia) and internal (muscle, liver, head kidney, spleen, intestines) hemorrhages follow 4–7 days the start of rhabdoviral infections [1,2]. Although such rhabdoviral-induced hemorrhages should include interferences with the host molecular coagulation pathways, those have not been explored yet. Therefore, we were interested in the study of transcriptional expression profiles along the coagulation-complement pathway induced by rhabdoviral early infections in the fish fins, their portal of entry [3], with the expectation of finding novel gene targets for preventive drugs. We chose the zebrafish *Danio rerio* because of its susceptibility to novirhabdoviruses like viral haemorrhagic septicemia virus (VHSV) [4,5] or infectious hematopoietic necrosis virus (IHNV), coding for non-virion NV genes and to rhabdoviruses like spring viremia of carp virus (SVCV) which lack the NV gene [6,7]. In addition, zebrafish transcriptomic data on fins and lymphoid organs have been previously reported by our research groups after immunization with VHSV [8,9] and SVCV [10].

Vertebrate coagulation starts when damaged blood vessels expose collagens of internal tissues to blood proteolytic cascade

factors (i.e. those coded by the *f3*, *f7*, *f8*, *f9*, *vwf* genes), leading to thrombin (*f2*) activation. Activated THROMBIN cleaves blood FIBRINOGEN (*fg*) generating the fibrin clots characteristic of coagulation (Fig. 1). The coagulation cascades are interconnected with parallel proteolytic cascades involved in complement-dependent cell lysis. Thus, the “complement and coagulation cascades” KEGG pathway (<http://www.genome.ad.jp/kegg/>) describes two proteolytic cascades interconnected by two proteins: FACTOR XII (endothelial secreted Hageman factor, coded by the *f12* gene) and PLASMINOGEN (a pro-enzyme, coded by *plg*). Proteolysis of FACTOR XII/PLASMINOGEN increase PLASMIN to reduce coagulation by fibrinolysis and to increase complement-dependent cell lysis (Fig. 1). To avoid tissue damage, excessive proteolysis are controlled by a variety of protein inhibitors (i.e.: coded by *serpind1*, *serpinc1*, *serpina1*, *serpinf2*, *serping1*, *serpine1*, *proc*, *tfpia*, *h*, *clu*, etc.). Among them, serine protease inhibitors (SERPIN proteins) could be grouped in those inhibiting, i) Coagulation by targeting THROMBIN coded by the *f2* gene (SERPIND1, SERPINC1, SERPINA1); ii) Both coagulation and complement-dependent cell lysis by targeting TISSUE PLASMINOGEN ACTIVATOR, coded by *tpa* or *plaub* (SERPINA1, SERPINE1) and/or PLASMIN (SERPINA1, SERPINF2); and iii) Complement-dependent cell lysis by targeting C1S/C1R, coded by *c1s/c1r* (SERPING1) (Fig. 1, red).

In this context, detailed re-analysis of previously published microarray gene results of the “complement and coagulation

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cascades” KEGG pathway of zebrafish fins infected with VHSV [8], discovered that most *serpins* were upregulated, except *serpine1*. Because the lack of modulation (up or downregulation) of *serpine1* could be explained by SERPIN1 interferences with VHSV infection, we then studied the effects of SERPIN specific inhibition on *in vitro* rhabdoviral infections. As demonstrated for human SERPINs [11,12], to specifically inhibit zebrafish SERPIN activities, we used the orthologous 14-mer amino-terminal amino acids of its Reactive Center Loops (RCL) (NH<sub>2</sub>P14.P13.P12...P3.P2.P1...P'1.P'2.P'3...P'12.P'13.P'14). Surprisingly, we discovered that only zebrafish SERPINe1-derived peptides and/or their corresponding specific low molecular weight inhibitors (i.e.: tannic acid, EGCG, tiplaxtinin) [13,14] inhibited not only VHSV but also SVCV and IHNV *in vitro* rhabdoviral infections and reduced zebrafish mortalities of VHSV infections. This is one novel example of how the identification of host genes targeted by viral infections using microarrays might facilitate the identification of novel prevention drugs in aquaculture and illuminate viral infection mechanisms.

## 2. Materials and methods

### 2.1. Fish rhabdoviruses and cell culture

The viral haemorrhagic septicemia virus VHSV- 07.71 [15] and/or the infectious hematopoietic necrosis virus IHNV-Cedar strain [16] novirhabdoviruses were grown at 14 °C in the *Epithelioma papulosum cyprini* (EPC) cells from the fathead minnow *Pimephales promelas* (ATCC, CRL-2872). Cells were grown in 25 cm<sup>2</sup> flasks at 28 °C in RPMI-Dutch modified cell culture medium buffered with 20 mM HEPES (Gibco, Invitrogen corporation, UK) and supplemented with 10% fetal calf serum (FCS), 1 mM pyruvate, 2 mM glutamine, 50 µg/ml gentamicin and 2.5 µg/ml fungizone. To prepare VHSV or IHNV, the cell culture media were the same as above, except for the use of 2% FCS, 10 mM Tris pH 8.0 and 14 °C. To assay for inhibition of infectivity, VHSV or IHNV were purified by pelleting infected supernatants by ultracentrifugation at 60,000 g for 60 min at 4 °C and then titrated by a focus forming unit (ffu) assay

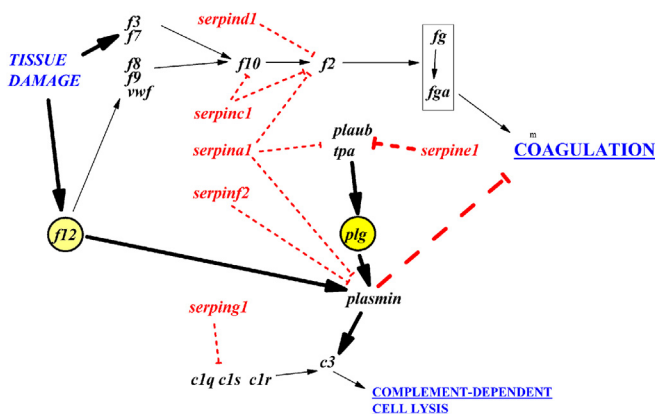
using monoclonal antibodies to recognize VHSV/IHNV-infected foci [17]. For the *in vivo* infections, supernatants from VHSV-infected cell monolayers were clarified by centrifugation at 4000 × g during 30 min and kept in aliquots at –70 °C. The spring viremia of carp virus SVCV isolate 56/70 [18] was grown in zebrafish embryonic fibroblast ZF4 cells [19] (ATCC, CRL-2050). ZF4 cells were maintained at 28 °C in a 5% CO<sub>2</sub> atmosphere in the same cell culture medium described above. To prepare SVCV, 2% FCS and 22 °C were used. To assay for inhibition of infectivity, SVCV was purified by pelleting SVCV-infected supernatants by ultracentrifugation at 60,000 g for 60 min at 4 °C and then titrated by methylcellulose plaque assays as described before [10].

### 2.2. Previously published experiments using zebrafish and its microarray data analysis

Zebrafish were maintained at 24–26 °C in 30 l aquaria with tap-dechlorinated carbon-filtered water with 1 g of CaCl<sub>2</sub>, 1 g of NaHCO<sub>3</sub> and 0.5 g of Instant Ocean sea salts added to water resulting in a conductivity of 200–300 µS pH of 7.8–8.2, as described before [8–10]. The aquaria were provided with biological filters and fish fed with commercial flakes (Tetramin, Tetra GmbH, Germany). For each experiment, groups of 10 zebrafish were moved to 2 l miniaquaria provided with biological filters for acclimation during 7 days. Groups of 10 zebrafish were infected-by-immersion with VHSV or mock infected, as previously reported [8–10]. Briefly, zebrafish were maintained in 2 × 10<sup>6</sup> ffu of VHSV per ml at 14 °C during 2 h in 50 ml containers with continuous aeration and then released to their 2 l miniaquaria at 14 °C. After 2-days, fins (dorsal, ventral and caudal) or lymphoid organs (head kidney plus spleen) were harvested and independently pooled for 6 zebrafish per group, n = 4 groups per treatment. Extraction, labeling of high quality RNA and hybridization were performed as described before [8,10]. Differential expression folds of genes of the “complement and coagulation cascades” KEGG pathway were compared from hybridization data deposited in the Gene Expression Omnibus (GSE19049) [8]. Gene differential expressions or modulations expressed were calculated in folds by the formula, fluorescences of VHSV-infected zebrafish/mean fluorescences of non-infected zebrafish. Then the means ± standard deviations of the folds were calculated for n = 4. Genes with differential expression fold values > 1.5 or <0.66 were defined as statistically significant according to the one sample 2-tail independent t-test at p < 0.05. Other details of sample processing were as previously described in detail [8,10].

### 2.3. Design and synthesis of SERPIN-derived peptides

To specifically inhibit serine protease inhibitors (*serpin* genes, SERPIN proteins) we used SERPIN-derived peptides, since mammalian SERPINs are specifically inhibited by 14-mer peptides derived from their 28 amino acid (P14...P14') Reactive Center Loops (RCL) [11,12]. To find the RCL sequences corresponding to zebrafish, P4.P3.P2.P1...P'2'.P'3'.P'4'. amino acid sequences (one single letter amino acid codes around the scissile P1–P1' bound) of each human SERPIN were retrieved [12] and aligned to the corresponding amino acid sequences in zebrafish. Orthologous peptide sequences corresponding to the 14-mer amino-terminal part of each zebrafish RCL SERPIN were then obtained from the P14.....P1 positions (Table 2). The 14-mer peptides were chemically synthesized by adding acetylation to its amino-terminal part as used before [11,12]. Cell penetrating peptide probabilities were estimated using predictive software at bioware.ucd.ie/CPPpred [24]. SERPIN-derived peptides and 4–7 amino acid subsequences of SERPINe1 (TKGSSAT, AAVIYSR, TKGS, SATA, and



**Fig. 1.** Simplified scheme of the “Complement and coagulation cascades” KEGG pathway. The zebrafish gene symbols in *italics* and their proposed relationships were obtained from the corresponding human KEGG-pathway (<http://www.genome.ad.jp/kegg/>). **Red italics.** Serine protease inhibitor (*serpin*) genes. All the human/zebrafish orthologous genes of the “Complement and coagulation cascades” pathway (Table 1) were assayed for transcript expression but only some of them have been drawn here for clarity. **Blue CAPITAL LETTERS.** main physiological inputs (cellular damage) and outputs (underlined, Coagulation and Complement-dependent cell lysis) of the pathway. Continuous black arrows, activation between gene products. **Discontinuous red arrows.** inhibition between gene products. Rectangle, fibrinogen conversion to fibrin. **Yellow circles.** f12 and plg genes linking coagulation to complement proteolytic cascades.

AVIYSR) of SERPINE1 (TKGSSATAAVIYSR) were synthesized by Proteogenix (Schiltigheim, France).

#### 2.4. Inhibition assay of in vitro rhabdoviral infection by SERPIN-derived peptides and low molecular weight drugs

Briefly, ZF4 zebrafish cells (50,000 cells per well) were grown at 28 °C in 96-well plates of polystyrene coated with poly-D-Lys (Corning, New York, NY, USA) as described before [17,20,21]. SERPIN-derived peptides (Table 2) and/or SERPIN1 inhibitors [13,14] were incubated overnight (~16 h) with 300 ffu of purified rhabdovirus (VHSV, SVCV or IHNV) per well in cell culture medium 2% FCS, 10 mM Tris pH 8.0 at 14 °C (VHSV, IHNV) or 22 °C (SVCV). ZF4 cell monolayers were then infected by adding the rhabdovirus + inhibitor mixtures during 2 h at 14 °C. Alternatively, SERPIN-derived peptides were incubated overnight with the ZF4 monolayers, washed and rhabdovirus added the next day during 2 h at 14 °C. After washing by flicking the plate and refilling with cell culture medium, rhabdovirus-infected cell monolayers were

incubated overnight at 14 °C before being fixed with 10% formaldehyde in phosphate buffered saline (PBS) during 20 min and permeabilized with 0.05% Saponin, 0.01% N<sub>3</sub>Na in PBS during 15 min. To detect rhabdoviral infection, the VHSV N protein was stained with MAb 2C9 [22], SVCV with a polyclonal Ab anti-SVCV (Bio-X Diagnostics) and IHNV with a mixture of anti-N MAbs [23]. The virus-specific Abs were diluted in 2% FCS, 0.05% Saponin, 0.01% N<sub>3</sub>Na in PBS (permeabilizing buffer) and rabbit FITC-labeled anti-mouse IgG (Nordic, Tilburg, The Netherlands) was used to fluorescently label the bound Abs. The monolayers were suspended by 10 min incubation with 0.25% trypsin 0.02% EDTA (Sigma, St. Louis, MS, USA) and pipetted after the addition of 1% BSA, 50 mM EDTA, 0.01% N<sub>3</sub>Na in PBS. The suspended cells were analyzed in a BD FACS Canto II apparatus (Beckton Dickinson, San Agustín de Guadalix, Madrid, Spain) provided with a high throughput sampler. Forward (FSC) and side (SSC) scatter threshold values corresponding to damaged cells, cellular debris and/or cellular aggregates were eliminated from the analysis. The number of fluorescent cells was determined over a threshold containing 95% (mean + 2 standard deviations) of non-

**Table 1**  
Differential expression of “coagulation and complement cascade” KEGG pathway gene transcripts in fins and lymphoid organs (head kidney plus spleen) from 2-day VHSV-infected zebrafish (GSE19049) [8].

Genes	Acc.numbers	Description	Fins mean	±sd	Organs mean	±sd
<b>COAGULATION:</b>						
<i>f3</i>	NM_001017728	Coagulation factor IIIb	0.79	0.17	0.96	0.07
<i>f7</i>	NM_173228	Coagulation factor VII	*2.05	0.01	*1.85	0.01
<i>f8</i>	XM_001922709	Coagulation factor VIII	*0.40	0.01	0.95	0.01
<i>f9</i>	NM_001040310	Coagulation factor IX	*6.98	0.01	1.08	0.01
<i>vwf</i>	XM_690650	von Willebrand factor type A	0.80	0.01	*0.40	0.02
<i>f10</i>	NM_201462	Coagulation factor X	*2.25	0.20	1.12	0.01
<i>f5</i>	NM_001007208	Coagulation factor V	*3.41	0.01	1.23	0.01
<i>serpina1</i>	NM_001077758	<b>Serpina1 protein inhibitor, clade A</b>	<b>*2.21</b>	<b>0.01</b>	<b>0.66</b>	<b>0.01</b>
<i>serpinc1</i>	NM_182863	<b>Serine proteinase inhibitor, clade C</b>	<b>*3.74</b>	<b>0.05</b>	<b>0.90</b>	<b>0.03</b>
<i>serpinf2</i>	NM_001080010	<b>Serine proteinase inhibitor, clade F</b>	<b>*5.35</b>	<b>0.79</b>	<b>1.15</b>	<b>0.08</b>
<i>a2m</i>	XM_001345402	Alpha-2-macroglobulin-like protein 1	0.81	0.45	1.11	0.01
<i>tfpia</i>	NM_182872	Tissue factor pathway inhibitor a	0.97	0.01	1.16	0.01
<i>proc</i>	NM_200356	Protein C (inactivator of Va and VIIIa)	*1.61	0.01	1.09	0.01
<i>pros1</i>	NM_001077323	Protein S (alpha)	0.97	0.01	0.70	0.01
<i>f12</i>	XM_002663051	Coagulation factor XII	1.00	0.01	1.00	0.01
<i>f2</i>	NM_213390	Coagulation factor II (thrombin)	*5.74	2.72	1.34	0.04
<i>f2r</i>	NM_001114846	Thrombin receptor	1.04	0.09	1.27	0.07
<i>f13</i>	NM_001077154	Coagulation factor XIII, A1 ( <i>f13a1</i> )	1.18	0.07	0.76	0.15
<i>fga</i>	NM_001194989	Fibrinogen alpha chain ( <i>fga</i> )	*5.56	0.68	1.20	0.04
<i>serpind1</i>	NM_182880	<b>Serine proteinase inhibitor, clade D</b>	<b>*4.75</b>	<b>0.87</b>	<b>1.31</b>	<b>0.29</b>
<i>serpine1</i>	NM_001114559	<b>Serine proteinase inhibitor, clade E</b>	<b>1.20</b>	<b>0.01</b>	<b>1.00</b>	<b>0.01</b>
<i>plaub</i>	NM_001252350	Tissue-type plasminogen activator urokinase b	1.04	0.21	0.98	0.13
<i>plg</i>	NM_201472	Plasminogen	*5.72	0.71	1.62	1.25
<b>COMPLEMENT:</b>						
<i>crp</i>	BC097160	Pentraxin	*13.54	0.36	1.19	0.01
<i>igm</i>	AY646262.1	Immunoglobulin heavy constant mu chain	1.23	0.58	1.25	0.70
<i>igz</i>	AY646263.1	Immunoglobulin zeta heavy chain	1.25	0.18	1.43	0.28
<i>hbl4</i>	NM_001114725	Hexose-binding lectin 4	*3.46	1.18	1.81	0.49
<i>c1q</i>	NM_001020527	Complement component 1q subcomponent	1.09	0.16	0.90	0.32
<i>c1s</i>	NM_001122858	Mannan-binding lectin serine peptidase 2	*5.05	0.01	1.04	0.01
<i>serping1</i>	NM_001123285	<b>Serine proteinase inhibitor, clade G</b>	<b>*4.65</b>	<b>0.56</b>	<b>*1.68</b>	<b>0.07</b>
<i>c4</i>	XM_689530	Complement component C4	*1.79	0.01	*1.98	0.01
<i>c2</i>	XM_001921497	Complement factor B/C2B	*2.13	1.59	1.36	0.18
<i>h</i>	XM_002666292.1	Complement factor H	*3.49	0.01	1.46	0.01
<i>d</i>	NM_001020532	Complement factor D adipsin (cfd)	1.76	1.07	1.18	0.71
<i>b</i>	NM_001083858	Complement factor B (cfb)	1.56	1.24	1.07	0.17
<i>c3</i>	NM_131243	Complement C3 Precursor	*6.14	0.01	*1.69	0.01
<i>clu</i>	NM_200802.1	Clusterin (complement lysis inhibitor)	0.90	0.08	1.04	0.01
<i>c6</i>	NM_200638	Complement component 6	*1.53	0.01	0.89	0.01
<i>c7</i>	XM_685854	Complement component 7	1.03	0.01	*0.59	0.01
<i>c8</i>	BC078409	Complement component 8, alpha polypeptide	*4.76	0.01	1.45	0.01
<i>c9</i>	NM_001024435.1	Complement component 9	*4.34	0.09	*2.18	0.13

Differential expression folds of genes of the “complement and coagulation cascades” KEGG pathway (simplified in Fig. 1) in fins and lymphoid organs (head kidney plus spleen) were compared from data obtained from zebrafish 2-days after infection with VHSV (GSE19049) [8]. **Bold**, highlighting the serine protease inhibitor genes (*serpins*). Gene modulations expressed in folds were calculated by the formula, hybridization fluorescence of infected zebrafish/mean fluorescence of non-infected zebrafish (n = 4). Means ± standard deviations, were then calculated from n = 4 biological replicates. \*, Genes with differential expression fold values > 1.5 or <0.66 were defined as statistically significant according to the one sample 2-tail independent t-test at p < 0.05. The sd < 0.001 have been rounded to 0.01 for clarity.

**Table 2**

Identification of human/zebrafish orthologous SERPIN-derived peptide sequences of their Reactive Center Loop (RCL) regions.

<i>serpins</i> target protease	Sp	accession numbers	amino	sequences of the RCL regions P14 P12 P10 highly conserved amino acids P4P3P2P1--P1'P2'P3'P4'	CPPP
<i>serpina1</i> αtrypsin	hsa dre	NM_01127707 NM_001077758	369 381	<b>TEAAG</b> AMFLE <b>AIPM</b> -- <b>SIP</b> PEVKFNKPFVF <b>TEAAA</b> ITTIE <b>IMPM</b> -- <b>SLPD</b> TVILNRPFV	0.093
<i>serpinc1</i> ATIII	hsa dre	NM_000488 NM_182863	412 397	<b>SEAAA</b> STAVV <b>IAGR</b> -- <b>SLNP</b> NRVTFKANRP <b>SEASA</b> ATAVV <b>ATGR</b> -- <b>SLNI</b> FREQFVADRP	0.289
<i>serpind1</i> heparin cofactor	hsa dre	NM_000185 NM_182880	450 458	<b>TQAT</b> TVTTVG <b>FMPL</b> -- <b>STQV</b> RFTVDRPFV <b>TEAA</b> AMTHIG <b>FMPL</b> -- <b>STQT</b> RFIVDRPFV	0.116
<i>serpine1</i> PAI-I	hsa dre	NM_000602 NM_001114559	356 343	<b>TVAS</b> SSTAVI <b>VSAR</b> -- <b>MAPE</b> EIIMDRPFV <b>TKGS</b> SATAAV <b>IYSR</b> -- <b>MAVE</b> EITLDRPFFV	0.184
<i>serpinf2</i> α2 anti plasmin	hsa dre	NM_000934 NM_001080010	390 407	<b>GVEAA</b> AATS <b>IAMS</b> R-- <b>MSLS</b> SFSVNRPFV <b>GAEVA</b> AATSV <b>VISR</b> -- <b>SNPS</b> FTVNQPFVFA	0.203
<i>serping1</i> C1s inhibitor	hsa dre	NM_001032295 NM_001123285	453 559	<b>GVEAA</b> AASAI <b>SVAR</b> -- <b>TLLV</b> FEVQPFV <b>GVEAA</b> AATS <b>ISFS</b> R-- <b>SFSS</b> FSALQPFVLI	0.113

Serine protease inhibitors (*serpin* genes, SERPIN proteins) target serine proteases to inhibit their activities by a SERPIN-"suicide" mechanism. Thus, target proteases recognize, bind and cleave the SERPIN scissile bond (--) located in its Reactive Center Loop (RCL) region

(NH<sub>2</sub>.....P14.P13.P12.P11.P10.P9.P8.P7.P6.P5.P4.P3.P2.P1.--

P1'.P2'.P3'.P4'.P5'.P6'.P7'.P8'.P9'.P10'.P11'.P12'.P13'.P14'.....). The serine proteases are then covalently bound to the RCL loop and inactivated. *Serpin* gene symbols (**bold** and *italics*) and their corresponding target proteases are given into the left column. The **P4P3P2P1--P1'P2'P3'P4'** amino acid sequences (one single letter amino acid codes ± 4 amino acids around the scissile bound in bold) of each human *serpin* were retrieved [12] and aligned to the corresponding sequences in zebrafish. Synthetic peptides of 14-mer corresponding to the amino-terminal part of each zebrafish SERPIN were obtained from the P14.....P1 positions (underlined). **Bold red**, highly conserved **P14**, **P12** and **P10** amino acid positions of the corresponding RCL regions. **Amino**, amino acid position of the RCL region in the corresponding SERPIN sequence. **Sp**, species. **hsa**, *Homo sapiens*. **dre**, *Danio rerio*. **CPPP**, cell penetrating peptide probability as estimated using predictive software at [bioware.ucd.ie/CPPpred](http://bioware.ucd.ie/CPPpred). 0.5, likely to be cell penetrating, 0, very unlikely to be cell penetrating [24].

infected cell controls (>50% of the cells infected). The BD FACSDIVA software calculated the percentage of fluorescent (N-positive, VHSV-infected) cells by the formula, 100 x number of cells with fluorescence > the threshold/total number of cells per well (n = 10,000 cells). Comparisons with the fluorescent values from infected cell monolayers without any peptide treatment were performed by using the Student-t test at the p < 0.05 level.

## 2.5. Tannic acid treatment of zebrafish and infection with VHSV

Adult zebrafish of 2–3 g (~4 cm in length, XL size) were obtained from a local fish pet shop to study a situation that more closely resembles the variability of natural populations. Tannic acid was delivered to zebrafish either by mouth with a micropipette or by

adding it to the feed. To deliver tannic acid fish-to-fish by mouth, groups of 10 zebrafish per treatment per experiment were moved to 14 °C aquaria and 3 days before the VHSV challenge were individually given 10 µl of PBS containing 50 µg of tannic acid each day by mouth with a micropipette. Mock-treated zebrafish were similarly given 10 µl of PBS each day per fish. To prepare the feed with tannic acid, commercial flakes (Tetramin, Tetra GmbH, Germany) were weighted and dissolved in water, 10% w/w of tannic acid added and mixed with an homogenizer. The mix was then dried at 37 °C during 2-days and kept at 4 °C until use. Groups of 10 zebrafish per treatment per experiment were moved to 14 °C aquaria for acclimatation and fed with 20 mg of feed containing 10% of tannic acid per day during 3 days before and 7 days after VHSV challenge. Mock-treated zebrafish were similarly fed with 20 mg of



feed each day per fish with feed prepared in a similar way but without tannic acid. In both cases, after tannic acid treatment, the groups of fish were moved to 50 ml containers and infected-by-immersion with aeration with  $10^7$  ffu/ml of VHSV during 120 min. Mock-infected zebrafish were incubated with cell culture medium in parallel experiments. From 1 to 15 days, fish were monitored for mortalities 2–4 times a day and those with external hemorrhages and alterations in swimming were euthanized by an overdose of MS222 (methanesulfonate 3-aminobenzoic acid ethyl ester) to minimize their suffering. After 15-days, the survivor fish were also euthanized by an overdose of MS222. Cumulative percentage mortalities for each group of 10 fish and treatment were calculated by the formula,  $100 \times \text{number of fish that die after 15-days}/10$ . Means of cumulative percent mortalities and its standard deviations from 3 different experiments for each treatment were then calculated. Means significantly lower at the  $p < 0.05$  level (Student t test) when compared to the PBS or feed control group (\*).

## 2.6. Zebrafish handling and ethics statement

Zebrafish were handled in accordance with the National and European guidelines and regulations. Animal work was approved by the INIA corresponding Ethic Committee (authorization CEEA 2011/022). All efforts were made to minimize zebrafish suffering during the course of these studies by monitoring for mortality 2–4 times a day. Those fish with external hemorrhages and alterations in swimming were euthanized by an overdose of MS222 (methanesulfonate 3-aminobenzoic acid ethyl ester) to minimize their suffering. To harvest fins and/or lymphoid organs, zebrafish were first euthanized by an overdose of MS222.

## 3. Results and discussion

### 3.1. Modulation of gene transcripts in the “complement and coagulation cascades” pathway in VHSV-infected zebrafish fins and lymphoid organs

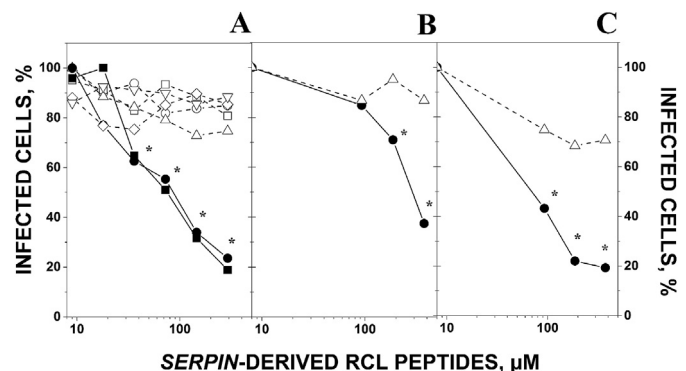
Two-days after infection-by-immersion of zebrafish with VHSV, more fin genes were differentially expressed (modulated) in the “complement and coagulation cascades” KEGG pathway than in many other immune-related pathways [8,10]. These published data suggested an important participation of this pathway in the early responses of zebrafish to rhabdoviral infections since fins are the portal of entry for these viruses [3]. A more detailed analysis of the corresponding gene-by-gene differential expressions in the above mentioned pathway, showed that more genes were modulated in fins (60.9%) than in lymphoid organs (17.1%) (Table 1). For example, while *f7*, *f9*, *f10*, *f5*, *proc*, *f2*, *fg*, *plg* (coagulation pathway) and *crp*, *hbl4*, *c1s*, *c4*, *c2*, *h*, *c3*, *plg*, *c6*, *c8*, *c9* (complement pathway) genes were upregulated in fins, only *f7*, *hbl4*, *c4*, *c3*, *c7* and *c9* were upregulated in lymphoid organs. In contrast, only *f8*, *vwf*, and *c7* were downregulated in fins or organs. The rest of the 41 genes in fins or organs were not modulated (Table 1).

In addition, many of the highest 4–6-fold gene upregulations in the fins were found on *serpins* (i.e. *serpina1*, *serpinc1*, *serpinf2*, *serpind1* and *serping1*) compared to lymphoid organs (Table 1, bold). Only *serpine1* remained not modulated, in sharp contrast to the rest of *serpins* in the fins (Table 1, bold). These data suggested that *serpins* as well as other genes coding for inhibitors in this pathway (i.e. *proc*, *h*), may be upregulated by the VHSV infection to favor viral spreading by increasing hemorrhaging and delaying complement-dependent cell lysis, except *serpine1*. These inhibitor upregulations could be part of the general immunosuppression described during early rhabdoviral infection of zebrafish with VHSV and other rhabdoviruses [8–10].

### 3.2. Inhibition of VHSV infection in vitro could only be obtained by SERPINE1-derived peptides

To investigate SERPIN-related mechanisms we inhibited their activities. To inhibit SERPIN activities, we used 14-mer synthetic peptides derived from the orthologous human/zebrafish SERPIN RCL loops (Table 2), since they demonstrated highly specific inhibition of SERPIN activities in mammals [11,12]. Fig. 2A (black circles) shows that only the peptides derived from SERPINE1 inhibited VHSV infection in a concentration-dependent manner (50–250  $\mu\text{M}$ ) when peptides and VHSV were incubated before being added to the cell monolayers. The rest of the SERPIN-derived peptides did not show any inhibition compared to VHSV-infected cell monolayers in the absence of any peptide (Fig. 2A open symbols). Because similar results were obtained when the SERPIN-derived peptides were added to the cells and washed before the addition of VHSV to the cell monolayers (SERPINE1 in Fig. 2A, black rectangles), the interactions of SERPINE1 inhibitory peptides must occur inside the cells rather than with VHSV virions. On the other hand, the absence of inhibition by other SERPIN-derived peptides was not due to lower cell penetrating activities because they have higher or similar predicted cell penetrating probabilities [24] (Table 2). In view that only the SERPINE1-derived peptide inhibited VHSV infection, we focused the rest of the experiments in exploring its possible practical applications. In addition, the SERPIND1-derived peptide having no effects on VHSV-infectivity was chosen as a negative control.

We then tested the two peptides for *in vitro* inhibition of other fish rhabdoviruses such as SVCV and IHNV. We found that both SVCV and IHNV were also inhibited by the SERPINE1-derived peptide (Fig. 2B and C, solid circles, respectively), but not by the SERPIND1-derived peptide (Fig. 2B and C, open triangles). SVCV but not IHNV (Fig. 2B and C, respectively) required 2–3-fold higher SERPINE1-derived peptide concentrations to obtain similar levels of



**Fig. 2.** Inhibition of VHSV-(A), SVCV-(B) and IHNV-(C) infection of ZF4 cells by peptides derived from zebrafish SERPIN RCLs. ZF4 cell monolayers were incubated overnight with SERPIN RCL-derived synthetic peptides (Table 2) previously incubated with VHSV, SVCV, or IHNV. To detect rhabdoviral infection, the VHSV N protein was stained with MAb 2C9 [22], SVCV was stained by a polyclonal Ab anti-SVCV (Bio-X Diagnostics) and the IHNV N protein was stained with a mixture of anti-N MAbs [23]. After staining with their corresponding FITC-labeled anti-viral antibodies, the suspended ZF4 cells were analyzed with a high throughput sampler in a BD FACS Canto II apparatus. The percentage of fluorescent (infected) cells was calculated by the formula,  $100 \times \text{number of cells with fluorescences above the threshold}/\text{total number of cells gated per well}$ . Means from 3 (VHSV) or 2 (SVCV, IHNV) different experiments are represented. Standard deviations ( $<30\%$  of the mean) were omitted for clarity. ●, SERPINE1-derived peptide. ○, SERPINA1-derived peptide. □, SERPINC1-derived peptide. ◇, SERPINF2-derived peptide. Δ, SERPIND1-derived peptide. T, SERPING1-derived peptide. ■, SERPINE1-derived peptide added to the monolayers 24 h before their infection, washed and then infected with VHSV. \*, significantly lower percentage of infected cells than those from rhabdoviral-infected ZF4 cell monolayers without peptide treatment at the  $p < 0.05$  level (Student t test).

inhibition than those obtained with VHSV (Fig. 2A). These results confirmed that the inhibition of rhabdoviral infections by the SERPINE1-derived peptide was caused by specific interactions with the cells rather than with the virions.

To investigate whether the SERPINE1-derived peptide (TKGSSATAAVIYSR) could be downsized and maintain its VHSV inhibitory effects, we designed shorter peptides derived from its amino-terminal, central or carboxy-terminal segments, corresponding to the amino acid sequences TKGSSAT, AAVIYSR, TKGS, SATA, and AVIYSR. However, none of these shorter peptides inhibited VHSV-infection when tested in a similar range of molar concentrations than the 14-mer SERPINE1-derived peptide. In contrast, simultaneously assayed 14-mer SERPINE1-derived peptide inhibited VHSV-infection (not shown). These results showed that the high specificity of viral inhibition by the SERPINE1-derived peptide required at least half of the 28-mer RCL sequence. Thus, despite their lower molecular weight and therefore their higher permeability, smaller SERPINE1-derived peptides were not capable of inhibiting VHSV-infection. Because the SERPINE1-derived 14-mer peptide requirement is highly specific, their inhibition of SERPINE1 cleavage is the most probable explanation for its mechanism of action, such as demonstrated before in mammals [11,12].

To confirm that SERPINE1 was the target of its derived peptides and to lower the costs of possible *in vivo* treatments, specific low molecular weight drug inhibitors of SERPINE1 previously described in the literature such as tannic acid, EGCG, and tiplaxtinin [13,14] were also tested for *in vitro* inhibition of VHSV infectivity. We found that VHSV infectivity was inhibited by all the low molecular weight drug inhibitors tested at concentrations ~50-fold lower than the SERPINE1-derived peptide and lower than those causing ZF4 cytotoxicity (Fig. 3). Therefore, since both the SERPINE1-derived peptide and SERPINE1 specific drug inhibitors inhibited VHSV-infection, an unknown serine protease X needs to be inhibited by SERPINE1 for successful VHSV infectivity. Thus, when SERPINE1 was inhibited, inhibition of VHSV infectivity was observed because the protease X inactivated VHSV infectivity. While unknown SERPINE1-targeted protease(s) X might be implicated in these mechanisms, other possible candidates are TPA, PLAUB and/or PLASMIN (Fig. 1). Although we have no evidences to favor any of these possibilities, PLASMIN, is a serine protease not only implicated in fibrinolysis but also in many other matrix protein breakdowns [25], which could include some of the rhabdoviral proteins. On this respect, *serpine1* knock-out mice has shown that *serpine1*, *tpa* and *plg* are implicated in controlling coronavirus infections in mammals [26]. However, in coronavirus [26] and in yellow fever [27] infections, *serpine1* levels were upregulated contrary to what occurred in fish rhabdoviruses. We can speculate that a dramatic fighting between host and rhabdovirus could exist to control *serpine1* transcript levels during the initial course of fish rhabdoviral infections. Thus, if *serpine1* levels are downregulated, VHSV infection will be inhibited but host hemorrhages will increase. On the contrary, if *serpine1* levels become upregulated to increase VHSV infection, host hemorrhages will be inhibited thus reducing the opportunities for viral spreading. During the first 2-days after infection, *serpine1* transcriptional levels remained not modulated (Table 1) because those 2 opposite effects.

### 3.3. Mortalities of zebrafish to VHSV infection were reduced after treatment with tannic acid

To investigate whether the VHSV infection could be inhibited *in vivo*, tannic acid was selected because its low costs could boost possible practical applications. Since initial experiments showed that a maximum of 50 µg of tannic acid could be administered to adult zebrafish without inducing any mortalities during the

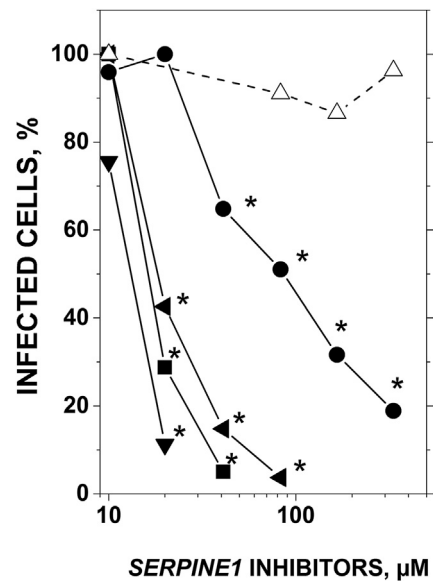
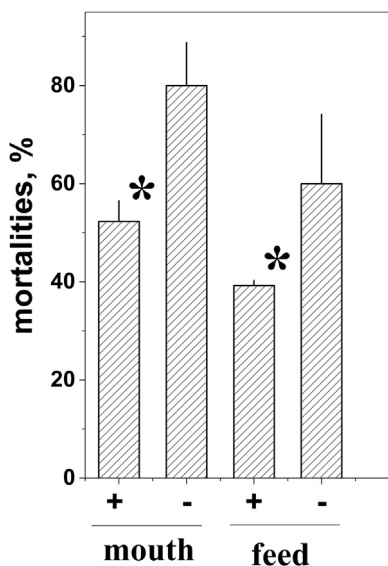


Fig. 3. Inhibition of VHSV-infection of ZF4 cells by low molecular weight SERPINE1 inhibitors. The assays were similar to those described in Fig. 3, except that some of the low molecular weight SERPINE1 inhibitors previously described [13,14] were used. ●, SERPINE1-derived peptide. ▼, EGCG. ◀, tiplaxtinin. ■, tannic acid. Δ, SERPINE1-derived peptide used as a control peptide. The experiments were made by duplicates. Standard deviations (<30% of the mean) were omitted for clarity. \*, significantly lower percentage of infected cells than those from VHSV-infected ZF4 cell monolayers without peptide treatment at the  $p < 0.05$  level (Student *t* test).

following days, that daily dose was delivered fish-to-fish by mouth. Therefore, 50 µg of tannic acid was administered per 2.5 g of fish per day during 3 days, corresponding to a total dose of 60 µg per g of fish. A similar dose of tannic acid of 68 µg per ml (~40 µM) caused ~95% inhibition of VHSV infectivity *in vitro* (Fig. 3). After being challenged with VHSV, 15-day mortalities of tannic acid-treated zebrafish were  $52.3 \pm 4.2\%$  while those in the absence of tannic acid treatment were  $80.0 \pm 8.8\%$  (Fig. 4). We then added 10% of tannic acid to the feed and fed zebrafish with feed containing 200 µg of tannic acid per fish per day during 10 days, corresponding a total dose of 800 µg per g of fish. Mortalities after VHSV challenge were  $39.2 \pm 1.0\%$  while those feed without tannic acid were  $60.0 \pm 14.1\%$  (Fig. 4).

## 4. Conclusions

We have identified most *serpins* of the “complement and coagulation cascades” pathway as one of the key gene classes upregulated after rhabdoviral infection in the fins of zebrafish. *Serpin* upregulation would inhibit many of the downstream serine proteases of that pathway confirming the immunosuppressive early effects of many viral infections including fish rhabdoviruses [7,8,10,28–30]. In contrast to the rest of *serpins* in this work, and in contrast to its upregulation in solid tumors [31], viral-induced hepatocarcinoma [32], and coronavirus [26] or yellow fever [27] infections in mammals, *serpine1* levels remained not modulated during fish rhabdoviral infections. To explain *serpine1* not modulated levels, we hypothesized on the existence of conflicting interests of SERPINE1 with fish rhabdoviral infectivity, since highly specific inhibition of VHSV infectivity was obtained *in vitro* with a 14-mer peptide derived from SERPINE1 and other specific drugs but not with those peptides derived from other SERPINS. In addition, drugs targeting SERPINE1 such as tannic acid, successfully but partially reduced mortalities to rhabdoviral infections of zebrafish *in vivo*. This is an example of how the identification of host genes



**Fig. 4.** Mortalities after VHSV infection of zebrafish treated with tannic acid by mouth or in the feed. Tannic acid described as one of the inhibitors of SERPINE1 [13] was delivered to groups of 10 zebrafish of 2.5 g per fish by mouth with a pipette or by adding it to the feed. To deliver tannic acid by mouth, 3-days before the VHSV infection, each zebrafish were given 10  $\mu$ l of PBS containing 50  $\mu$ g of tannic acid each day by mouth. Mock-treated fish were given 10  $\mu$ l of PBS. To deliver tannic acid with the feed, 3-days before and 7- days after VHSV infection, groups of 10 zebrafish were fed daily with 20 mg of feed containing 10% of tannic acid (equivalent to 200  $\mu$ g of tannic acid per fish per day). Mock-fed fish were given the same amounts of feed. All the zebrafish groups were finally infected with  $10^7$  ffu/ml of VHSV. Mock-infected zebrafish were incubated with cell culture medium in parallel experiments. Percentages of cumulative mortalities for each group of 10 fish and treatment were calculated by the formula,  $100 \times$  number of fish that die/10 fish. Means of cumulative percent mortalities and its standard deviations from 3 experiments per treatment were then calculated ( $n = 3$ ). +, treated with a total of 60  $\mu$ g of tannic acid per g of fish (by mouth) or 800  $\mu$ g of tannic acid per g of fish (by feed). –, treated with PBS (by mouth) or feed without tannic acid (by feed). \*, means significantly lower at the  $p < 0.05$  level (Student t test) when compared to the PBS (by mouth) or to the feed without tannic acid (by feed) control groups.

targeted by fish *rhabdoviral* infections obtained by using expression microarrays, offered an alternative to explore possible drugs for seasonal prevention of these diseases. Further work could allow for higher reduction of VHSV infection with low molecular weight drugs in larger fish.

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