Antiviral Research 96 (2012) 187–195

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A small molecule screen in yeast identifies inhibitors targeting protein–protein interactions within the vaccinia virus replication complex

Olivier Flusin [a], Laurent Saccucci [a,b], Céline Contesto-Richefeu [a], Amel Hamdi [c], Carine Bardou [d], Thomas Poyot [e], André Peinnequin [e], Jean-Marc Crance [a], Pierre Colas [c], Frédéric Iseni [a,*]

[a] Unité de virologie, Institut de Recherche Biomédicale des Armées (IRBA), 24 avenue des Maquis du Grésivaudan, 38702 La Tronche, France
[b] Protein'eXpert SA, zone Minatec Entreprises, 7 Parvis Louis Néel BP50, 38040 Grenoble, France
[c] Station Biologique, CNRS, USR 3151, P2I2 Group, Place Georges Teissier, 29680 Roscoff, France
[d] IMAXIO SA, 181-203 avenue Jean Jaurès, 69007 Lyon, France
[e] Plateforme de Génomique, Institut de Recherche Biomédicale des Armées (IRBA), 24 avenue des Maquis du Grésivaudan, 38702 La Tronche, France

ARTICLE INFO

Article history:
Received 26 April 2012
Revised 23 July 2012
Accepted 24 July 2012
Available online 2 August 2012

Keywords:
Vaccinia virus
Small molecules
Yeast two-hybrid
Protein interaction inhibitors
Anti-orthopoxvirus drugs

ABSTRACT

Genetic and biochemical data have identified at least four viral proteins essential for vaccinia virus (VACV) DNA synthesis: the DNA polymerase E9, its processivity factor (the heterodimer A20/D4) and the primase/helicase D5. These proteins are part of the VACV replication complex in which A20 is a central subunit interacting with E9, D4 and D5. We hypothesised that molecules able to modulate protein–protein interactions within the replication complex may represent a new class of compounds with anti-orthopoxvirus activities. In this study, we adapted a forward duplex yeast two-hybrid assay to screen more than 27,000 molecules in order to identify inhibitors of A20/D4 and/or A20/D5 interactions. We identified two molecules that specifically inhibited both interactions in yeast. Interestingly, we observed that these compounds displayed a similar antiviral activity to cidofovir (CDV) against VACV in cell culture. We further showed that these molecules were able to inhibit the replication of another orthopoxvirus (i.e. cowpox virus), but not the herpes simplex virus type 1 (HSV-1), an unrelated DNA virus. We also demonstrated that the antiviral activity of both compounds correlated with an inhibition of VACV DNA synthesis. Hence, these molecules may represent a starting point for the development of new anti-orthopoxvirus drugs.

1. Introduction

Variola virus (VARV), the etiologic agent of smallpox, was responsible of the most devastating of all infectious diseases. After an intense campaign of vaccination to eliminate the virus worldwide, the last natural case of smallpox was reported in 1977 in Somalia (Geddes, 2006). In the late 70's, the vaccination program against smallpox had ceased in most countries which results in a high proportion of non-immune people in today's population. Therefore, if used as a biological weapon, VARV could represent a serious threat to the civilian population since no specific antiviral treatment is yet available against orthopoxvirus infection and vaccination is a source of morbidity and mortality (Berche, 2001). As a consequence, important efforts have been made, in recent years, to develop effective new anti-poxviral agents. These compounds would not only be precious in response to the malicious release of VARV but could also be used to treat complications due to vaccination or infections caused by monkeypox virus (MPXV) and cowpox virus (CPXV).

Currently, for treatment of human poxvirus infections, the food and drug administration (FDA) only allows the use of cidofovir (CDV), a nucleoside analogue approved for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients. CDV was shown to block the replication of a broad range of DNA viruses in cell culture (De Clercq, 2003) and is effective against orthopoxviruses such as VARV, vaccinia virus (VACV), CPXV, and MPXV (De Clercq, 2002). The efficacy of CDV was also proven, *in vivo*, in several animal models challenged with lethal doses of either VACV, CPXV or MPXV (Bray et al., 2002, 2000; Smee et al., 2001a,b; Stittelaar et al., 2006). Unfortunately, in case of a renewed smallpox outbreak, the large-scale use of CDV is unrealistic because of its poor oral bioavailability (i.e. it must be administered intravenously), and its significant kidney toxicity. However, a promising CDV chemical analogue (CMX001), that could be delivered orally, was shown to retain a potent antiviral activity while being less nephrotoxic (Parker et al., 2008). This molecule is currently under development (phase II clinical trials) for the treatment of poxvirus infections. Recently, ST-246, another efficient anti-orthopoxviral compound

* Corresponding author. Tel.: +33 4 76 63 97 72; fax: +33 4 76 63 69 17.
E-mail address: fiseni@crssa.net (F. Iseni).

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http://dx.doi.org/10.1016/j.antiviral.2012.07.010

active in cell culture and mice has been identified (Duraffour et al., 2007; Yang et al., 2005). Unlike CDV which targets the viral DNA polymerase (Kornbluth et al., 2006), ST-246 interfere with the function of F13, a viral protein involved in virion assembly and dissemination (Blasco and Moss, 1991; Yang et al., 2005). Clinical studies in healthy human volunteers have shown that ST-246 does not induce adverse effects. SIGA Technologies, Inc. will produce and sell 1.7 million doses of the antiviral to the US Strategic National Stockpile under Advanced Research and Development Authority (BARDA) contract. However, despite their efficacy in animal models, CMX001 and ST-246 may not successfully pass the clinical trials, where attrition rates are generally high. Hence, as recommended by the WHO, it is crucial to find new treatments and to develop new strategies to design potent inhibitors against orthopoxviruses (WHO, 2010).

Protein-protein interactions (PPI) play a major role in most biological processes, including the successive events leading to the production of viruses from infected cells. In that particular case, protein interactions occur between cellular and virus-encoded components or between viral proteins themselves. Theses interactions are involved in all steps of the virus life cycle such as entry, replication (see below), assembly, egress, and many of them are potential targets for antiviral agents (Loregian et al., 2002). Inhibitors of such interactions present some advantages over more classical compounds designed to inhibit the enzymatic activity of a protein target. Indeed, the complementarity of protein–protein interfaces is such that it gives the possibility to discover small molecules that will interfere with the interaction in a highly specific manner (Laudet et al., 2007). Furthermore, resistance to these molecules is unlikely since a mutation on the binding interface of one of the partners would require a simultaneous compensatory mutation on the other partner to restore the interaction (Loregian and Palu, 2005). In contrast, a single mutation often suffices to lead to drug resistance against active site-directed inhibitors. This is what was observed with CDV where mutations at position 314 or 684 in the VACV DNA polymerase induced viral resistance (Andrei et al., 2006).

Poxviruses are unique among DNA viruses in that they replicate exclusively in the cytoplasm of the host cell. DNA synthesis takes place in so-called 'viral factories' which consist of cytoplasmic foci located at the periphery of the nucleus (Moss, 2007). Because they do not rely on nuclear enzymes, poxviruses encode most if not all the proteins necessary for genome replication. Among these proteins, four were shown to be essential and directly involved in the synthesis of the ~200 kbp genome. For VACV these are: E9, the catalytic subunit of the DNA polymerase, D5, a DNA-independent nucleoside triphosphatase (NTPase) that contains a helicase domain (Boyle et al., 2007) and a primase activity (De Silva et al., 2007), D4, an uracil DNA glycosylase and A20, a central component whose role is likely to assemble and/or stabilize the complex formed by these four proteins. The heterodimer formed by A20 and D4 was shown to act as a processivity factor for the viral DNA polymerase (Stanitsa et al., 2006). Through the direct interaction between A20 and E9 (Klemperer et al., 2001), the three proteins assemble the processive DNA polymerase holoenzyme. Yeast-two hybrid experiments have also revealed that D5 interacts with a central domain of A20 (Ishii and Moss, 2002; McCraith et al., 2000). Thus, the discovery of molecules able to disrupt PPI within the replication complex, and particularly the interactions between A20 and its viral partners, could represent an excellent strategy to develop effective antiviral drugs against orthopoxviruses.

In this study, a high-throughput (HTP) screening of small molecules was carried out to identify compounds capable of modulating A20 interaction with D4 and/or D5 proteins. The screen was based on an automated dual-luminescence yeast two-hybrid assay, performed in 384-well plates (Baines and Colas, 2006; Nieuwenhuijsen et al., 2003). This duplex assay performed with two luciferase reporter genes (firefly and *Renilla*) enabled us to screen, simultaneously, two different yeast strains, each one hosting a distinct pair of interacting proteins (i.e. A20/D4 and A20/D5).

Screening of a collection of ~27,000 compounds from diverse commercial libraries led to the identification of two molecules that inhibit VACV in cell culture. These compounds display a similar antiviral activity than CDV and are also good inhibitors of CPXV replication. However, in contrast to CDV, they do not inhibit HSV-1 growth, thus demonstrating a good specificity. We further showed that, as expected, the two molecules interfere with VACV DNA synthesis.

2. Materials and methods

2.1. Materials

Small-molecule compounds used in this study were purchased from Prestwick (Illkirch), Nanosyn (Santa Clara), Tripos (St. Louis) and Chembridge (San Diego) chemical libraries. Compounds 10,295 and 8776 were purchased from ASDI Biosciences (Newark, DE) (vendor ID, 150003007) and LifeChemicals (Burlington, Canada) (vendor ID, F0035-0015) respectively. Cidofovir (HPMPC, Vistide™) was synthesized at, and provided by Gilead Sciences (Foster City, CA). The yeast strains and yeast two-hybrid plasmids used in this study were described in detail elsewhere (Bardou et al., 2009).

2.2. Cells and viruses

Vero cells (African green monkey kidney cells, ATCC CCL-81) and A549 cells (human lung carcinoma cell line, ATCC CCL-185) were grown in M199 medium and F12K medium (Gibco, Invitrogen Corporation, UK), respectively, supplemented with 10% foetal calf serum (FCS) and maintained at 37 °C in a 5% CO_2 atmosphere. BHK21 cells (baby hamster kidney, ATCC CCL10) were cultured in Glasgow minimum essential medium GMEM (Gibco, Invitrogen Corporation) containing 10% FCS, 10% tryptose phosphate (Sigma–Aldrich, St Quentin-Fallavier, France) and 50 mM HEPES (Gibco, Invitrogen Corporation) at 37 °C in 5% CO_2 atmosphere. HeLa cells (human cervical carcinoma, ATCC CCL-2) were propagated in Minimum essential medium (MEM, Gibco, Invitrogen Corporation) with 1% non essentials amino acids (Gibco, Invitrogen Corporation), 1% L-glutamine (Gibco, Invitrogen Corporation), 1% sodium pyruvate (Gibco, Invitrogen Corporation), 10% FCS and were incubated at 37 °C with 5% CO_2.

VACV strain Lister (provided by Sanofi-Pasteur, batch number X55-33), CPXV strain Brighton (ATCC VR 302) and HSV-1 (kindly provided by Pr. Jean-Marie Seigneurin, La Tronche, France), were grown on Vero cells.

2.3. Plasmid constructions

pLP4-D4R plasmid, which carries a CEN/ARS origin of replication, was constructed by PCR amplification of the D4R gene from VACV (Copenhagen strain) DNA using the primers 5′-TCT GAA TTC ACC ATG AAC TCA GTG ACT GTA TCA CAC G-3′ and 5′-CTC CTC GAG TCA ATA AAT AAA CCC TTG AGC CCA ATT TAT AG-3′. The PCR fragment was cleaved and ligated into the EcoRI/XhoI-cleaved pLP4, to obtain a plasmid that directs the galactose-inducible expression of a B112–NLS-D4 fusion protein (prey). pLP2-D5R, a high-copy number plasmid (2μ replication origin), was constructed by subcloning the D5R gene from pWP2-D5 plasmid (Saccucci et al., 2009) into the XhoI/EcoRI-cleaved pLP2, to obtain a plasmid that directs the galactose-inducible expression of a B112–NLS-D5 fusion protein (prey). pHA1-A20R plasmid (CEN/

ARS) was obtained by subcloning the VACV A20R gene from previously described pEG202-A20 vector (Saccucci et al., 2009) into the Xho1/EcoR1-digested pHA1, to obtain a plasmid that directs the constitutive expression of a LexA-A20 fusion protein (bait).

2.4. Construction of luciferase reporter yeast strains

All yeast transformations were performed as described (Bardou et al., 2009). To generate the firefly luciferase reporter strain, TB50α *erg6* yeast were sequentially transformed with pRAP-Col (Bardou et al., 2009), pLP4-D4R and pHA1-A20R vectors. Similarly, the *Renilla* luciferase reporter strain was obtained by co-transformation of TB50α *erg6* yeast with pRAP-Ren (Bardou et al., 2009), pLP2-D5R and pEG202-A20R vectors.

2.5. High-throughput dual luciferase screening

Screening was performed on a PerkinElmer robotic automated platform composed of the following parts: an Evolution™ P^3 precision pipetting platform, a FlexDrop™ Precision Reagent Dispenser and an EnVision® Multilabel plate readers (Perkin Elmer, Shelton, CT). Screening was carried out in 384-well plates, essentially as previously described (Bardou et al., 2009). Yeasts were incubated for 6 h in presence of galactose and small-molecules (10 μM final concentration). Using both positive and negative controls the statistical parameter Z′, assessing the quality of the screen was calculated (Bardou et al., 2009). For each plate tested during the course of the screen, Z′ values were always >0.5.

2.6. Yeast two-hybrid inhibition specificity assays of compounds 8776 and 10,295

Two TB50α *erg6* yeast strains carrying the plasmid combinations pLP2-D5R/pEG202-A20R/pRAP-COL and pLP4-D4R/pHA1-A20R/pRAP-COL were tested together with a yeast strain hosting an unrelated control interaction and also carrying the pRAP-COL reporter plasmid (A. Hamdi and P. Colas, unpublished results). Compounds 8776, 10,295 and 3C11 (specific inhibitor of the control interaction) were incubated at 10 μM with yeast. After 4 h incubation in the presence of 2% galactose the luminescence signal was quantified as described (Bardou et al., 2009). The percentage of luminescence inhibition was calculated by the ratio between the signals obtained in presence of compounds and the signals obtained without molecule. Each point was done in triplicate.

2.7. Cytotoxicity assay

Cytotoxicity assays were performed using the Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. Briefly, cells were seeded in 96-well plates at a density of 5×10^4 cells/well in 100 μl cultured medium. One day after, cell supernatants were removed and cells were treated for 24 h at 37 °C with different concentrations of compounds diluted in medium. As a control, cells were grown in M199 medium with DMSO at an equivalent concentration to that present in the medium containing the highest concentration of compound tested. Then, 10 μl of WST-1 reagent were added to each well and the plates were incubated at 37 °C for 30 min. Absorbance was measured at 450 nm. Each compound was tested in triplicate in 2-fold serial dilutions. Cytotoxicity was determined as the ratio of absorbance between treated cells and non-treated cells. The CC_{50} (concentration required to reduce cell viability by 50%) was calculated as the mean of two independent experiments using the GraphPadPrism version 4.00 software (GraphPad Software, San Diego California, USA) for non linear regression.

2.8. Antiviral assays

2.8.1. Viral plaque reduction assay

Confluent monolayers of Vero cells in 24-well plates were infected with 50 plaque forming units (PFU)/well of VACV in M199 medium. After 1 h of incubation, cells were washed and then cultivated with M199 medium supplemented with 0.4% FCS or treated with 0.4% FCS M199 medium containing various concentrations of compounds. One day post-infection, cells were fixed and stained in a solution of 1.95 g/l of crystal violet, 4% formaldehyde and 7% ethanol. Each well was rinsed, dried and plaques were counted under the light microscope. Each compound was tested in quadruplicate in 2-fold serial dilutions. The 50% effective concentration (EC_{50}), defined as the concentration of molecule required to reduce plaque number by 50%, was calculated as the means from two independent experiments.

2.8.2. Virus yield reduction assay

Confluent Vero cells grown in 24-well plates were infected with VACV, CPXV or HSV-1 at an MOI of 0.01 in M199 medium. The viral inoculums were removed 1 h post-infection, cells were rinsed in medium and then treated with different concentrations of compound 10,295 or 8776 or cultivated in M199 medium supplemented with 0.4% FCS at 37 °C in a 5% CO_2 atmosphere. Each compound was tested in quadruplicate in 2-fold serial dilutions. Virus titration was performed 24 h post-infection, on Vero cells, in 96-well microtiter plates as already described (Ferrier et al., 2004). The EC_{50} was calculated as the means from two independent experiments.

2.9. Quantification of viral DNA synthesis

Vero cells, seeded in 24-well plates, were infected with VACV at an MOI of 1. One hour post-infection, monolayers were washed twice and treated with antiviral compounds (50 μM of compound 8776, 25 μM of compound 10,295 or 50 μM of CDV) or cultivated with fresh media supplemented with 0.4% FCS (and with 0.1% DMSO) at 37 °C in a 5% CO_2 atmosphere. Infected cells were harvested at 3, 6, 9 and 15 h post-infection. Medium was removed and cells were lysed following 3 cycles of freezing and thawing in 200 μl of PBS 1X. Total DNA was extracted using the QIAamp DNA mini kit (Qiagen) with a proteinase K digestion step (56 °C, 30 min), according to the manufacturer's instructions. Lysates were loaded on a Qiacube instrument (Qiagen). DNA was eluted in 200 μl DNase free water and was stored at −20 °C until PCR amplification. qPCR were performed in duplicate on a Lightcycler (Roche Applied Science) using 5 μl of diluted total DNA (1/200) in a 20 μl final volume. VACV qPCR assay was described elsewhere (Scaramozzino et al., 2007). DNA standards, primer design, optimization and specificity checking were done as described previously (Peinnequin et al., 2004). Normalization was carried out using the geometrical mean of 2 validated standards targeting mitochondrial DNA (mtDNA) and a repeated chromosomic DNA sequence (aluSx) (Poyot et al, manuscript in preparation). qPCR were carried out with LC Fast Start DNA Master SYBR Green kit (Roche Applied Science) using 4 mM $MgCl_2$, 0.4 μM of each primer. PCR were performed for 50 cycles at 95 °C for 20 s, 59 °C for 5 s and a final step of 8 s at 72 °C, with the following primers: mtDNA, 5′-GGA CTA ACC CCT ATA CCT TCT GCA T-3′ and 5′-CGG GTG TGC TCT TTT AGC TGT-3′ (AY612638); aluSx 5′-ACA TGG TGA AAC CCC GTC TTT AC-3′ and 5′-TGA CGT GAT CTC AGC TCA CTG TAA C-3′ (consensus of AF190116, AF190117 and AY497013). Quantification cycles (Cq) values were calculated from the Light Cycler Software v.3.5 (Roche Applied Science) using the second derivative maximum method. Data are presented as means ± SEM. A 1-way analysis of variance was used to evaluate the global effect. When

appropriate, the Newman-Keuls post hoc test was used for inter-group comparisons. Statistical significance was accepted for $p < 0.05$.

3. Results

3.1. Yeast two-hybrid small-molecule screening against A20/D4 and A20/D5 interactions

The duplex assay developed in this study allowed the quantification, in a single well, of two interaction phenotypes, produced by the A20/D4 and A20/D5 protein interactions that are formed in two yeast strains carrying the firefly (*luc*) and the *Renilla* (*ruc*) luciferase reporter genes, respectively. As depicted in Fig. 1A, the originality of this assay relies on the fact that both interaction phenotypes are screened at once. Thus, a small molecule interfering selectively with the *luc* signal would be retained as a potential A20/D4 interaction inhibitor, while a hit candidate for A20/D5 interaction inhibition would specifically inhibit the *ruc* signal. Usually, a compound interfering with both bioluminescent reporters would be discarded without further analysis and considered as toxic to yeast (Bardou et al., 2009). However, in this particular assay, one cannot exclude the possibility that molecules interfering with both signals could interact with A20, induce a conformational change and alter both interactions (Fig. 1A). Since yeast permeability to drugs is a recurrent concern, an *erg6 Saccharomyces cerevisiae* strain, carrying a mutation in the ergosterol biosynthetic pathway (which enhances membrane permeability) was used (Mukhopadhyay et al., 2002).

Prior to starting the screening, a yeast two-hybrid pilot assay was performed in order to quantify the luciferase signals produced upon A20/D4 and A20/D5 interactions in yeast. The aim of this preliminary experiment was to select yeast two-hybrid expression plasmids that produced similar two-hybrid phenotypes (i.e. comparable *luc* and *ruc* signals) (Bardou et al., 2009). Indeed, we and others reported a strong interaction phenotype for A20/D4 and a weak yeast two-hybrid phenotype for A20/D5 (Ishii and Moss, 2002; Saccucci et al., 2009). To adjust the bioluminescent signals, a series of constructs expressing A20, D4 and D5 yeast two-hybrid fusion proteins were built in plasmids containing either a high-copy (2μ) or a low-copy *CEN/ARS*-replication origin and the interaction phenotypes obtained from the different combinations were tested (data not shown). This effort allowed us to identify two plasmid combinations that produced comparable yeast two-hybrid phenotypes and were thus suitable for the following small-molecule screening (Fig. 1B).

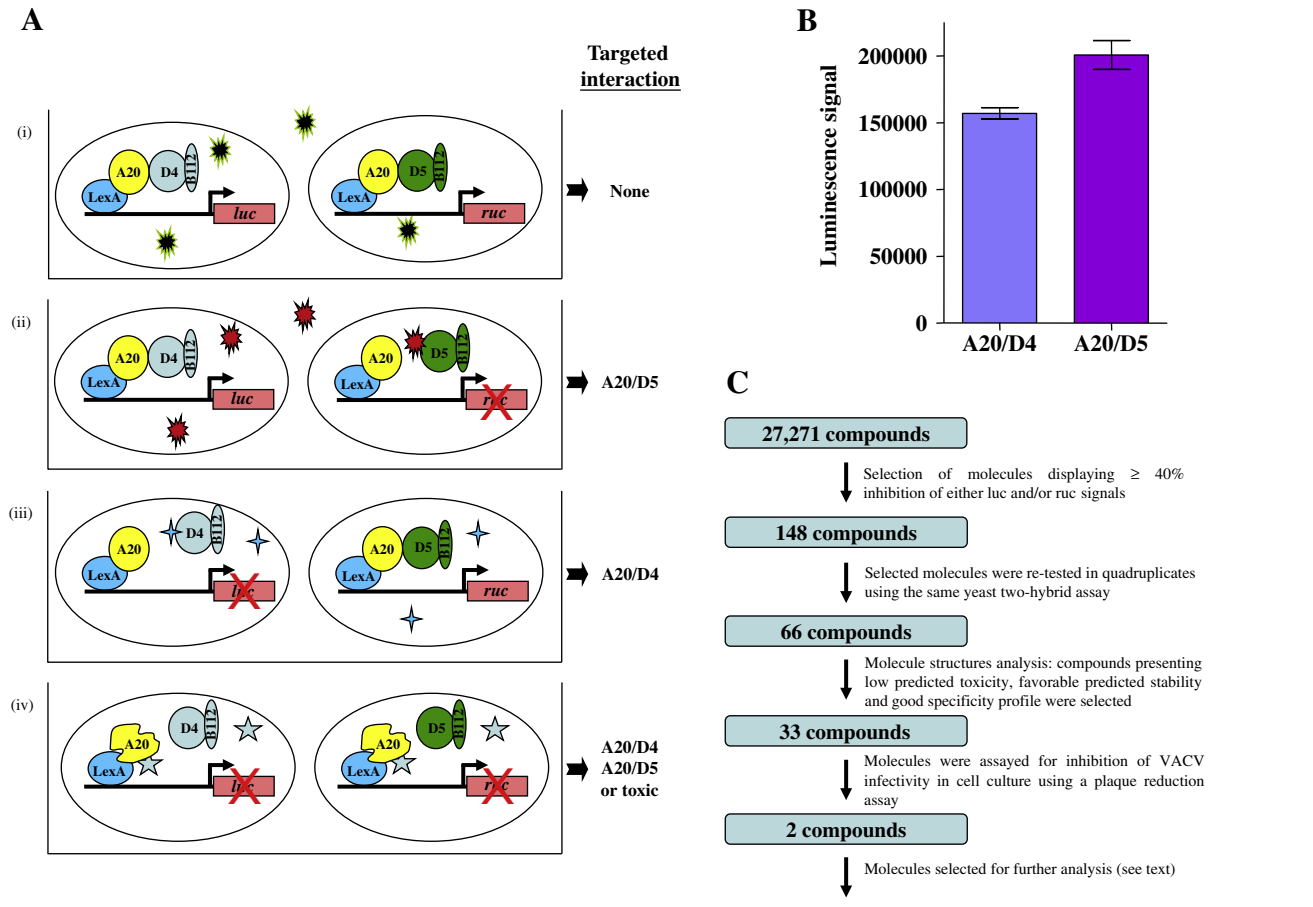


Fig. 1. Dual luminescence yeast two-hybrid screen. (A) An equal volume of two TB 50α *erg6* yeast populations expressing A20/D4 and A20/D5 were loaded in 384-well plates. A20 is fused to the DNA-binding domain of LexA transcription factor while D4 and D5 are fused to the B112 transcriptional activation domain. Protein interactions were allowed to form in presence of compounds during 6 h at 30 °C. Light emission catalyzed by firefly (*luc*) and R*enilla* (*ruc*) luciferases, upon reporter gene activation, was then monitored. In panel (i) the compound tested produces no effect on either yeast two-hybrid phenotype and is discarded. In (ii and iii), the molecules inhibit selectively A20/D5 and A20/D4 and are retained as potential inhibitors of A20/D5 and A20/D4 interaction respectively. The yeast two-hybrid phenotype observed in (iv) maybe the result of a toxic compound or a molecule interacting with A20 and thus interfering with both A20/D4 and A20/D5 interactions. This compound must be selected for further analysis. (B) Selection of yeast strains expressing two couples of interactions that produced similar two-hybrid phenotypes (i.e. similar *luc* and *ruc* signals). Two yeast strains carrying the plasmid combinations pLP4-D4R/pHA1-A20R/pRAP-COL and pLP2-D5R/pEG202-A20R/pRAP-Ren showed comparable luminescence signals when tested in conditions similar to the ones used for small molecules screening (i.e. 6 h galactose induction). (C) Flowchart highlighting the different steps leading to the selection of two compounds with anti-orthopoxvirus activities.

3.2. High-throughput yeast two-hybrid screening

A total of 27,271 compounds selected from commercial libraries (i.e. Prestwick, Nanosyn, Tripos and Chembridge) were chosen in order to test a wide range of chemically diverse molecules. All inhibitors were tested at a single concentration of 10 μM. Using both positive and negative controls, the statistical parameter Z′, assessing the quality of the screen, was calculated. A value >0.5 validates the screening assay (Zhang et al., 1999). For each plate tested during the course of the screen, Z′ values always ranged between 0.6 and 0.8, thus establishing the very good quality of the HTP yeast two-hybrid screening presented here. 148 compounds displaying ⩾40% inhibition of either *luc* and/or *ruc* signals were selected and re-tested in quadruplicates. Inhibition of yeast two-hybrid phenotypes was confirmed for 66 molecules, which corresponds to a rather common attrition rate for such a HTP cellular screening assay. Among the 66 compounds, 12 inhibited both yeast two-hybrid interaction phenotypes. Upon analysis of molecule structures, we retained 33 compounds that presented low predicted toxicity, favorable predicted stability, and a good specificity profile (i.e. molecules that produced significant inhibition of unrelated PPI, in previous screening campaigns, were discarded). These compounds were then tested for inhibition of VACV infectivity in cell cultures and two of them (8776 and 10,295) were selected for further analysis.

To confirm the inhibition of A20/D4 and A20/D5 interaction phenotypes in yeast and to further verify their specificity, both molecules were 'manually' tested against our two protein interaction targets and against a control protein interaction, for which an inhibitor, 3C11, has been identified using a similar screen (A. Hamdi and P. Colas, unpublished results). As shown in Fig. 2A, both A20/D4 and A20/D5 interaction phenotypes were inhibited by the two compounds, each compound inhibiting both phenotypes to a similar extent (∼75% for 8776 and ∼50% for 10,295). As expected, both compounds did not interfere notably with the control interaction (∼20% inhibition). Accordingly, compound 3C11 did not interfere with A20/D4 and A20/D5 interaction phenotypes (∼20% inhibition) but inhibited its own target interaction phenotype to >90%. Thus, using the yeast two-hybrid screen described above, we identified two candidate molecules able to interfere with A20/D4 and A20/D5 interactions. Since the inhibition of both interactions was tested independently, these results suggest that the two molecules interact with A20 and consequently inhibit its interaction with both D4 and D5. The structures of compounds 8776 and 10,295 are shown in Fig. 2B.

3.3. Antiviral activity of compounds 8776 and 10,295 on VACV

We first investigated the activity of both compounds in a plaque reduction assay. To obtain readily countable plaques, Vero cells were infected with 50 PFU of VACV for 24 h. Fig. 3A shows the dose-response curves obtained with the two selected molecules and with CDV, used as a control. As expected, CDV inhibited VACV growth and in our assay, we determined that the effective concentration of CDV needed to inhibit plaque formation by 50% (EC_{50}) was 23 μM (Fig. 3C). Interestingly, we observed that compounds 8776 and 10,295 both inhibited VACV replication with a comparable efficiency as CDV, exhibiting EC_{50} values of 14.8 and 18.6 μM, respectively (Fig. 3C). We then evaluated the inhibitory effect of compounds on virus yield. In this experiment, Vero cells were infected at a multiplicity of infection of 0.01 in the presence of inhibitors. Again, we showed that in this assay, compounds 8776, 10,295 and CDV have similar inhibitory activities on VACV replication with EC_{50} values of 15.8, 19.1 and 15.4 μM, respectively (Fig. 3B and C).

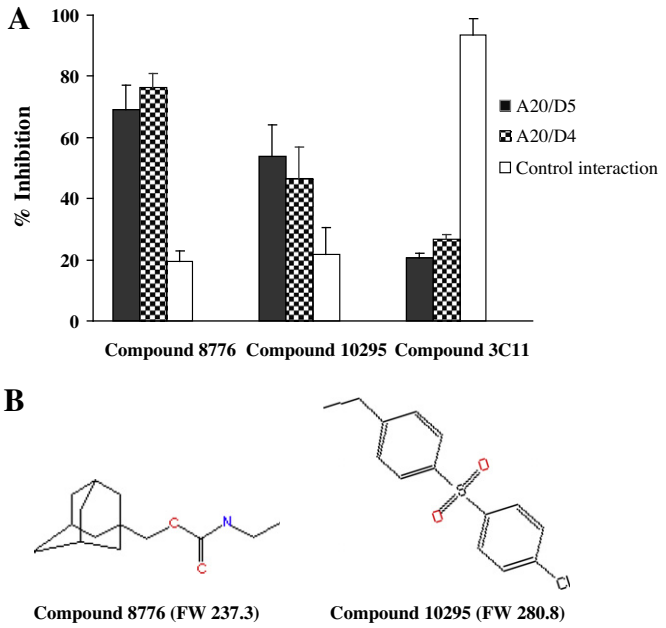
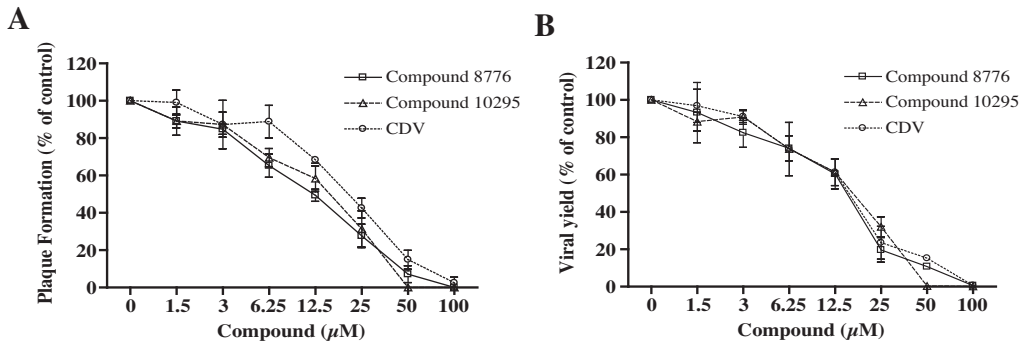


Fig. 2. Identification of inhibitors targeting the VACV replication complex. (A) Inhibition of yeast two-hybrid luminescent phenotypes of yeast expressing A20/D4, A20/D5, or two unrelated interacting proteins in response to compounds 8776, 10,295 or 3C11 (specific inhibitor of control interaction). Each molecule was used at 10 μM. Quantification of luminescence signals was performed 4 h after galactose induction. This experiment was performed in triplicates and results are expressed as the ratio of luciferase activity in presence of molecule relative to luciferase activity without compound and reported as mean ± SD from three samples. NB: in this experiment, interactions were formed in yeast strains carrying the firefly (*luc*) luciferase reporter gene (see Section 2). (B) Compound 8776 (FW 237.3): 1-adamantylmethyl ethycarbamate. Compound 10,295 (FW 280.8): 1-chloro-4-[(4-ethylphenyl) sulfonyl] benzene.

Compound toxicity was measured in various cell lines and the CC_{50} values were determined 24 h post-treatment. As highlighted in Table 1, for both compound, toxicity varies significantly across cell lines. However, CC_{50} values for both selected molecules are lower than that of CDV for all tested cell lines. The selectivity index (SI) in Vero cells, calculated as the ratio of the CC_{50} for cell viability and EC_{50} for virus replication is reported in Fig. 3C.

3.4. Antiviral activity of compounds 8776 and 10,295 on CPXV and HSV-1

Our two inhibitors were identified for their ability to interfere with VACV A20/D4 and A20/D5 interactions. Among the *Orthopoxvirus* genus, these proteins share 98% sequence identity. Thus, compounds 8776 and 10,295 should retain their antiviral activities against other orthopoxviruses and therefore be inefficient against unrelated viruses. To test this hypothesis, CPXV or HSV-1 infected Vero cells were treated with our molecules or with CDV. Fig. 4A shows that compound 8876 efficiently inhibited CPXV replication with an EC_{50} value of 20.5 μM, similar to the value observed for VACV (15.8 μM, Fig. 3C). However, it did not interfere with HSV-1 growth, even at the highest tested concentration (50 μM). Similar results were obtained with compound 10,295 (Fig. 4B). It inhibited CPXV at an estimated EC_{50} value of 16.5 μM and had no antiviral activity on HSV-1. In contrast, CDV, as expected, potently inhibited HSV-1 growth with an EC_{50} value of 0.4 μM (Fig. 4C), which is in accord with values previously reported (Hockova et al., 2011). Finally, we showed that CPXV replication was inhibited to the same extent as VACV upon treatment with CDV (EC_{50} value of 14.3 μM, Fig. 4C). These experiments highlight the specificity of compounds 8776 and 10,295 as anti-orthopoxvirus inhibitors.



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Compound	Plaque formation		Viral yield	
	$EC_{50}{}^{a}$ (μM)	SI[c]	$EC_{50}{}^{b}$ (μM)	SI[c]
8776	14.8 ± 1.8	5.8	15.8 ± 2.2	5.4
10295	18.6 ± 2.1	1.7	19.1 ± 2.9	1.6
CDV	23 ± 3.1	>17.4	15.4 ± 3.5	>26

Fig. 3. Inhibition of VACV growth by compounds 8776, 10,295 and CDV. (A) Vero cells infected with VACV were treated with either molecule at the concentrations indicated. Plaques were counted 24 h post-infection. (B) Vero cells were infected with VACV and treated with either molecule at the indicated concentrations. Virus yields were determined by virus titration 24 h post-infection. Results are reported as number of plaques (or virus yield) obtained in treated cells relative to number of plaques (or virus yield) obtained in non-treated infected cells. Experiments were performed in quadruplicate and repeated at least twice. (C) EC_{50} and selectivity index (SI) values calculated from the experiments described in (A and B). (a) concentration required to reduce plaque number by 50%. (b) Concentration required to reduce viral yield by 50%. (c) Selectivity index (i.e. CC50/EC50).

Table 1
Toxicity of compounds 8776, 10,295 and CDV on various cell lines.

Compound	CC_{50}[a] (μM)			
	Vero cells	BHK-21 cells	A549 cells	Hela cells
8776	85.2 ± 3.5	>200	23.1 ± 2.8	38.9 ± 10.3
10,295	31.3 ± 2.2	65.3 ± 8.8	9.6 ± 0.4	16.2 ± 1.3
CDV	>400	>200	>200	>200

[a] CC_{50}, concentration required to reduce cell viability by 50%.

3.5. Inhibition of VACV DNA synthesis by compounds 8776 and 10,295

We next wanted to investigate if the inhibition of VACV growth was correlated with a decrease in viral genome synthesis. Vero cells were infected at an MOI of one and genome synthesis was quantified by qPCR at different time points up to 15 h post-infection (p-i). The results shown in Fig. 5 indicate that in cells treated with compounds 8776 and 10,295, a decrease in viral genome accumulation was detectable as soon as 6 h p-i. The level of DNA synthesis inhibition remained then relatively constant up to 15 h p-i reaching a maximum of ~50% and ~40% inhibition with molecules 8776 and 10,295, respectively. As expected, CDV, which targets the VACV DNA polymerase E9, produced also an inhibitory effect on genome synthesis. However, we observed that this effect was delayed compared to that induced by inhibitors 8776 and 10,295 and was only detectable 15 h p-i. In our experiment, at 15 h p-i, inhibition of VACV replication by CDV reached ~40%, which is comparable with data published earlier (Jesus et al., 2009) and with the inhibition induced by our inhibitors.

4. Discussion

There is still a need to develop new strategies to design inhibitors with different and original mechanisms of action that would enlarge the armamentarium of therapeutic compounds against orthopoxviruses and particularly smallpox virus. Currently, except for CDV which has been used off-label, there is no licensed antiviral molecule to treat orthopoxvirus infections. CDV as well as other inhibitors targeting VACV DNA polymerase (E9) are potent inhibitors of VACV replication both *in vivo* and *in vitro*. However, treatment of infected cells with increasing concentrations of these compounds leads to the selection of drug-resistant viruses (at least *in vitro*) bearing mutations in the E9L gene (Andrei and Snoeck, 2010). Genetic and biochemical data have demonstrated that several VACV proteins are involved in viral genome replication. Among these proteins E9, A20, D4 and D5 have been shown to be essential subunits of the viral polymerase complex (Moss, 2007). Therefore, interfering with the PPIs within the VACV replication complex should inhibit DNA synthesis and offer a valuable strategy to develop a new class of anti-orthopoxvirus drugs.

Inhibitors of PPIs have recently emerged as new tools to modulate protein functions within various classes of targets, and it has become clear that they have a great therapeutic potential. A couple of these molecules are already in clinical use (Cheok et al., 2011; Veselovsky et al., 2007). Inhibition of PPI with small molecules has also been proved to represent a valid strategy to interfere with virus infections. The targeted interactions may occur between a viral and a host protein (Betzi et al., 2007; Briz et al., 2006) or between two viral proteins (White et al., 2011). The discovery of compounds targeting the subunit interactions of HSV and CMV viral DNA polymerase has been reported (Loregian and Coen, 2006; Pilger et al., 2004). In these studies, a HTP screen based on fluorescence polarization was developed. This allowed the selection of inhibitors targeting the interaction between the processivity subunit and a peptide corresponding to the C-terminal catalytic domain of both viral DNA polymerases. These compounds were further shown to be potent inhibitors of viral replication.

In our study, we have adapted a duplex forward yeast two-hybrid assay that was initially designed to discover small-molecule inhibitors of interactions between target proteins and peptide aptamers (Baines and Colas, 2006). Our successful screening vali-

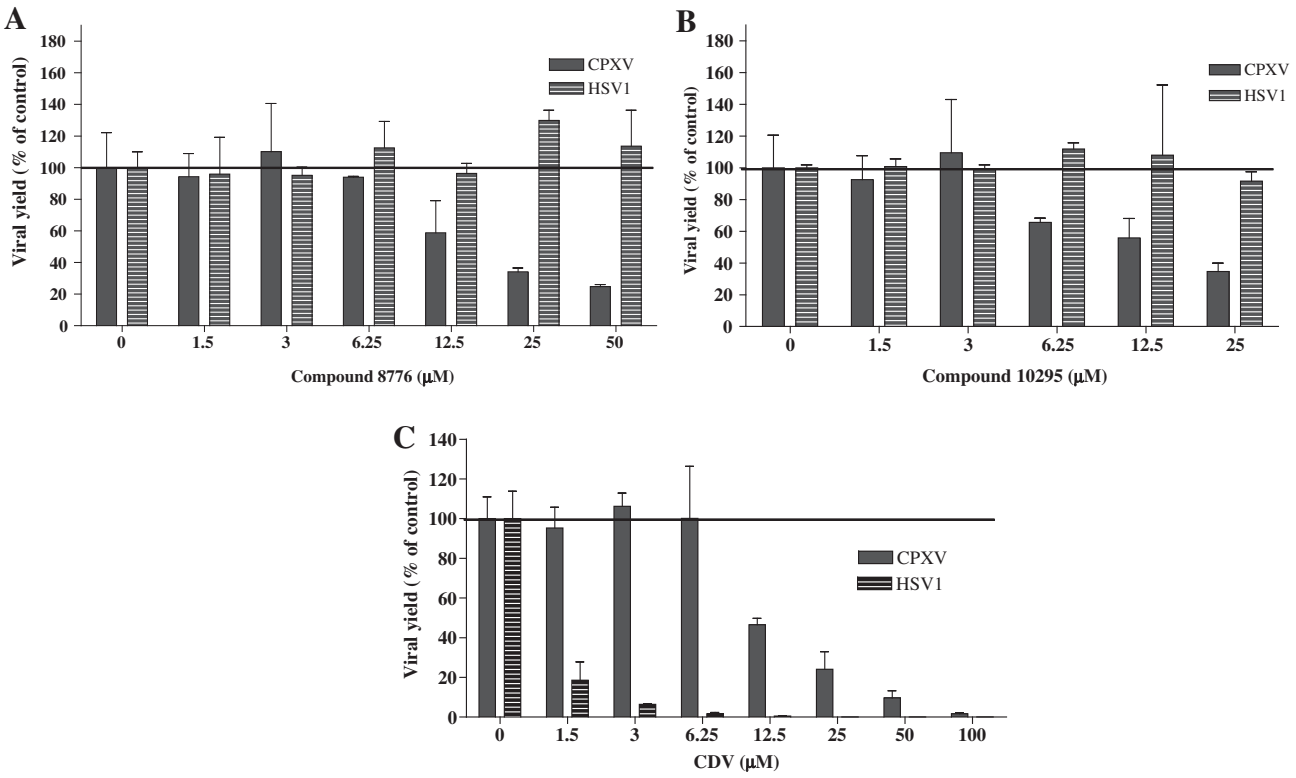

Fig. 4. Antiviral activity of compounds 8776, 10,295 and CDV on CPXV and HSV-1. Vero cells infected with CPXV or HSV-1 were treated at the indicated concentrations with compound 8776 (panel A), compound 10,295 (panel B) or CDV (panel C). Virus yields were determined by virus titration 24 h post-infection. Results are reported as virus yield obtained in treated cells relative to virus yield obtained in non treated infected cells. Experiments were performed in quadruplicate and repeated at least twice.

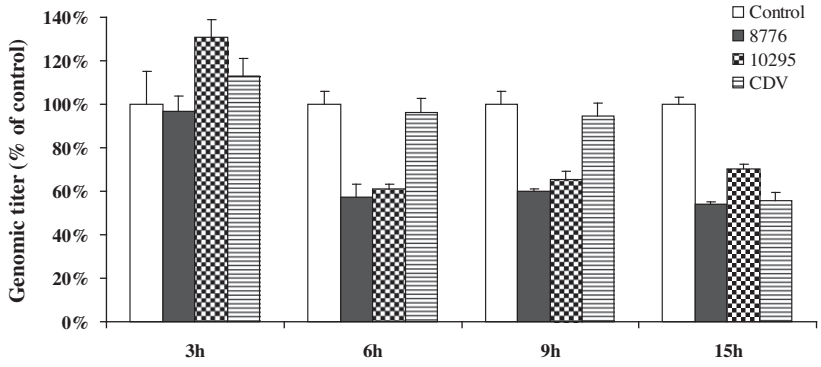

Fig. 5. Inhibition of VACV replication by compounds 8776, 10,295 and CDV. Vero cells infected with VACV at an MOI of 1 were incubated with either 25 μM of molecule 10,295, 50 μM of compound 8776 or 50 μM of CDV. Cells were harvested at the indicated time points. VACV DNA was then extracted and quantified by qPCR using primers specific to VACV A27L gene. Normalization was performed using mtDNA and aluSx genes. Results are expressed as copy number of viral DNA in treated samples relative to non treated samples and reported as mean ± SD from three samples.

dates this assay as a powerful method to discover inhibitors of PPIs. It presents valuable advantages as compared to other *in vitro* or cellular screening methods (Colas, 2008). It entails a rather short and easy development effort for each PPI to be screened, its cost is competitive and the high sensitivity of the luminescent reporters enables the use of small volumes of yeast suspensions, compatible with HTP screening. Moreover, as compared to other forward yeast two-hybrid assays, it allows to screen against non-preformed PPIs, since the expression of one of the two interacting partners (the prey) is induced immediately after adding the small-molecules. This key feature enhances hit rates, since it has been shown that some molecules can prevent the formation of protein complexes but cannot disrupt preformed complexes (Corbel et al., 2011). More generally, our successful screening brings yet another demonstration of the usefulness of yeast two-hybrid methods in drug discovery, and more specifically in the discovery of small-molecule modulators of PPIs (Hamdi and Colas, 2012).

Usually, this duplex screening assay involves two different pairs of interacting proteins, which facilitates the selection of potential hit candidates, on the basis of the selective inhibition of one of the two interaction phenotypes. However, here, A20 was a common interaction partner for the two interactions, and we could not immediately discard molecules that inhibited both interaction phenotypes, in the hypothesis that some of them could bind to A20 and trigger a conformational change that may affect both interactions with D4 and D5. The only hit molecules retained from this

first screening campaign seem to exhibit this mechanism of action and we did not identify molecules that specifically inhibit A20/D4 or A20/D5 interactions.

The two identified molecules described in this report inhibited VACV growth *in vitro* with an EC_{50} value similar to CDV. In another recent study, Schormann et al., used a different screening assay (i.e. AlphaScreen), to identify compounds inhibiting the interaction between A20 and D4 (Nuth et al., 2011; Schormann et al., 2011). Remarkably, they also observed that their molecules, although chemically unrelated to ours, had an antiviral activity on VACV comparable to CDV (Schormann et al., 2011). This reinforces the idea that developing inhibitors of PPIs involved in VACV DNA synthesis may represent a promising alternative to the more classical inhibitors of the viral DNA polymerase activity. However in both studies, it was observed that the selected compounds, belonging to commercial libraries, were significantly more cytotoxic than CDV. Thus, further medicinal chemistry efforts including lead optimization, pharmacokinetic and toxicologic studies will be necessary before their use as potential therapeutic agents.

As shown in Fig. 5, the selected molecules like CDV displayed a relatively low but significant inhibition of VACV DNA synthesis. The weak reduction of virus DNA accumulation upon CDV treatment has already been documented and is associated with defects in VACV morphogenesis (Jesus et al., 2009). It has been hypothesized, that incorporation of CDV in the DNA strands could induce alteration in DNA structure leading to genome encapsidation inhibition. Although molecules 8776 and 10,295 differ from CDV in their mechanism of action, we can not exclude the possibility that their effect on virus DNA synthesis may also affect later steps of the VACV life cycle.

In our assay, A20 is a common subunit of the two targeted PPIs. Since the identified molecules seemed to interfere with both interactions, it is tempting to hypothesise that they interact with A20 and cause structural perturbations that inhibit the binding of D4 and D5. Inhibitors of PPIs may act either by direct blocking of the protein–protein interface or through binding to an allosteric site inducing conformational changes in the protein target (Arkin and Wells, 2004). Our data favour the latter hypothesis. Proteins with multiple interacting partners are known to be flexible and to undergo conformational changes when forming interactions (Higurashi et al., 2008). A20, which interacts with D4 and D5, was also shown to bind to E9 and another viral protein H5 (Ishii and Moss, 2002; Klemperer et al., 2001). Our results further validate A20 as a pertinent target of VACV replication inhibitors (Saccucci et al., 2009).

The two compounds described herein add to a short but rapidly expanding list of small molecules that can specifically modulate PPIs (Morelli et al., 2011). They represent a good starting point for the future development of new anti-orthopoxvirus drugs. Screening for inhibitors of PPIs within the viral replication machinery is a promising strategy to identify inhibitors against other viral infections. More generally, the systematic exploration of viral interactomes and of viral-host interactomes by proteome-wide yeast two-hybrid screening efforts keep unveiling PPIs that play essential roles in viral pathogenicity and thus represent potential therapeutic targets (Khadka et al., 2011; McCraith et al., 2000; Shapira et al., 2009; von Brunn et al., 2007). It can be anticipated that this vast, promising reservoir of targets will be increasingly exploited to discover next-generation anti-viral therapeutics.

Acknowledgements

We thank Dr C. Peyrefitte for critical reading of the manuscript. A.H. is the recipient of a doctoral fellowship awarded by the Région Bretagne. This work was supported by research grants from the Service de Santé des Armées, the Délégation Générale pour l'Armement, the ARAMI association and the GenHomme network.

References

Andrei, G., Gammon, D.B., Fiten, P., De Clercq, E., Opdenakker, G., Snoeck, R., Evans, D.H., 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. 80, 9391–9401.
Andrei, G., Snoeck, R., 2010. Cidofovir activity against poxvirus infections. Viruses 2, 2803–2830.
Arkin, M.R., Wells, J.A., 2004. Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. Nat. Rev. Drug Discov. 3, 301–317.
Baines, I.C., Colas, P., 2006. Peptide aptamers as guides for small-molecule drug discovery. Drug Discov. Today 11, 334–341.
Bardou, C., Borie, C., Bickle, M., Rudkin, B.B., Colas, P., 2009. Peptide aptamers for small molecule drug discovery. Methods Mol. Biol. 535, 373–388.
Berche, P., 2001. The threat of smallpox and bioterrorism. Trends Microbiol. 9, 15–18.
Betzi, S., Restouin, A., Opi, S., Arold, S.T., Parrot, I., Guerlesquin, F., Morelli, X., Collette, Y., 2007. Protein protein interaction inhibition (2P2I) combining high throughput and virtual screening: application to the HIV-1 Nef protein. Proc. Natl. Acad. Sci. USA 104, 19256–19261.
Blasco, R., Moss, B., 1991. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. J. Virol. 65, 5910–5920.
Boyle, K.A., Arps, L., Traktman, P., 2007. Biochemical and genetic analysis of the vaccinia virus d5 protein: multimerization-dependent ATPase activity is required to support viral DNA replication. J. Virol. 81, 844–859.
Bray, M., Martinez, M., Kefauver, D., West, M., Roy, C., 2002. Treatment of aerosolized cowpox virus infection in mice with aerosolized cidofovir. Antiviral Res. 54, 129–142.
Bray, M., Martinez, M., Smee, D.F., Kefauver, D., Thompson, E., Huggins, J.W., 2000. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge. J. Infect. Dis. 181, 10–19.
Briz, V., Poveda, E., Soriano, V., 2006. HIV entry inhibitors: mechanisms of action and resistance pathways. J. Antimicrob. Chemother. 57, 619–627.
Cheok, C.F., Verma, C.S., Baselga, J., Lane, D.P., 2011. Translating p53 into the clinic. Nat. Rev. Clin. Oncol. 8, 25–37.
Colas, P., 2008. High-throughput screening assays to discover small-molecule inhibitors of protein interactions. Curr. Drug Discov. Technol. 5, 190–199.
Corbel, C., Wang, Q., Bousserouel, H., Hamdi, A., Zhang, B., Lozach, O., Ferandin, Y., Tan, V.B., Gueritte, F., Colas, P., et al., 2011. First BRET-based screening assay performed in budding yeast leads to the discovery of CDK5/p25 interaction inhibitors. Biotechnol. J. 6, 860–870.
De Clercq, E., 2002. Cidofovir in the treatment of poxvirus infections. Antiviral Res. 55, 1–13.
De Clercq, E., 2003. Clinical potential of the acyclic nucleoside phosphonates cidofovir, adefovir, and tenofovir in treatment of DNA virus and retrovirus infections. Clin. Microbiol. Rev. 16, 569–596.
De Silva, F.S., Lewis, W., Berglund, P., Koonin, E.V., Moss, B., 2007. Poxvirus DNA primase. Proc. Natl. Acad. Sci. USA 104, 18724–18729.
Duraffour, S., Snoeck, R., de Vos, R., van Den Oord, J.J., Crance, J.M., Garin, D., Hruby, D.E., Jordan, R., De Clercq, E., Andrei, G., 2007. Activity of the anti-orthopoxvirus compound ST-246 against vaccinia, cowpox and camelpox viruses in cell monolayers and organotypic raft cultures. Antiviral Ther. 12, 1205–1216.
Ferrier, A., Garin, D., Crance, J.M., 2004. Rapid inactivation of vaccinia virus in suspension and dried on surfaces. J. Hosp. Infect. 57, 73–79.
Geddes, A.M., 2006. The history of smallpox. Clin. Dermatol. 24, 152–157.
Hamdi, A., Colas, P., 2012. Yeast two-hybrid methods and their applications in drug discovery. Trends Pharmacol. Sci. 33, 109–118.
Higurashi, M., Ishida, T., Kinoshita, K., 2008. Identification of transient hub proteins and the possible structural basis for their multiple interactions. Protein Sci. 17, 72–78.
Hockova, D., Holy, A., Andrei, G., Snoeck, R., Balzarini, J., 2011. Acyclic nucleoside phosphonates with a branched 2-(2-phosphonoethoxy)ethyl chain: efficient synthesis and antiviral activity. Bioorg. Med. Chem. 19, 4445–4453.
Ishii, K., Moss, B., 2002. Mapping interaction sites of the A20R protein component of the vaccinia virus DNA replication complex. Virology 303, 232–239.
Jesus, D.M., Costa, L.T., Goncalves, D.L., Achete, C.A., Attias, M., Moussatche, N., Damaso, C.R., 2009. Cidofovir inhibits genome encapsidation and affects morphogenesis during the replication of vaccinia virus. J. Virol. 83, 11477–11490.
Khadka, S., Vangeloff, A.D., Zhang, C., Siddavatam, P., Heaton, N.S., Wang, L., Sengupta, R., Sahasrabudhe, S., Randall, G., Gribskov, M., et al. 2011. A physical interaction network of dengue virus and human proteins. Mol. Cell Proteomics 10, M111 012187.
Klemperer, N., McDonald, W., Boyle, K., Unger, B., Traktman, P., 2001. The A20R protein is a stoichiometric component of the processive form of vaccinia virus DNA polymerase. J. Virol. 75, 12298–12307.
Kornbluth, R.S., Smee, D.F., Sidwell, R.W., Snarsky, V., Evans, D.H., Hostetler, K.Y., 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob. Agents Chemother. 50, 4038–4043.
Laudet, B., Prudent, R., Filhol, O., Cochet, C., 2007. Therapeutic agents targetting protein–protein interactions: myth or reality? Med. Sci. (Paris) 23, 273–278.

Loregian, A., Coen, D.M., 2006. Selective anti-cytomegalovirus compounds discovered by screening for inhibitors of subunit interactions of the viral polymerase. Chem. Biol. 13, 191–200.
Loregian, A., Marsden, H.S., Palu, G., 2002. Protein–protein interactions as targets for antiviral chemotherapy. Rev. Med. Virol. 12, 239–262.
Loregian, A., Palu, G., 2005. Disruption of protein-protein interactions: towards new targets for chemotherapy. J. Cell. Physiol. 204, 750–762.
McCraith, S., Holtzman, T., Moss, B., Fields, S., 2000. Genome-wide analysis of vaccinia virus protein–protein interactions. Proc. Natl. Acad. Sci. USA 97, 4879–4884.
Morelli, X., Bourgeas, R., Roche, P., 2011. Chemical and structural lessons from recent successes in protein–protein interaction inhibition (2P2I). Curr. Opin. Chem. Biol. 15, 475–481.
Moss, B., 2007. Poxviridae: The viruses and their replication. In Fields Virology. In: Knipe, D.M., Howley, P.M. (Eds.), Philadelphia. Lippincott, Williams, & Wilkins, pp. 2905–2945.
Mukhopadhyay, K., Kohli, A., Prasad, R., 2002. Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob. Agents Chemother. 46, 3695–3705.
Nieuwenhuijsen, B.W., Huang, Y., Wang, Y., Ramirez, F., Kalgaonkar, G., Young, K.H., 2003. A dual luciferase multiplexed high-throughput screening platform for protein–protein interactions. J. Biomol. Screen. 8, 676–684.
Nuth, M., Huang, L., Saw, Y.L., Schormann, N., Chattopadhyay, D., Ricciardi, R.P., 2011. Identification of inhibitors that block vaccinia virus infection by targeting the DNA synthesis processivity factor D4. J. Med. Chem. 54, 3260–3267.
Parker, S., Touchette, E., Oberle, C., Almond, M., Robertson, A., Trost, L.C., Lampert, B., Painter, G., Buller, R.M., 2008. Efficacy of therapeutic intervention with an oral ether-lipid analogue of cidofovir (CMX001) in a lethal mousepox model. Antiviral Res. 77, 39–49.
Peinnequin, A., Mouret, C., Birot, O., Alonso, A., Mathieu, J., Clarencon, D., Agay, D., Chancerelle, Y., Multon, E., 2004. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. BMC Immunol. 5, 3.
Pilger, B.D., Cui, C., Coen, D.M., 2004. Identification of a small molecule that inhibits herpes simplex virus DNA polymerase subunit interactions and viral replication. Chem. Biol. 11, 647–654.
Saccucci, L., Crance, J.M., Colas, P., Bickle, M., Garin, D., Iseni, F., 2009. Inhibition of vaccinia virus replication by peptide aptamers. Antiviral Res. 82, 134–140.
Scaramozzino, N., Ferrier-Rembert, A., Favier, A.L., Rothlisberger, C., Richard, S., Crance, J.M., Meyer, H., Garin, D., 2007. Real-time PCR to identify variola virus or other human pathogenic orthopox viruses. Clin. Chem. 53, 606–613.
Schormann, N., Sommers, C.I., Prichard, M.N., Keith, K.A., Noah, J.W., Nuth, M., Ricciardi, R.P., Chattopadhyay, D., 2011. Identification of protein–protein interaction inhibitors targeting vaccinia virus processivity factor for development of antiviral agents. Antimicrob. Agents Chemother. 55, 5054–5062.
Shapira, S.D., Gat-Viks, I., Shum, B.O., Dricot, A., de Grace, M.M., Wu, L., Gupta, P.B., Hao, T., Silver, S.J., Root, D.E., et al., 2009. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell 139, 1255–1267.
Smee, D.F., Bailey, K.W., Sidwell, R.W., 2001a. Treatment of lethal vaccinia virus respiratory infections in mice with cidofovir. Antiviral Chem. Chemother. 12, 71–76.
Smee, D.F., Bailey, K.W., Wong, M.H., Sidwell, R.W., 2001b. Effects of cidofovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice. Antiviral Res. 52, 55–62.
Stanitsa, E.S., Arps, L., Traktman, P., 2006. Vaccinia virus uracil DNA glycosylase interacts with the A20 protein to form a heterodimeric processivity factor for the viral DNA polymerase. J. Biol. Chem. 281, 3439–3451.
Stittelaar, K.J., Neyts, J., Naesens, L., van Amerongen, G., van Lavieren, R.F., Holy, A., De Clercq, E., Niesters, H.G., Fries, E., Maas, C., et al., 2006. Antiviral treatment is more effective than smallpox vaccination upon lethal monkeypox virus infection. Nature 439, 745–748.
Veselovsky, A., Archakov, A.I., 2007. Inhibitors of protein–protein interactions as potential drugs. Curr. Comput.-Aid. Drug Des. 3, 51–58.
von Brunn, A., Teepe, C., Simpson, J.C., Pepperkok, R., Friedel, C.C., Zimmer, R., Roberts, R., Baric, R., Haas, J., 2007. Analysis of intraviral protein–protein interactions of the SARS coronavirus ORFeome. PLoS One 2, e459.
White, P.W., Faucher, A.M., Goudreau, N., 2011. Small molecule inhibitors of the human papillomavirus E1–E2 interaction. Curr. Top. Microbiol. Immunol. 348, 61–88.
WHO, 2010. Scientific review of variola virus research, 1999–2010. <(http://www.who.int/csr/resources/publications/WHO> HSE GAR BDP 2010 3/en/index.html).
Yang, G., Pevear, D.C., Davies, M.H., Collett, M.S., Bailey, T., Rippen, S., Barone, L., Burns, C., Rhodes, G., Tohan, S., et al., 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. J. Virol. 79, 13139–13149.
Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 4, 67–73.