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Identification of selective inhibitors of VHSV from biased combinatorial libraries of *N*,*N*′-disubstituted 2,5-piperazinediones

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Abstract

A combinatorial strategy has been used to design and identify inhibitors of viral haemorragic septicemia virus (VHSV), a salmonid rhabdovirus of economic importance. Two libraries of *N*,*N*′-disubstituted 2,5-piperazinediones (DKP), DKP-I and DKP-II were screened for inhibition of VHSV infectivity. Among the 98 DKP-derivatives (R1-DKP-R2) screened, a novel class of VHSV in vitro inhibitors was identified. Evidences are presented showing that the selected DKP-derivatives cause dose-dependent inhibition of VHSV infectivity in the absence of cellular toxicity. Preliminary characterization of its inhibition mechanism ruled out direct inactivation of the virus (virucidal effect) or interference with early viral replication steps. Furthermore, analysis of infection foci sizes, virus titers, viral protein accumulation and presence of cell free virus derived from VHSV-infected cell cultures in the presence of DKP-derivates suggested that virus assembly/release was impaired leading to a reduced virus spread in cell culture. New DKP-derivatives with a significant higher specific activity need to be developed to start testing its possible practical use but the selected DKP-derivatives described here may contribute to their further development as well as being tools to improve our knowledge on the fish rhabdovirus infection cycle.

Keywords: VHSV; Rhadovirus; Piperazinediones; Combinatorial libraries; Inhibitors; Antivirals

1. Introduction

Peptides derived from viral surface glycoprotein sequences are potent inhibitors of many enveloped viruses (Jiang et al., 1993; Wild et al., 1993; Lambert et al., 1996; Judice et al., 1997; Wild and Buckland, 1997; Yao and Compans, 1996; Kilby, 1998; Eckert, 1999; Eckert and Kim, 2001; Kliger et al., 2001). In contrast, no similar inhibitory peptides have been found for rhabdoviruses (Mas et al., 2002). Moreover, in an attempt to find anti-rhabdoviral peptides and using viral hemorrhagic septicaemia rhabdovirus (VHSV) as a model, we have previously reported (Mas et al., 2002) the screening of a large number of 17-mer L-peptides from an α-helix-restricted positional-scanning combinatorial peptide library and paradoxically, we only found peptides enhancing rather than inhibiting VHSV infectivity (Mas et al., 2002). This absence of inhibitors might be due to the different mechanisms involved in the entry/fusion steps in rhabdoviruses compared to other enveloped virus (Mas et al., 2002; Yao et al., 2003) or simply because of the restricted nature of the viral peptide sequences and/or the peptide library. In addition, L-peptides often have limitations as drugs due to their rapid enzymatic degradation (Nefzi et al., 1998) and immune clearance (Boggiano et al., 2003).

Because of those reasons in order to find effective antivirals against these viruses, both the inhibition of other events of rhabdovirus replication cycle, different from entry/fusion steps and the search of alternate drug candidate to L-peptides should be explored.

In the present work, we screened two combinatorial libraries of discrete compounds based upon a 2,5-piperazinedione (DKP) scaffold (Fig. 1A) to identify possible inhibitors of VHSV infection. The chemical diversity of DKP libraries was biased toward distinct amines, R1 and R2 (Fig. 1B), in 1,4 *N*,*N*′ positions of the 2,5-piperazinedione scaffold. DKPs libraries were chosen since: (i) DKPs are conformationally defined scaffolds that exhibit remarkable stability towards proteolysis; (ii) DKP-derivates have proven to be broadly useful as therapeutic agents (Szardenings et al., 1988; Carbonell et al., 2000; Einholm et

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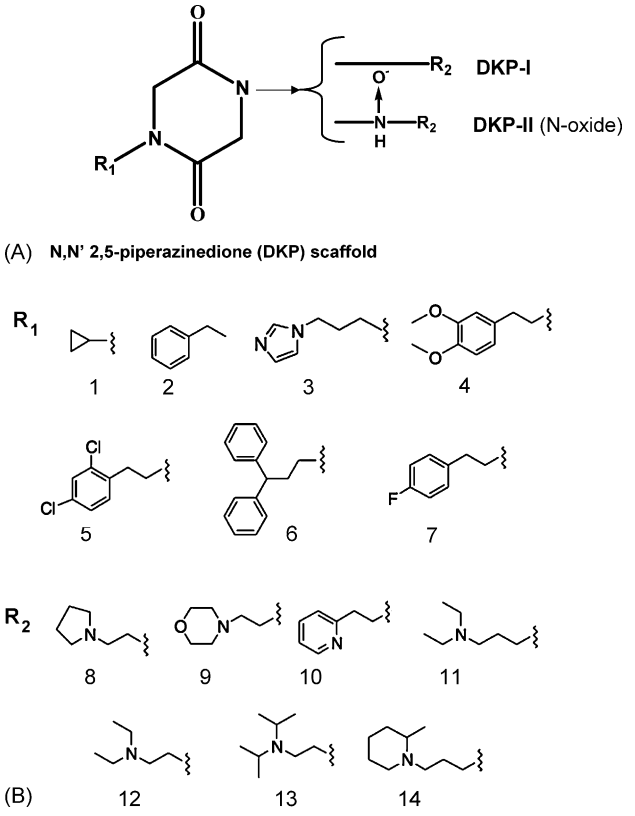

Fig. 1. Structure of the of the 2,5-piperazinedione (DKP) scaffold (A) and diversity set of primary amines used for generate the libraries (B). R1: (1) *N*-cyclopropyl-; (2) *N*-phenethyl-; (3) *N*-[3-(1-imidazolyl) propyl]; (4) *N*-(3,4-dimethoxyphenethyl)-; (5) *N*-(2,4-dichlorophenethyl)-; (6) *N*-(3,3-diphenylpropyl)-, (7) *N*-(4-fluorophethyl)-. R2: (8) *N*-[2-(1-pyrrolidinyl) ethyl]-; (9) *N*-[2-(4-morpholinyl) ethyl]-; (10) *N*-[2-(2-pyridyl) ethyl]-; (11) *N*-[3-(diethylamino) propyl]-; (12) *N*-[2-(diethylamino)ethyl]-; (13) *N*-[2-(diisopropylamino) ethyl]-; (14) *N*-[3-(2-methylpiperidyl)propyl]-.

al., 2003); (iii) a natural substituted 2,6-piperazinedione, called flutimide, and a synthetically designed aromatic analogue have been shown to selectively inhibit influenza virus transcriptase (Tomassini et al., 1996, Singh and Green, 2001) and (iv) the utility of DKP-derivates in the treatment of aquaculture infections to protect cultured larvae has been the subject of a recent patent application (Fdhila et al., 2002, 2003).

On the other hand, we continue to focus on VHSV, a fish rhabdovirus, because of the enormous economic and social impact of this and related rhabdovirus in the expansion of aquaculture industry. Aquaculture appears to have strongest potential to meet the increasing demands for aquatic products in most regions of the world (Subasinghe, 2005). Moreover, except a DNA vaccine against infectious haematopoietic necrosis virus (IHNV) commercialised in Canada, no licensed vaccines or specific inhibitors against any fish rhabdovirus are yet available. In this regards, the search for antivirals in natural sources is also a promising research field (Micol et al., 2005).

After a preliminary screening, we selected among 98 DKP-derivatives (R1-DKP-R2) from DKP libraries those which have the strongest inhibition activity against VHSV in absence of toxicity to cells. Regarding the mode of action of selected DKP-derivatives, we show that the inhibition activity is dose-dependent and not due to a direct inactivation of the virus (no virucidal effect). Moreover, time-of-addition experiments suggest that DKP-derivative may not act upon the early viral replication step but interfere with an intracellular step of virus multiplication. Because of the smaller size of VHSV-infected cell foci and the lower titer of released VHSV observed when DKP-derivative are present in VHSV-infected cell cultures, the virus spread in cell culture may be affected by the inhibitors.

For the practical use new DKP-derivatives with significant higher specific activity need yet to be developed. Nevertheless, the selected DKP-derivatives may contribute to the further development of antiviral agents for fish rhabdovirus as well as tools to improve our knowledge on fish rhabdovirus infection cycle.

2. Materials and methods

2.1. *N,N′-disubstituted 2,5-piperazinedione combinatorial libraries*

Two previously described (Carbonell et al., 2000) libraries of discrete compounds based upon a 2,5-piperazinedione (DKP) scaffold (Fig. 1A), DKP-I and DKP-II (a *N*-oxide library generated from DKP-I library), were screened. The chemical diversity of DKP libraries is biased toward distinct amines, R1 and R2, in 1,4 *N,N′* positions of the 2,5-piperazinedione scaffold (Fig. 1A). In order to simplify the terminology, R1 amines were numbered from 1 to 7 (Fig. 1B) and R2 amines from 8 to 14 (Fig. 1B). The resulted DKP-derivates were named R1-DKP-R2. The total number of DKP-derivates screened was 98.

2.2. *EPC cell cultures and VHSV*

The fish cell line "*Epithelioma papulosum cyprini*" (EPC) (Fijan et al., 1983) used in this work derived from a skin tumour of carp (*Cyprinus carpio* L.) and was purchased from the European collection of cell cultures (ECACC no. 93120820). EPC cell monolayers were maintained at 28 °C as previously reported (Basurco et al., 1989, Perez et al., 2002). The virus used to infect the EPC cell monolayers at 14 °C was the viral haemorrhagic septicaemia virus (VHSV) 07.71 isolated in France from rainbow trout, *Oncorhynchus mykiss*. (LeBerre et al., 1977). Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × g during 20 min and kept in aliquots at −70 °C. Viruses from clarified supernatants were concentrated to 10^{10} foci forming units (f.f.u.) per ml by ultracentrifugation at 100,000 × g during 45 min (Basurco and Coll, 1989).

2.3. *Antiviral assays*

To test the direct influence of the DKP-derivates on VHSV infectivity, a previously developed immunostaining focus assay was used (Lorenzo et al., 1996; Mas et al., 2002; Perez et al., 2002). Briefly, prior to infection, DKP-derivates were pre-incubated at different concentrations with VHSV during 4 h at 14 °C in 100 μl of volume in RPMI-1640 cell culture medium supplemented with 2% fetal calf serum (FCS), 1 mM

sodium piruvate, 2 mM L-glutamine, 500 μg/ml gentamicin and 25 μg/ml amphotericin B. After the pre-incubation period, the mixtures containing 100 f.f.u. of VHSV plus each of the DKP-derivative were added to EPC cell monolayers, grown in 96-well plates, during 2 h at 4 °C (adsorption period). Then, the infected EPC cell monolayers were not washed (or washed when indicated) and further incubated during 24 h at 14 °C. After incubation, the EPC cell monolayers were fixed during 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) was added to the wells (100 μl/well) and incubated during 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 during 30 min. After three washings by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) in PBS containing H_2O_2 were added (Sanz and Coll, 1992; Lorenzo et al., 1996) and the reaction allowed to proceed until brown foci were detected with an inverted microscope. Once washed with water and air-dried, brown foci of DAB stained cells (VHSV-infected cell foci) or brown DAB stained cells (VHSV-infected single cells) were counted with an inverted microscope (Leica Ltd., Cambridge, UK) 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 a DKP−derivate/total number of VHSV−infected cell foci in the absence of DKP-derivate) × 100. The concentration at which an inhibition of 50% was observed, the 50% inhibition concentration (IC_{50}), was defined as the concentration of DKP-derivate, which reduced the percentage of VHSV-induced foci by 50% with respect to untreated virus and expressed in μM. For IC_{50} determination, the values were interpolated from dose–response data.

2.4. Cytotoxicity assays

The cytotoxic effects of the selected DKP-derivates on EPC cells were determined by quantifying cell viability using an MTS/PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl) 2H-tetrazolium] Assay (Cell Titer 96; Promega) according to the manufacturer's instructions. Cytotoxicity was examined following 2 days of cell exposure to different concentrations of DKP-derivates (from 25 to 400 μM). Results were expressed as the percentage of cell viability in the control (untreated EPC cells in culture medium) by the formula, (cell viability in the presence of DKP-derivate/cell viability in the absence of DKP-derivates) × 100.

2.5. Time-of-addition study

Briefly, EPC cells were grown on 96-well plates and infected with VHSV (100 f.f.u./well). Then, at infection time ($t = 0$) or at different times post-infection (p.i.) (4 and 12 h p.i.) active DKP-derivates (100 μM) were added or not to the infected cells and 24 h later, the presence of VHSV-infected cell foci were determined by the immunostaining focus assay described above. The results were expressed as the percentage of infectivity and calculated by the formula: (number of VHSV-infected cell foci in the presence of DKP/total number of VHSV-infected cell foci in absence of DKP) × 100.

2.6. Analysis of the presence and infectivity of VHSV particles in the supernatants of VHSV-infected EPC cells in the presence of DKP derivates

EPC cells were grown on 96-well plates and infected with VHSV (30 f.f.u./well). Then, at infection time ($t = 0$) 5-DKP-8 and 6-DKP-14 (both at 100 μM) was added to the infected EPC cells and the plates incubated at 14 °C. The presence of infectious virus in culture supernatants was evaluated at 24 and 48 h post-infection by a virus yield assay. For that, EPC cells were grown on 96-well plates to 100% confluence and then infected with serial dilutions of the supernatants. After adsorption, (2 h at 4 °C), the viral inoculum was removed, the cells washed with PBS and fresh medium was added to the infected cells. Plates were incubated at 14 °C during 24 h and the viral titters were determined by the immunostaining focus assay described above and expressed as foci forming units (f.f.u.) per ml. The results were expressed as the percentage of reduction of VHSV titers in the supernatants of VHSV-infected EPC cells in the presence of 5-DKP-8 or 6-DKP-14 and calculated by the formula 100 − [(f.f.u per ml in the presence of DKP derivate/f.f.u per ml in the absence of DKP derivate) × 100].

Moreover, in these culture supernatants, the presence of total virus particles (infectious and no infectious) was evaluated at 24 h post-infection by western blot. Briefly, the supernatants of VHSV-infected EPC cell cultures in the presence of 6-DKP-14 were harvested at 24 h p.i. and clarified by centrifugation at 3000 × g during 10 min. SDS-polyacrylamide gels at 15% were loaded with 20 μl of samples in buffer containing ß-mercaptoethanol. The proteins in the gel were transferred during 3 h at 125 mM in 2.5 mM Tris, 9 mM glycine, 20% metanol 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 with a PAb anti-VHSV, (Fernandez-Alonso et al., 1998), (1:200 dilution) before incubation with a peroxidase-conjugated rabbit anti-IgG mouse antibody (SIGMA) (1:500). Finally, the peroxidase activity was detected using the ECL chemiluminescence reagents (Amersham Biosciences, UK) and revealed by exposure to X-rayfilms (Amersham). The bands were quantified by densitometry with the Scion Program (http:ww.w.scionorg.com). Relative peak areas were calculated by the formula, (area under protein in the presence of 6-DKP-14/area under protein in the absence of 6-DKP-14) × 100.

2.7. VHSV protein detection in VHSV-infected cells in the presence of DKP-derivates by western blot

The influence of DKP compounds on VHSV proteins was estimated in VHSV-infected EPC cells by western blot using the 6-DKP-14. To prevent some effect of the compound on

viral adsorption/entry, EPC cell monolayers grown on 48-well plates were infected with 5 f.f.u. of VHSV per cell and incubated during 2 h at 4 °C, Then, the viral inoculum was removed, the cells washed with PBS and fresh medium containing 6-DKP-14 (100 μM) or devoid of inhibitors was added to the infected cells. Twenty-four hour p.i., cell culture supernatants were removed and 200 μl of PBS per well were added to the cells. Then, cells were frozen and thawed and supernatants from cell lysates clarified by centrifugation at 1000 × *g*. during 10 min and resuspended in electrophoresis buffer. SDS-polyacrylamide gels, western blots and band densitometries were performed as described above.

3. Results

3.1. Selection of DKP-derivatives from N,N-disubstituted 2,5-piperazinedione combinatorial libraries inhibiting VHSV infectivity in vitro

In order to identify VHSV inhibitors, each of the 98 DKP-derivatives (R1-DKP-R2) from the DKP-I and DKP-II libraries (Fig. 1) were screened for their ability to inhibit VHSV infectivity on EPC cell monolayers. The DKP-derivatives (400 μM) were pre-incubated with the VHSV inoculum and the mixture added to and incubated with the EPC cell monolayers until analysis. The preliminary screening of these libraries led to the identification of several DKP-I-derivatives with varying degrees of VHSV inhibiting capacity (from none to 100%) (supplementary information, Table 1). In contrast, no DKP-derivatives inhibiting more than 66.2% of VHSV infectivity were found among those obtained from the DKP-II library, the *N*-oxide library (supplementary information, Table 1). Since library screening was performed at high compound concentration, only the seven DKP-I derivatives inhibiting VHSV infectivity more than 99.7% were selected for further analysis. Two of them had R1 = *N*-(2,4-dichlorophenethyl)- (5-DKP-8 and 5-DKP-14) and four had R1 = *N*-(3,3-diphenylpropyl)-, (6-DKP-8, 6-DKP-11, 6-DKP-12 and 6-DKP-14).

3.2. Evaluation of the cellular toxicity of the DKP-derivatives with the highest inhibition capacity

To exclude non-specific antiviral activities due to cellular toxicity, the cell viability after an exposure during 48 h of the EPC cell monolayers to different concentrations of each of the selected DKP-derivatives was then studied (Fig. 2). At 400 μM, the concentration used for the screening of the libraries, only, 5-DKP-8 and 6-DKP-14 (Fig. 3A), did not shown any cytotoxic effects (Fig. 2) and consequently studies were only pursued with these DKP-derivatives.

3.3. Dose-dependent inhibition of VHSV infectivity by 5-DKP-8 and 6-DKP-14

To determine the minimum concentration required to detect in vitro inhibition, the effect of increasing concentrations (from 25 to 200 μM) of 5-DKP-8 and 6-DKP-14 on VHSV infectiv-

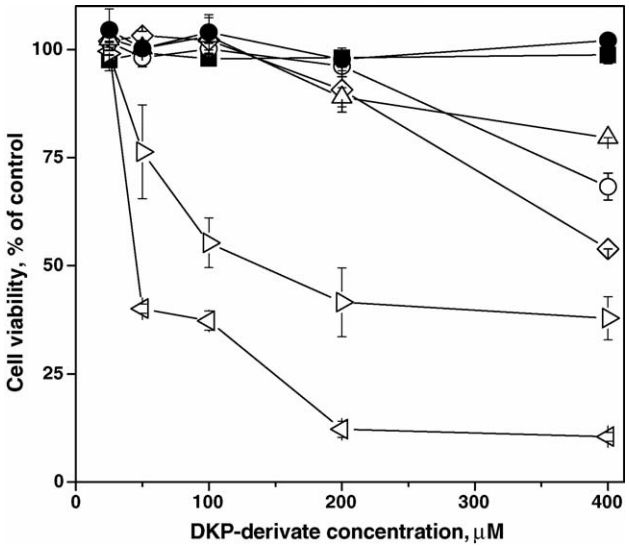

Fig. 2. Viability of EPC cells exposed to DKP-derivates causing 100% inhibition of the VHSV infectivity. EPC cells were seeded in 96-well plates and then exposed to different concentrations of DKP-derivates at 28 °C during 48 h. Cellular survival was assessed using a MTS/PMS assay (see Section 2). Average values and standard deviations from two different experiments each by triplicate were used. (Ξ) 3-DKP-12; (■) 5-DKP-8; (△) 5-DKP-14; (◯)6-DKP-8; (◊) 6-DKP-11; (χ) 6-DKP-12; (●) 6-DKP-14.

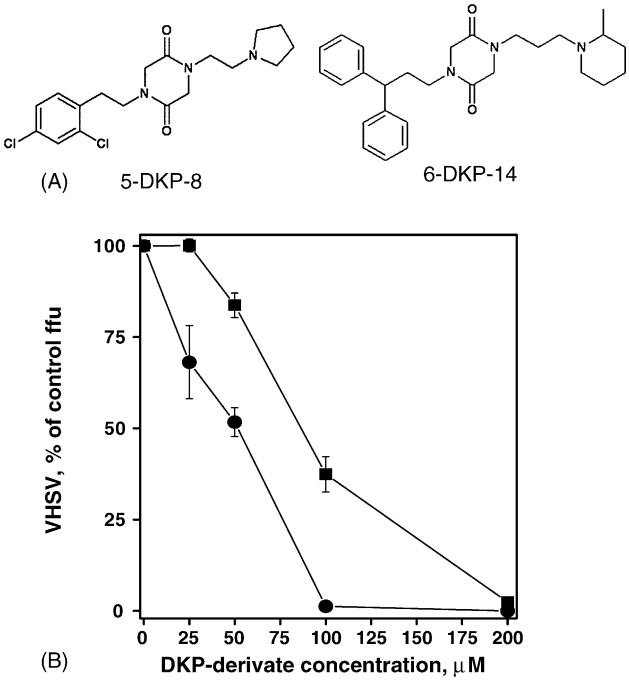

Fig. 3. Chemical structure of 5-DKP-8 and 6-DKP-14 and its dose-dependant anti-VHSV activity. (A) The structures of the selected DKP-derivates as follow: 5-DKP-8; *N*-(2,4-dichlorophenethyl)-*N*′-[2-(pyrrolidin-1-yl)ethyl]piperazine-2,5-dione; 6-DKP-14, *N*-(3-(2-methylpiperidin-1-yl)propyl)-*N*′-(3,3-diphenylpropyl)piperazine-2,5-dione. (B) Dose-dependent inhibition of VHSV infectivity was estimated by pre-incubation of VHSV with each of the DKP-derivatives at the indicated concentrations before infection of the EPC cell monolayers. Foci of VHSV-infected cells were quantified 24 h post-infection as indicated in materials and methods. Average values and standard deviations from two different experiments each by triplicate were represented. (■) 5-DKP-8 and, (●) 6-DKP-14.

ity, was estimated by the VHSV-infected cell foci formation assay in EPC cell monolayers. Both DKP-derivatives inhibited VHSV-infected cell foci formation in a dose-dependent manner (Fig. 3B) when pre-incubated with VHSV before the infection. The IC_{50} values obtained were 86.2 and 51.1 μM, for 5-DKP-8 and 6-DKP-14, respectively, suggesting that 6-DKP-14 had a higher inhibitory specific activity than 5-DKP-8. Moreover, at 100 μM, a four-fold lower concentration than that used for libraries screening, 6-DKP-14 inhibited VHSV infection nearly 100% whereas 5-DKP-8 inhibited only 62.5% (Fig. 3B).

3.4. Reduction of VHSV-infected cell foci size by 5-DKP-8 and 6-DKP-14

Confirming preliminary visual observations, the analysis of infection foci size (number of VHSV-infected cells per infection foci) demonstrated that not only a reduction in number of VHSV-infected cell foci but also a reduction in number of VHSV-infected cells per foci could be observed in EPC cell monolayers infected with VHSV pre-incubated with DKP-8 or 6-DKP-14. Thus, after 24 h post-infection (p.i.) in EPC cell monolayers infected with non-treated VHSV, more than 41% of the VHSV-infected cell foci contained 16–20 cells per foci (Fig. 4A), and significative amounts of larger foci of 21–25 and 26–30 cells per foci were also present. In contrast, when cells where infected with VHSV pre-incubated with 50 μM of either 5-DKP-8 or 6-DKP-14, most of the foci were single VHSV-infected cells or small VHSV-infected cell foci of 2–5 cells per foci (Fig. 4A). Both 5-DKP-8 and 6-DKP-14 showed a similar distribution of VHSV-infected foci sizes. These results suggest that virus spreading from initially infected cells is very inefficient.

3.5. Influence of the time of addition of 5-DKP-8 and 6-DKP-14 in their inhibiting activity

All the previously commented inhibitory assays were performed by pre-incubating the VHSV inoculum with the DKP-derivatives (inoculum mixture), adding the inoculum mixture to the EPC cell monolayers and maintaining their presence throughout all the time of incubation until analysis. However, if the inoculum mixture was removed after a VHSV adsorption step (2 h, 4 °C), the cell monolayers washed (to remove both DKP-derivates and unadsorbed virus) and incubation proceeded with fresh medium devoid of DKP-derivatives, the antiviral effects of either 5-DKP-8 or 6-DKP-14 were ∼90% reduced in each case (data not shown). Therefore, pre-incubation of VHSV with DKP-derivatives did no alter virus particles and cell binding properties of the virus and virus adsorption step did not appear to be affected.

To further investigate the time period required for inhibition, 5-DKP-8 and 6-DKP-14 were added at 0 h (from the beginning of infection), at 4 and at 12 h p.i. The results showed that a pre-incubation period was not necessary to obtain inhibition since a similar extent of VHSV-infectivity inhibition was obtained whether there was a pre-incubation period or not (0 h) (data not shown). Moreover, the inhibitory activity of 5-DKP-8 and 6-DKP-14 was also similar when they were added to culture medium at 0 or 4 h p.i. Thus, 6-DKP-14 reduced VHSV infectivity by ∼95% both at 0 and 4 h p.i. and still caused ∼50% inhibition when added at 12 h p.i. (Fig. 4B). 5-DKP-8 showed a parallel temporal inhibition pattern but with a much lower inhibitory effect than 6-DKP-14. These results demon-

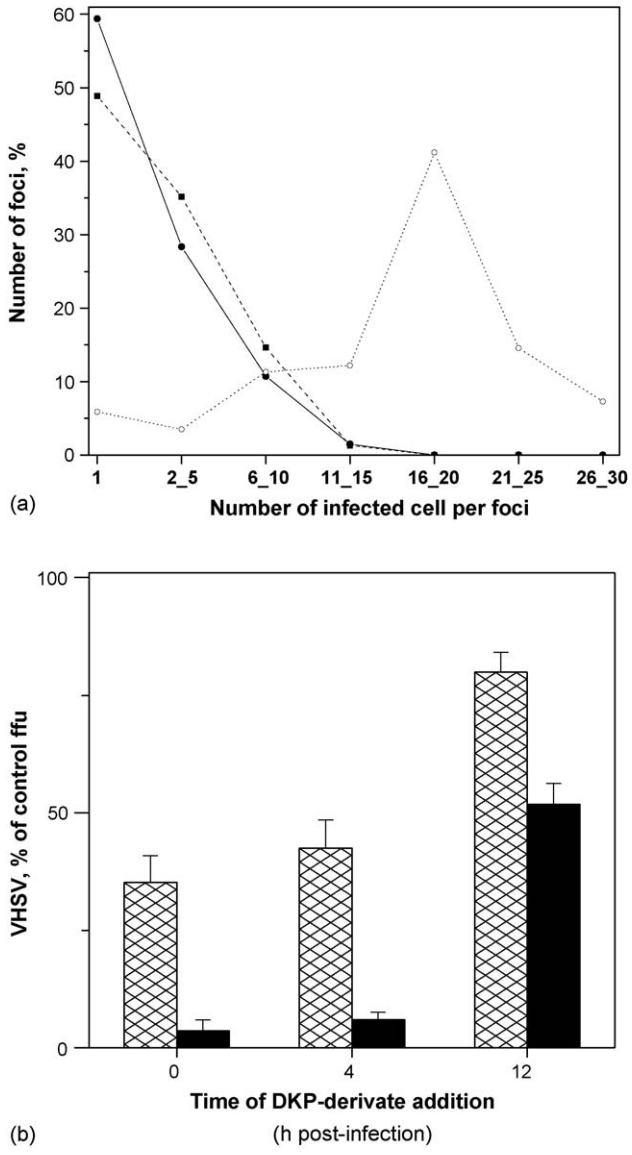

Fig. 4. Influence of the addition of 5-DKP-8 and 6-DKP-14 in VHSV-infected foci size (A) and of their time of addition in VHSV infectivity (B). (A) Influence of DKP-derivatives on the VHSV-infected foci sizes were estimated by pre-incubation of VHSV with each of the DKP-derivatives at the indicated concentration before infection of the EPC cell monolayers. Foci of VHSV-infected cells were quantified 24 h post-infection as indicated in materials and methods. (◯) sizes in the absence of DKP-derivatives; (■) sizes in the presence of 5-DKP-8; (●) sizes in the presence of 6-DKP-14. (B) Influence of the time of addition of DKP-derivatives in the VHSV-infectivity. In this assay the VHSV was not pre-incubated with the DKP-derivatives before infection of the EPC cell monolayers. The effects of adding 100 μM 5-DKP-8 or 6-DKP-14 at 0, 4 and 12 h after infection of EPC cell monolayers with VHSV were studied. Averages and standard deviations from two different experiments each by triplicate were used. Hatched bars, VHSV infectivity in the presence of 5-DKP-8; black bars, VHSV infectivity in the presence of 6-DKP-14.

Table 1
Titers of VHSV in supernatants from EPC cells infected with VHSV in the presence of 5-DKP-8 or 6-DKP-14 compound

Time post-infection (h)	VHSV titer in cell culture supernatants[a] (% of reduction)	
	5-DKP-8	6-DKP-14
24	76.1 ± 12.7	98.6 ± 21.2
48	96.4 ± 14.1	99.4 ± 5.6

[a] The results were expressed as the percentage of reduction of VHSV titers in supernatants of VHSV-infected EPC cells in the presence of DKP compound compared to VHSV titers in supernatants of VHSV-infected EPC cells in the absence of DKP compound. The data represent means ± standard deviations of two independent experiments each performed by triplicate.

strate that both DKP-derivates affected VHSV-infectivity in a time-dependent manner and they may not interfere with an early step of virus multiplication cycle.

3.6. Infectivity of recovered VHSV from the supernatants of VHSV-infected EPC cells in the presence of 5-DKP-8 or 6-DKP-14

EPC cell monolayers infected with VHSV in the presence of 100 μM of 5-DKP-8 or 6-DKP-14 showed a reduction of the VHSV relative titers to those obtained in the absence of DKP-derivatives of 76.1 or 98.6% 24 h p.i. and of 96.4 or 99.4% 48 h p.i., respectively (Table 1). These results confirmed again the higher inhibitory activity of the 6-DKP-14 relative to the 5-DKP-8, estimated now by the reduction in the titer of VHSV released in the supernatant of VHSV-infected EPC cell monolayers. These results also showed that the inhibition of virus spread in cell culture in the presence of active DKP-derivatives correlates with a lower amount of infective-VHSV released in the supernatants.

3.7. Presence of VHSV particles in VHSV-infected EPC cell supernatants infected in the presence of 6-DKP-14

To determine if the observed inhibition of VHSV titers in cell culture supernatants was due to a decrease in the amount of virus particles released (infective or not) from the infected cells, the culture supernatants from VHSV-infected EPC cells in the presence of an active DKP-derivate were analyzed by western blot at 24 h p.i. Western blot were only performed with 6-DKP-14 because of its stronger in vitro inhibitory activity against VHSV. The results showed an overall decrease >85% in the levels of VHSV proteins in the supernatants derived from cell infected in the presence of 6-DKP-14 compared to those present in control supernatants (supernatants derived from cell infected in the absence of 6-DKP-14) (Fig. 5A). Thus, the substantial reduction of VHSV particles detected in supernatants from VHSV-infected EPC cells in the presence of 6-DKP-14, indicate that the observed reduction of viral titers seems to correlate with an overall reduction in the number of viral particles released rather than the production of viruses with diminished infectivity.

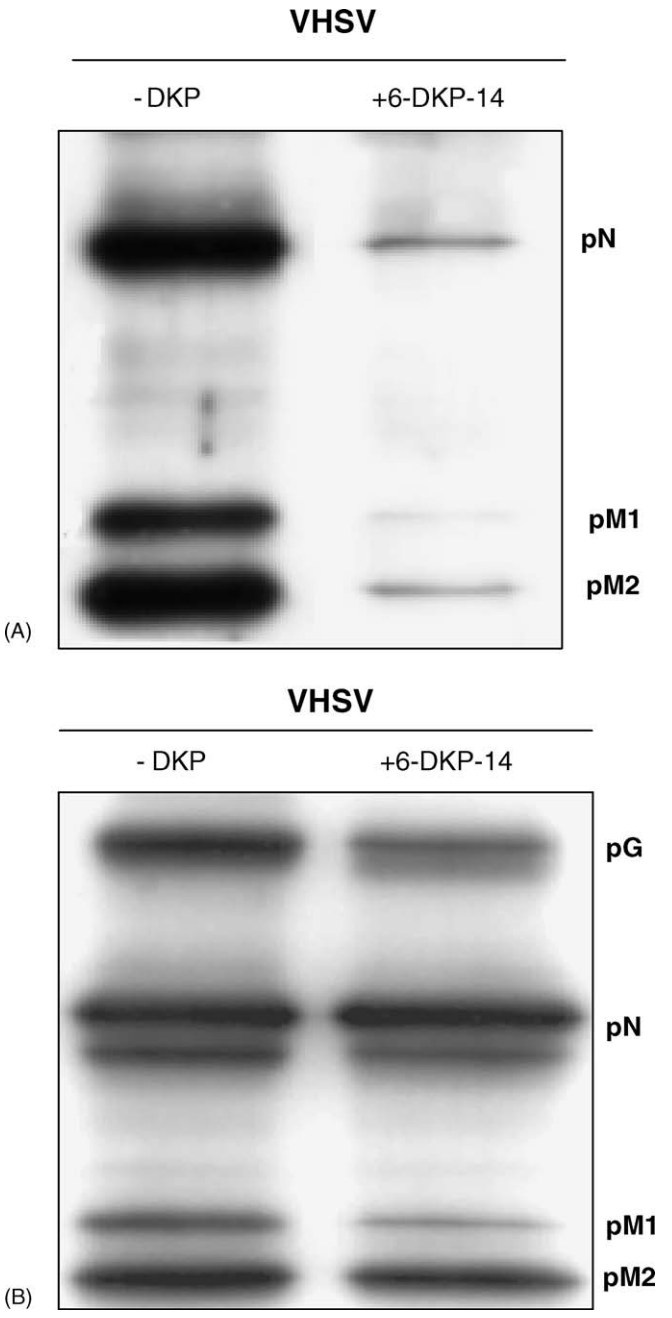

Fig. 5. Influence of 6-DKP-14 on VHSV protein accumulation in VHSV-infected cells. (A) Supernatants from EPC cells infected with VHSV in the presence of 6-DKP-14 or in the absence of compound collected 24 h p.i. and (B) EPC cell monolayers infected with VHSV in the presence of 6-DKP-14. In this case, 6-DKP-14 was added to infected cells after adsorption period. VHSV-infected cells were harvested and lysed at 24 h p.i., Samples from supernatants and cell lysates were separated by SDS-PAGE, transferred to nitro-cellulose membranes and then they were recognised with a PAb against VHSV. The blots are representative of three independent experiments. pG, VHSV-G glycoprotein; pN, VHSV-N nucleoprotein; pM1, VHSV-M1 phosphoprotein; pM2, VHSV-M2 membrane protein.

3.8. VHSV protein accumulation in VHSV-infected EPC cells in the presence of 6-DKP-14

Western blotting was also used to analyze the levels of viral protein accumulation in VHSV-infected EPC cell resid-

ual monolayers at 24 h p.i. No such a large differences as those obtained with the supernatants were observed in the viral proteins of VHSV-infected cells in the presence of 6-DKP-14, except a slight relative reduction of the G and M1 (Fig. 5B). The densitometry of the bands corresponding to the immunoblotted G and M1 proteins confirmed the reduction observed visually in the western blot estimating 31.2% and 42.9%, respectively lower levels than in EPC cells infected in the absence of 6-DKP-14.

4. Discussion

A combinatorial strategy has been used to identify VHSV inhibitors. For this task, two libraries of *N*,*N*′-disubstituted 2,5-piperazinediones, DKP-I and DKP-II were screened by a infectious-foci forming assay and a novel class of VHSV inhibitors identified. Similar combinatorial approaches have been used successfully to find inhibitory compounds against human immunodeficiency virus type I (HIV-I) (Ferrer et al., 1999; Rinnova et al., 2000; Park and Raines, 2000; Hwang et al., 2003; Srivastava et al., 2004; Frecer et al., 2005), picornavirus (Tsang et al., 2001), influenza virus (Hochgurtel et al., 2002) and adenovirus (Horne et al., 2005).

The preliminary screening of DKP-I library led to identification of seven DKP-derivates which completely abolished VHSV infectivity (supplementary information, Table 1) but no inhibitors of VHSV infectivity were found among compounds of DKP-II library, the N-oxide library. Since the only difference between the libraries was the introduction of an oxide group in R2, which introduces a negative charge, that might be a possible explanation for the abolishment of the inhibitory activity found in the corresponding members of the partner library. On the other hand, that result also makes clear the specificity of the inhibition found in the DKP-I library compounds.

Among the DKP-derivatives with the highest inhibitory activity two of them had R1 = *N*-(2,4-dichlorophenethyl)- (5-DKP-8 and 5-DKP-14) and four had R1 = *N*-(3,3-diphenylpropyl)- (6-DKP-8, 6-DKP-11, 6-DKP-12 and 6-DKP-14), which might indicate a requirement for the presence of phenyl rings in R1 to strongly inhibit VHSV infectivity.

On the other hand, among the seven compounds identified after the screening of DKP-I library only two, the 5-DKP-8 and 6-DKP-14 (Fig. 3A), were selected for further studies. The selection procedure was based on the absence of toxicity of these compounds on EPC cells at any of the tested concentrations (Fig. 2). The discarded DKP-derivatives showed toxic effects at concentrations above 100 μM (Fig. 2). In contrast, no cytotoxic effects were found up to 250 μM for the same DKP-derivatives of the DKP-I library when tested on HeLa human cell cultures (Carbonell et al., 2000). Therefore, EPC carp cells seem to be more susceptible to DKP-derivatives than human cells.

When the ability of the selected DKP-derivates to inhibit VHSV was further characterized, we observed that these compounds cause dose-dependent inhibition of VHSV infectivity showing activity at four-fold lower concentrations that those used for the library screening. Thus, at 100 μM, the compound 6-DKP-14 reduced VHSV infectivity by 100% (Fig. 3B). Similar concentrations were required to completely inhibit influenza virus with the N substituted 2,6-piperazinedone, flutimide (Tomassini et al., 1996).

Regarding the mode of action of 5-DKP-8 and 6-DKP-14, we show that the inhibition of infectivity is not due to a direct inactivation of the VHSV (no virucidal effect). Moreover, the time-of-addition experiments (Fig. 4B) showed that both compounds affect VHSV infectivity in time-dependent manner, exerting similar antiviral activity when added to infected cells at 0 or 4 h p.i. Thus, these results suggest that 5-DKP-8 and 6-DKP-14 may not act upon the early viral replication steps such as adsorption, entry and uncoating and interfere with an intracellular step of virus multiplication. As consequence of this interference very low amounts of virus particles are released from infected cells in the presence of active DKP-derivates (Table 1 and Fig. 5A) and virus spread in cell culture is prevented (Fig. 4A). However, virus replication occurred to some degree in the presence of inhibitors as viral protein accumulation could be detected in infected cells (Fig. 5B).

The proposed mechanism of action of the N substituted 2,6-piperazinedone, flutimide, is the inhibition of primary transcription of influenza virus, specifically the cap-dependent endonuclease of transcriptase (Tomassini et al., 1996). If VHSV transcriptase were also the target of 5-DKP-8 or 6-DKP-14, the level of accumulation of all VHSV proteins would be expected to be affected to a similar degree. However, the effect of 6-DKP-14 on the G glycoprotein and M1 phosphoprotein levels were more pronounced than the effect on the other VHSV proteins (Fig. 5B), suggesting some other mechanism.

On the other hand, the level of accumulation of VHSV proteins in VHSV-infected cells in presence of 6-DKP-14 (Fig. 5B) did not account for the observed inhibition of infectious virus release by the cells (∼76 and 98% for 5-DKP-8 and 6-DKP-14, respectively, Table 1). However, a potential defect in virus assembly/budding may explain this discrepancy. After analyzing the presence of VHSV in supernatants from cell cultures infected with VHSV in the presence of 6-DKP-14 could be determined that the decrease in infectivity was due to an overall suppression of VHSV particles release in these supernatant (Fig. 5A). Thus, virus assembly/budding seems to be compromised in the cells infected in the presence of these inhibitors. A similar phenomenon was observed in neuroblastoma cells pretreated with INF-β and then infected VSV (vesicular stomatitis virus, a mammalian rhabdovirus) (Trottier et al., 2005). A specific inhibition of some of the assembly stages of VSV infection by INF-β was proposed as an explanation in that case. Although a direct inhibition of virus assembly could also be caused by the 6-DKP-14, since both phosphoprotein and glycoprotein are critical for assembly and budding process of infectious rhabdovirus (La Ferla and Peluso, 1989; Green et al., 2000; Das and Pattnaik, 2005; Maillard et al., 2003), it is plausible that the decrease in the steady state levels of G and M1 proteins will alter the correct stoichiometry required for efficient virus assembly/budding. Additional studies are in progress to determine the molecular mechanics underlying the selective effect of DKP-derivates on VHSV G and M1 proteins and how the impaired ratio of these proteins in VHSV-infected cells makes diminish the efficiency of virus assembly/release.

The findings obtained in the present study do not yet allow the use of DKP-derivates for the treatment of VHSV because for the practical utility of this new class of antivirals agents, new DKP-derivates with significant higher activity need to be fashioned. Nevertheless, they may contribute to the further development of antiviral agents for rhabdovirus as well as tools to improve our knowledge on rhabdovirus infection cell cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2006.04.005.

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