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Review

Inhibition of herpesvirus and influenza virus replication by blocking polymerase subunit interactions

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ABSTRACT

Protein–protein interactions (PPIs) play a key role in many biological processes, including virus replication in the host cell. Since most of the PPIs are functionally essential, a possible strategy to inhibit virus replication is based on the disruption of viral protein complexes by peptides or small molecules that interfere with subunit interactions. In particular, an attractive target for antiviral drugs is the binding between the subunits of essential viral enzymes. This review describes the development of new antiviral compounds that inhibit herpesvirus and influenza virus replication by blocking interactions between subunit proteins of their polymerase complexes.

Contents

1. Introduction

Protein–protein interactions (PPIs) are intrinsic to virtually every biological process ranging from cell cycle control, DNA replication, transcription, splicing and translation, to intermediary metabolism, secretion, formation of protein macrostructures and enzymatic complexes. Moreover, formation or alteration of PPIs is known to contribute to many diseases. As an example, PPIs between virus-encoded components or between viral proteins and cellular factors occur during every step of the replication of human viruses in the host cell (Loregian et al., 2002). Hence, the modulation of PPIs that contribute to disease is a potential antiviral strategy. The contact surfaces of the protein complexes have usually unique structure and properties, thus in a number of cases they can represent potential targets for new drugs. During the past 30 years, numerous investigations were undertaken to find or design compounds that block protein dimerization or heterologous PPIs (Garner and Janda, 2011; Loregian and Palù, 2005a; Valkov et al., 2012).

Inhibitors of PPIs potentially have several favorable features over other classes of antivirals. First, the specificity of such interactions offers the possibility of interfering with them in a highly specific manner. Design of inhibitors binding to the active site of

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enzymes of pathogen viruses can sometime be limited by high structural similarity between the human and the viral enzyme, whereas the greater structural variability of protein–protein interfaces may provide the possibility of an effective differentiation between the host and the virus (Valkov et al., 2012). Another advantage of targeting viral PPIs is that dissociative inhibitors may be less prone to drug resistance than compounds acting by other mechanisms. Because inhibitors directed at enzyme active sites usually establish a few high-affinity interactions with the target protein, a single residue change often leads to resistance. In contrast, a single mutation in one subunit of a protein–protein interface is likely to require a complementary mutation in the other subunit to maintain intact the complex. As simultaneous coupled mutations in different subunits are unlikely, the probability that the target protein complex can acquire resistance is reduced. However, other possible mechanisms of resistance to PPI inhibitors cannot be excluded, for example substitutions strengthening the target interaction or mutations creating new high-affinity binding sites for the inhibitor, which thus sequester it.

During the last two decades, the interest of many research groups including ours has been focused on the development of new antiviral inhibitors which act by disrupting the interactions between the subunits of viral enzymes, e.g., the DNA polymerase of herpesviruses and the RNA polymerase of influenza virus. Research efforts have been focused on characterizing PPIs between the enzyme subunits and their functional role and on identifying peptides and small molecules that are able to disrupt the viral protein complexes. Here we review past and recent studies aimed at identifying and designing peptides and small-molecule compounds which block these PPIs.

2. Inhibiting PPI of herpesvirus DNA polymerases

Antiviral agents currently approved for the treatment of herpesvirus infections include acyclovir and its prodrug valacyclovir, ganciclovir and its prodrug valganciclovir, foscarnet, and cidofovir, all of which inhibit the catalytic activity of the viral DNA polymerase. These drugs have provided a major advance in anti-herpesvirus therapy, but they suffer from poor bioavailability, significant toxicity, and limited effectiveness. In addition, the emergence of drug-resistant viral strains is becoming an increasing problem for disease management, and since these anti-herpesvirus compounds have similar mechanisms of action, mutant viruses resistant to one drug are sometime cross-resistant to others. Thus, new anti-herpesvirus agents, possibly acting by other mechanisms (e.g., inhibition of PPIs), are still needed.

Among the members of *Herpesviridae* family, a two-subunit DNA polymerase appears to be a common theme, being composed of a catalytic subunit and an accessory protein which is not necessary for polymerase activity *per se* but increases the processivity of the holoenzyme (Coen, 1996). For example, the DNA polymerase of HSV-1 exists as a heterodimer of the UL30 catalytic subunit and the UL42 accessory protein (Gallo et al., 1989; Gottlieb et al., 1990). The human cytomegalovirus (HCMV) DNA polymerase also consists of two proteins, UL54 and UL44 (Ertl and Powell, 1992); VZV polymerase interacts with a DNA-binding protein (encoded by gene 16) which shows sequence similarity with UL42 (Davison and Scott, 1986). Other examples include the two proteins of PRV DNA polymerase (Berthomme et al., 1995), the BALF5/BMRF1 complex of EBV (Zhang et al., 1996), the Pol-6/p41 proteins of human herpesvirus 6 (Agulnick et al., 1993), and the ORF8(Pol-8)/ORF59(PF-8) subunits of human herpesvirus 8 (HHV-8) polymerase (Lin et al., 1998).

2.1. HSV UL30/UL42 interactions

Some of the herpesvirus DNA polymerases have been subject to detailed analysis, characterizing the subunit interactions (Loregian and Palù, 2005b). As far as the DNA polymerase of HSV-1, mutations of UL30 and of UL42 which specifically disrupt interaction between the two enzyme subunits were shown to inhibit the replication of *UL30* or *UL42* null mutant viruses in plasmid-based complementation assays, thus suggesting the essential role of the UL30/UL42 association (Digard et al., 1993a,b; Hamatake et al., 1993; Stow, 1993; Tenney et al., 1993). This led to growing interest in its potential as an antiviral drug target and fostered the characterization of the regions in UL30 and UL42 responsible for their physical and functional interactions. Several studies clearly demonstrated that the extreme C-terminus of UL30 is necessary and sufficient for specific interaction with UL42 (Digard et al., 1993a; Digard and Coen, 1990; Marsden et al., 1994; Stow, 1993). The crystal structure of UL42 with an UL30 C-terminal peptide has been solved, and important contact features identified (Zuccola et al., 2000). The three-dimensional structure showed that the C-terminal 18 residues (ATAEETRRMLHRAFDTLA) of the catalytic subunit form an -helix that binds in a deep groove in UL42. Importantly, mutational and biophysical studies on UL30/UL42 binding interface demonstrated that, although numerous hydrophobic interactions are observed in the crystal structure of the UL30 peptide bound to UL42, only few specific hydrogen bonds are crucial determinants of binding energy (Bridges et al., 2001).

Attempts to disrupt the interaction of UL30 with UL42 first resulted in the identification of peptides corresponding to the C-terminus of UL30 (Table 1) which inhibit the ability of UL42 to stimulate UL30 activity *in vitro* with IC_{50}s of 2–30 μM (Digard et al., 1995; Marsden et al., 1994). One such peptide, when intracellularly delivered by a protein carrier, could also inhibit HSV replication in cell culture with an EC_{50} of 11 μM (Table 1; (Loregian et al., 1999)). Interestingly, a nuclear localization signal was identified in the inhibitory peptide that could mediate its intranuclear import (Loregian et al., 2000). Taken together, these studies established proof-of-principle for blocking the UL30/UL42 interaction as a feasible antiviral strategy. In addition, the observation that single UL30 and UL42 residues are crucial for their binding (Bridges et al., 2000, 2001; Zuccola et al., 2000) suggested that a small molecule might be able to disrupt such an interaction. This hypothesis found support with the discovery, from a library of ~16,000 small molecules, of a compound, termed BP5 (Table 1), which specifically inhibits the interaction of UL30 and UL42 *in vitro* and also blocks virus replication in infected cells with EC_{50}s of 0.3–2 μM (Pilger et al., 2004). However, this compound also exhibited significant cytotoxicity (CC_{50} of ~20 μM), thus having a low selectivity index (Pilger et al., 2004). In addition, no data demonstrating that viral inhibition by this molecule indeed occurs through interference with the UL30/UL42 interaction in HCMV-infected cells have been provided.

2.2. HCMV UL54/UL44 interactions

A similar strategy has also been proposed to inhibit the DNA polymerase of HCMV. The interaction between the UL54 and UL44 subunits of HCMV DNA polymerase has been well characterized (Fig. 1) and it has been shown that it presents both similarities and differences compared with the interaction between HSV-1 UL30 and UL42 proteins. UL44, like HSV-1 UL42, possesses a characteristic structural element, the 'connector loop' (residues 129–140), that connects two topologically similar domains of the protein (Appleton et al., 2004). It has been demonstrated that this region of UL44 is involved in the interaction with UL54, similarly to what happens for UL42 and UL30 proteins of HSV-1 (Appleton

Table 1
Inhibitory peptides or small molecules directed against subunit interactions of herpesvirus DNA polymerases.

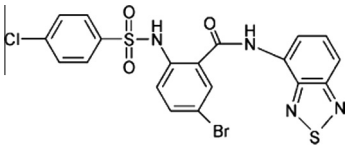
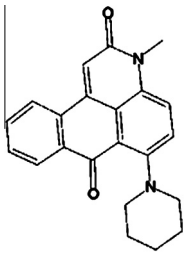
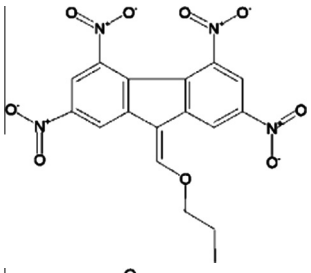
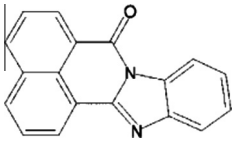
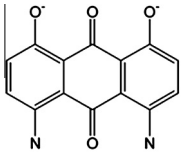
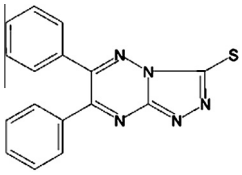
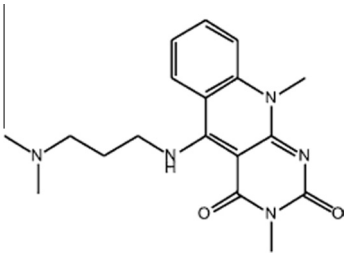
Inhibitor	Viral Target	Sequence/structure	Activity[a]		Ref.
			In vitro assays IC_{50} (μM)	Cell culture assays EC_{50} (μM)	
UL30 C-terminal peptide (aa 1209–1235)	HSV-1 UL30/ UL42 interaction	AGFGAVGAGATAEETRRMLHRAFDTLA	25 (ELISA, DNA pol assay)	n.d.[b]	Marsden et al. (1994)
UL30 C-terminal peptide (aa 1200–1235)	HSV-1 UL30/ UL42 interaction	DDVAARLRAAGFGAVGAGATAEETRRMLHRAFDTLA	2–5 (FP assay, DNA pol assay)	n.d.[b]	Digard et al. (1995) and Pilger et al. (2004)
UL30 C-terminal peptide (aa 1218–1235)	HSV-1 UL30/ UL42 interaction	ATAEETRRMLHRAFDTLA	30 (DNA pol assay)	n.d.[b]	Digard et al. (1995)
EtxB-UL30 peptide (aa 1209–1235)	HSV-1 UL30/ UL42 interaction	EtxB-AGFGAVGAGATAEETRRMLHRAFDTLA	10–15 (ELISA, DNA pol assay)	11 (PRA)	Loregian et al. (1999)
BP5	HSV-1 UL30/ UL42 interaction		15–26 (FP assay, DNA pol assay)	0.3–2 (VYRA, PRA)	Pilger et al. (2004)
UL54 C-terminal peptide (aa 1221–1242)	HCMV UL54/ UL44 interaction	LPRRLHLEPAFLPYSVKAHECC	11–20 (ELISA, DNA pol assay)	n.d.[b]	Loregian et al. (2003)
AL5	HCMV UL54/ UL44 interaction		5–7 (FP assay, ELISA, DNA pol assay)	1.0–2.2 (VYRA, PRA)	Loregian and Coen (2006)
AL9	HCMV UL54/ UL44 interaction		10–21 (FP assay, ELISA, DNA pol assay)	3.1–10 (VYRA, PRA)	Loregian and Coen (2006)
AL12	HCMV UL54/ UL44 interaction		12–19 (FP assay, ELISA, DNA pol assay)	1.4–3.2 (VYRA, PRA)	Loregian and Coen (2006)
AL18	HCMV UL54/ UL44 interaction		5–30 (FP assay, ELISA, DNA pol assay)	0.3–1.1 (VYRA, PRA)	Loregian and Coen (2006)
AL21	HCMV UL54/ UL44 interaction		5–10 (FP assay, ELISA, DNA pol assay)	1.7–3.5 (VYRA, PRA)	Loregian and Coen (2006)

Table 1 (*continued*)

Inhibitor	Viral Target	Sequence/structure	Activity[a]		Ref.
			In vitro assays IC_{50} (μM)	Cell culture assays EC_{50} (μM)	
NSC 373989	HHV-8 Pol-8/PF-8 interaction		9.7 (DNA pol assay)	1.4–2.7 (qPCR of viral DNA)	Dorjsuren et al. (2006)

[a] The activities in *in vitro* assays (IC_{50}: inhibitor concentration to give 50% inhibition *in vitro*) and in cell culture assays (EC_{50}: inhibitor concentration to give 50% inhibition in cells) are reported. The assays employed to evaluate the antiviral activity *in vitro* or in cell culture are reported in parenthesis underneath the IC_{50} or EC_{50} values, respectively (FP, Fluorescence Polarization; ELISA, enzyme-linked immunosorbent assay, PRA, plaque reduction assay, VYRA, virus yield reduction assay, qPCR, quantitative polymerase chain reaction).

[b] n.d., Not determined.

et al., 2006; Loregian et al., 2004b; Zuccola et al., 2000). In addition, like in HSV-1 UL30, in UL54 the region involved in the interaction with the accessory subunit is the extreme C-terminus (Loregian et al., 2003). Moreover, like for HSV-1 UL30 and UL42, single residues have been identified in UL54 and UL44 that are crucial for their physical and functional (Loregian et al., 2004a,b). However, although the residues most important for HCMV DNA polymerase subunit interactions lie in regions analogous to those of the HSV-1 counterparts, the UL54–UL44 interaction appears to be more dependent upon hydrophobic interactions, whereas, in the HSV-1 UL30–UL42 interaction, hydrogen bonds between polar residues are crucial (Bridges et al., 2001; Zuccola et al., 2000). In addition, while the HSV-1 UL30/UL42 interaction involves a groove to one side of the UL42 connector loop, the HCMV UL54/UL44 interaction involves a crevice near the UL44 connector loop. Furthermore, the HCMV UL44 crevice is on the opposite side of the connector loop with respect to the HSV-1 UL42 groove. The UL54/UL44 interactions also presents similarities and differences with the interaction between the eukaryotic processivity factor called proliferating cell nuclear antigen (PCNA) and PCNA-interacting proteins (e.g. Bruning and Shamoo, 2004; Gulbis et al., 1996; Sakurai et al., 2005). In fact, PCNA also interacts with its binding partners via a hydrophobic crevice, which is in a position analogous to the crevice on UL44. In both cases, the bound peptide that contacts the hydrophobic crevice assumes a roughly helical conformation, with the important hydrophobic side chains clustered on the face of the helix facing the crevice. However, the molecular details that define the interactions with their binding partners are not identical, as some of the residues most important for the UL54/UL44 interaction have no counterpart in the PCNA complex.

The observations that both UL54 and UL44 are essential for viral DNA replication (Kim and Ahn, 2010; Pari and Anders, 1993; Pari et al., 1993; Ripalti et al., 1995; Silva et al., 2010) and that antisense inhibition of UL44 synthesis in HCMV-infected cells strongly inhibits viral DNA replication (Ripalti et al., 1995) suggested that the UL54/UL44 interaction might also be a valid target for antiviral drugs. A peptide corresponding to C-terminal 22 residues of UL54 has been shown both to disrupt the physical interaction between UL54 and UL44 and to specifically inhibit the stimulation of UL54 activity by the accessory protein (Table 1; (Loregian et al., 2003). More recently, from ~50,000 small molecules five compounds, termed AL5, AL9, AL12, AL18, and AL21 (Table 1) have been identified that specifically interfere with the physical and functional interactions of UL44 with UL54 (Loregian and Coen, 2006). These five compounds also inhibited HCMV replication with sub- to low micromolar potency and at concentrations up to 500-fold lower than those at which they exhibited cytotoxicity, thus having a selectivity index significantly higher than that of BP5, the compound directed against the subunit interactions of HSV-1 DNA polymerase. Studies aimed at isolating and characterizing virus strains resistant to the most active of these anti-HCMV compounds, AL18, in order to demonstrate that it indeed inhibits virus replication by disrupting the UL54/UL44 interaction, are in progress (Loregian et al., unpublished data). These new compounds could represent a promising starting point for the development of new anti-HCMV drugs, especially if the proposed mechanism of action will be confirmed.

2.3. HHV-8 Pol-8/PF-8 interactions

The molecular complex formed by the HHV-8 DNA polymerase subunits, Pol-8 and PF-8, may also serve as an excellent antiviral target, as both Pol-8 and PF-8 are indispensable for lytic viral DNA synthesis. In addition, HHV-8, also called Kaposi's sarcoma-associated herpesvirus (KSHV), is an important human pathogen with tumorigenic potential. It has not only been strongly implicated in the development of Kaposi's sarcoma but also has been associated with primary effusion (body cavity-based) lymphoma, multifocal Castleman's disease, and multiple myeloma (Ganem, 1997). From a therapeutical point of view, no drug to treat KSHV infections does exist as yet, although some studies in infected cells have shown that HHV-8 exhibits a certain degree of susceptibility to ganciclovir and foscarnet (Kedes and Ganem, 1997; Medveczky et al., 1997). Therefore, the discovery of new, effective and specific antiviral agents against KSHV is still in great demand.

It has been shown that the physical interaction between the two subunits of HHV-8 DNA polymerase is essential for the viral replication, as the PF-8 protein binds Pol-8 and confers processivity by enabling the DNA polymerase to elongate the viral genome continuously without dissociating from the DNA template (Lin et al., 1998). Thus, the interaction between PF-8 and Pol-8 may represent a potential target for antiviral strategies. Mutational analyses suggested that two regions of PF-8 (aa 10–27 and aa 277–396) might be involved in the interaction with Pol-8 (Chan and Chandran, 2000; Chen et al., 2004). However, based on the crystal structure of PF-8 (Baltz et al., 2009), these regions are critical for the overall fold of the protein, thus it can be predicted that deletions of these portions most likely affect the folding of PF-8. Indeed, by analogy with the binding of UL42 and UL44 to their respective catalytic subunits, one would expect the binding between Pol-8 and PF-8

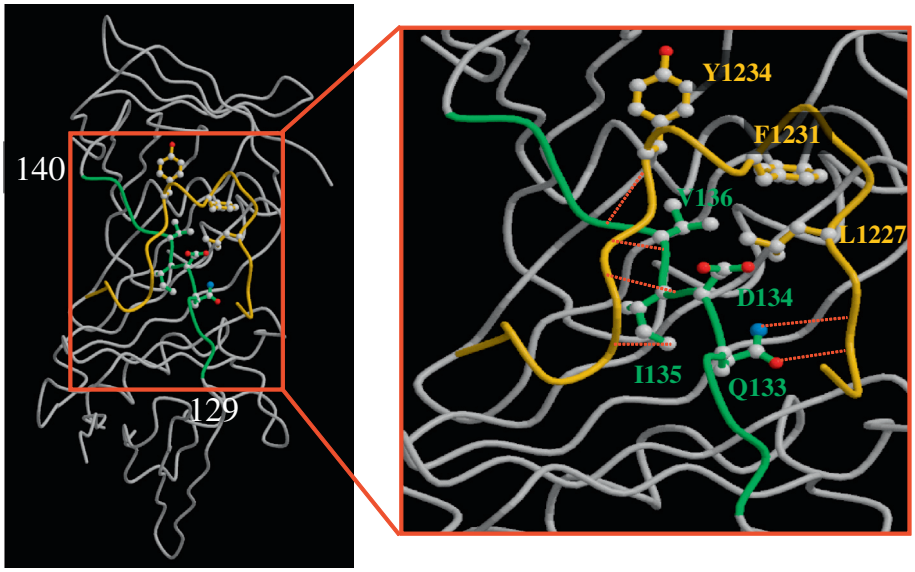


Fig. 1. The UL54/UL44 binding interface. The connector loop of UL44 (*green*, residues 129–140) and the C-terminal region of UL54 (*yellow*, residues 1221–1242) are joined by an extensive network of hydrogen bonds (*red dots*). Residues of UL44 and UL54 most important for their interaction are shown in stick representation and labelled. Ile135 of UL44 connector loop forms a critical hydrophobic anchor below the hydrogen-bonding network. Leu1227, Phe1231, and Tyr1234 of UL54 form a hydrophobic plug that packs against a hydrophobic crevice composed of Val136 from the UL44 connector loop. Fig. based on data reported in Appleton et al. (2006).

to involve the extreme C-terminus of Pol-8 and the connector loop of PF-8. In line with this hypothesis, a peptide corresponding to the 28 C-terminal residues of Pol-8 inhibited with an IC_{50} of 50 μM (Baltz et al., 2009). In addition, a model of the ternary complex of Pol-8, PF-8, and DNA recently predicted a number of amino acids as important for the interaction between Pol-8 and PF-8 (Baltz et al., 2009), and several of these residues were localized in the C-terminal region of Pol-8 and in or around the connector loop of PF-8.

By screening the NCI Diversity Set that includes 1992 synthetic compounds, Dorjsuren and colleagues identified 28 compounds able to inhibit Pol-8/PF-8-mediated DNA synthesis with IC_{50}s ranging from 0.12 to 10.83 μM (Dorjsuren et al., 2006). Eighteen of the active compounds also efficiently blocked HHV-8 processive DNA synthesis *in vitro*. Importantly, one of the hit compounds, NSC 373989 (Table 1), a pyrimidoquinoline analog, was shown to reduce in a dose-dependent manner the levels of virus yield and viral DNA in lytically induced HHV-8-infected BCBL-1 cells, suggesting that the compound blocked lytic HHV-8 DNA synthesis (Dorjsuren et al., 2006). These findings suggest that the search for Pol-8/PF-8 interaction inhibitors is feasible and may lead to the discovery of novel anti-HHV-8 drugs.

2.4. Inhibiting polymerase subunit dimerization

Another potential antiviral strategy could the inhibition of the dimerization of the accessory subunit of some herpesvirus DNA polymerases. HCMV UL44 displays an overall fold strikingly similar to that of other processivity factors, such as HSV-1 UL42, however, differently from UL42, which is a monomer, UL44 forms a head-to-head C-clamp-shaped homodimer (Appleton et al., 2004). UL44 dimerizes across one of the crystallographic 2-fold axes and the interaction between the two monomers entails an antiparallel β interaction which involves six main-chain-to-main-chain hydrogen bonds and extensive packing of hydrophobic side chains at the interface. F121 of each monomer is buried against a hydrophobic loop composed of P85, L86, and L87 of the opposite monomer (Appleton et al., 2004). The dimerization of UL44 appears to occur in the cytoplasm (Alvisi et al., 2006) and some phosphorylation sites in UL44 have been shown to regulate the protein nuclear import (Alvisi et al., 2011; Silva et al., 2011). Interestingly, substitution of highly phosphorylated sites adjacent to the nuclear localization signal abolished viral replication, suggesting a crucial role for phosphorylation-mediated nuclear localization of UL44 for HCMV replication (Silva et al., 2011).

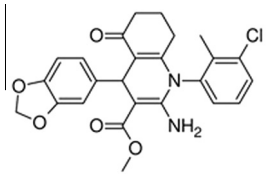
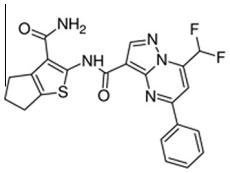
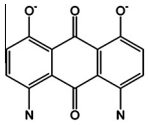
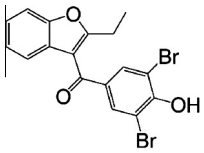
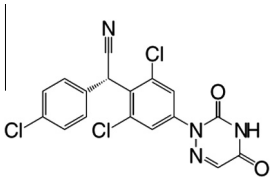
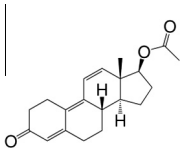
Biophysical studies have demonstrated that UL44 binds to duplex DNA as a dimer (Loregian et al., 2007). Intriguingly, point mutations (i.e., L86A/L87A and F121A) that interfere with dimerization *in vitro* also result in decreased DNA binding (Appleton et al., 2004), leading to the hypothesis that formation of UL44 dimers might be important for its function in viral DNA replication. In keeping, we recently showed that UL44 point mutants that are impaired for dimerization, but not for nuclear localization or interaction with the C-terminus of the polymerase catalytic subunit, are not capable of supporting HCMV *ori*Lyt-dependent DNA replication in cells (Sinigalia et al., 2008). These data suggest that disruption of UL44 homodimers could be a potential anti-HCMV strategy (Loregian and Palù, 2005b; Mercorelli et al., 2008).

Similarly to HCMV UL44, HHV-8 PF-8 forms homodimers in solution and is observed as a dimer on the DNA (Zhou et al., 2010). The recent crystal structure of residues 1–304 of PF-8 has revealed that each monomer of PF-8 shares a fold common to other processivity factors and that like UL44, PF-8 forms a head-to-head dimer in the form of a C clamp (Baltz et al., 2009). In addition, PF-8 can also dimerize in cells and like UL44, PF-8 dimerizes in the cytoplasm before being translocated to the nucleus (Zhou et al., 2010). The accessory subunit of EBV DNA polymerase, called BMRF1, also forms a C-shaped head-to-head dimer, and dimer formation is required for DNA binding (Murayama et al., 2009). Although the importance of PF-8 and BMRF1 dimerization for virus replication remain to be established, both protein–protein interfaces could represent new targets for drug discovery.

2.5. Other approaches

Finally, other than UL54, a plethora of viral or cellular proteins have been reported to associate with HCMV UL44 (Gao et al., 2008; Kim and Ahn, 2010; Krosky et al., 2003; Marschall et al., 2003;

Table 2
Peptide and small molecule inhibitors of influenza virus RNA polymerase subunit interactions.

Inhibitor	Viral Target	Sequence/structure	Activity[a]		Ref.
			In vitro assays IC_{50} (μM)	Cell culture assays EC_{50} (μM)	
PB1 N-terminal peptide (aa 1-15) fused to aa 47-59 of HIV-1 Tat	Influenza A virus PA/PB1 interaction	MDVNPTLLFLKVPAQ	0.04–35.5 (ELISA)	15.5–48.4 (Minireplicon assay, PRA)	Muratore et al. (2012a) and Wunderlich et al. (2009)
Compound 1	Influenza A virus PA/PB1 interaction		30.4 (ELISA)	1.5–22.5 (VYRA, PRA)	Muratore et al. (2012a)
Compound 5	Influenza A virus PA/PB1 interaction		25.4 (ELISA)	30.7–75.5 (VYRA, PRA)	Muratore et al. (2012a)
AL18	Influenza A virus PA/PB1 interaction		20.3 (ELISA)	2.5–14.5 (VYRA, PRA)	Muratore et al. (2012b)
Benzbromarone	Influenza A virus PA/PB1 interaction		n.d.[b]	39 (PRA)	Fukuoka et al. (2012)
Diclazuril	Influenza A virus PA/PB1 interaction		n.d.[b]	31 (PRA)	Fukuoka et al. (2012)
Trenbolone acetate	Influenza A virus PA/PB1 interaction		n.d.[b]	51 (PRA)	Fukuoka et al. (2012)
PB2 N-terminal peptide (aa 1–37)	Influenza A virus PB1/PB2 interaction	MERIKELRNLMSQSRTREILTKTTVDHMAIIKKYTSG	0.37 (ELISA)	n.d.[b]	Chase et al. (2011)
PB1 C-terminal peptide (aa 731–757)	Influenza A virus PB1/PB2 interaction	ESGRIKKEEFTEIMKICSTIEELRRQK	n.d.[b]	n.d.[b]	Li et al. (2013)

[a] The activities in *in vitro* assays (IC_{50}: inhibitor concentration to give 50% inhibition *in vitro*) and in cell culture assays (EC_{50}: inhibitor concentration to give 50% inhibition in cells) are reported. The assays employed to evaluate the antiviral activity *in vitro* or in cell culture are reported in parenthesis underneath the IC_{50} or EC_{50} values, respectively (ELISA, enzyme-linked immunosorbent assay; PRA, plaque reduction assay; VYRA, virus yield reduction assay).
[b] n.d., Not determined.

Prichard et al., 2005; Ranneberg-Nilsen et al., 2008; Sinigalia et al., 2012; Strang et al., 2009, 2010a,b). However, only for very few of these partners a direct PPI with UL44 has been conclusively demonstrated and indeed some of these interactions have been called into question (Strang et al., 2009). In addition, at the moment for most of these associations no clear functional role in virus replication has been reported and for several of the UL44 partners the binding site is still unknown. Nevertheless, at least some of these PPIs might provide novel antiviral targets.

In general, since both the catalytic subunit and the accessory protein of herpesvirus DNA polymerases are essential for viral DNA replication and since they cannot be replaced by any other cellular or viral proteins, they are potentially excellent antiviral targets. As the residues most important for subunit interactions of herpesvirus DNA polymerases are not conserved, small molecule inhibitors targeting the residue side chains could be significantly more virus-specific than most of the drugs currently licensed for anti-herpesvirus chemotherapy. In addition, given the extreme specificity PPIs, the discovery of inhibitory agents against herpesvirus DNA polymerase subunit interactions may lead to specific antiviral therapies with minimal toxicity to host cells.

3. Inhibiting PPI of the influenza virus RNA polymerase

Inhibitors that act by preventing formation of active enzyme oligomer complexes or by dissociating such complexes have also

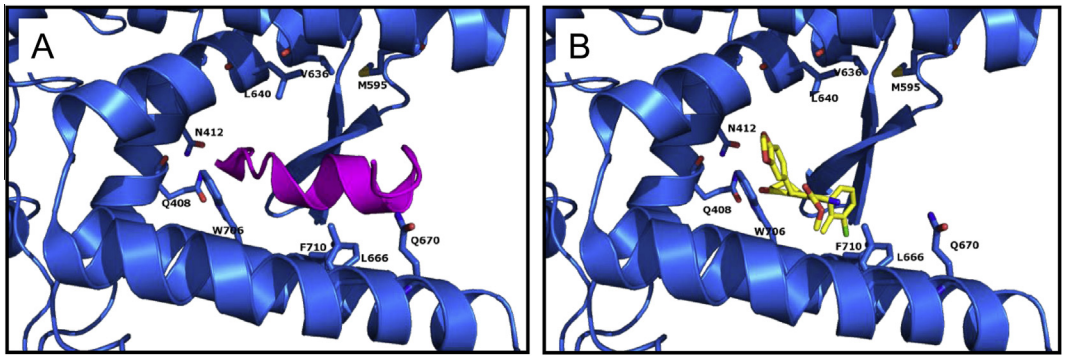


Fig. 2. A small molecule inhibitor of influenza virus RNA polymerase subunit interactions. (A) Interaction between PA and PB1 subunits. The C-terminal groove of PA (*blue*) and the amino-terminal region (aa 1–25) of PB1 (*magenta*) are shown in ribbon representation. Residues of PA interacting with the PB1 peptide are shown in stick representation and labelled. (B) Interaction of compound 1 with PA. The best pose of compound 1 (*yellow*) in the C-terminal groove of PA (*blue*) is shown. Based on data reported in He et al. (2008) and Muratore et al. (2012a).

been discovered for the RNA polymerase of influenza virus. Currently, two classes of anti-influenza drugs are available: adamantanes, which block the M2 ion channel and inhibit virus entry, and neuraminidase inhibitors, which prevent the release of virions from the host cell (De Clercq, 2006). However, they all suffer from limited efficacy, adverse side effects, and emergence of drug-resistance. Vaccines also exist, but must be reformulated annually and give limited protection. Thus, there is still a considerable need for new anti-influenza drugs.

The influenza virus RNA polymerase provides an underexploited drug target. The viral polymerase is a heterotrimeric complex of three virus-encoded proteins (PB1, PB2, and PA), all essential for viral RNA synthesis (Palese and Shaw, 2007). PB1 is the nucleic acid polymerase and forms the backbone of the complex (Biswas and Nayak, 1994; Digard et al., 1989). PB2 and PA play accessory roles, mainly in viral transcription (Dias et al., 2009; Guilligay et al., 2008; Yuan et al., 2009). The three subunits bind each other non-covalently in a set of interactions that are essential for polymerase function (Perez and Donis, 2001; Sugiyama et al., 2009). Although the polymerase forms a globular structure (Torreira et al., 2007), the primary PPIs are via the N-terminus of PB1 with the C-terminus of PA (Gonzalez et al., 1996; Ohtsu et al., 2002; Perez and Donis, 1995) and the C-terminus of PB1 with the N-terminus of PB2 (Gonzalez et al., 1996; Poole et al., 2007). In contrast to the viral glycoproteins, the polymerase is highly conserved between different viral strains (Palese and Shaw, 2007). Thus, inhibition of these interactions represents an attractive strategy for the development of drugs with broad efficacy against all influenza virus strains.

The feasibility of this approach was first proved by a study showing that a 25-amino-acid peptide corresponding to the PA-binding domain of PB1 is able to block the polymerase activity of influenza A virus and inhibit viral spread when intracellulary delivered by the translocating domain of human immunodeficiency virus Tat protein (Ghanem et al., 2007). A shorter PB1 peptide (N-terminal residues 1–15, Table 2) also showed inhibitory activity (Muratore et al., 2012a; Wunderlich et al., 2009). Recently, two crystallographic structures of a truncated form of PA bound to a PB1-derived peptide have been published (He et al., 2008; Obayashi et al., 2008). These structures revealed that the PA/PB1 binding interface consists of an N-terminal 3_{10} helix from PB1 that binds into a hydrophobic groove in the C-terminus of PA (Fig. 2). Importantly, the structures showed that relatively few residues drive binding of PB1 to PA, suggesting the potential for small molecule-mediated inhibition. In support of the structural data, point mutations in the C-terminal domain of PA (e.g., V636S, L640D, L666D, W706A, and Q670A/W706A) and in the N-terminal region of PB1 (e.g., substitution of Pro 5, Leu 7, Leu 8, Phe 9 or Leu 10 with aspartatic acid) greatly weakened or abolished PA/PB1 binding and also reduced viral RNA synthesis or virus production (He et al., 2008; Obayashi et al., 2008; Perez and Donis, 2001).

Taking advantage of the crystallographic information, we recently conducted an *in silico* screening of 3 million small molecule structures to search for inhibitors of the PA/PB1 interaction. From this, 32 compounds emerged as candidates. Among these small molecules, two compounds – 1 and 5 (Table 2) – were able to interfere with the interaction between PB1 and PA both *in vitro* and in cells, to inhibit nuclear import of a binary PA/PB1 complex as well as transcription by the full viral ribonucleoprotein complex (Muratore et al., 2012a). Of these, compound 1 (Fig. 2) also acted as a potent replication inhibitor of a variety of influenza A virus strains in infected cells, including H3N2 and H1N1 seasonal and 2009 pandemic strains, with EC_{50} values in the low micromolar range and negligible cytotoxicity. Importantly, this included an Oseltamivir resistant isolate. Furthermore, compound 1 also inhibited the replication of influenza B viruses but not that of other RNA or DNA viruses (Muratore et al., 2012a). The lack of activity against other viruses strongly suggests a selective antiviral mechanism of action, however, further work such as isolation and characterization of compound 1-resistant viruses will be required to demonstrate that this compound indeed inhibits influenza A virus replication as proposed.

Serendipitously, during these studies one of the compounds previously shown to inhibit subunit interactions of HCMV DNA polymerase and HCMV replication in infected cell cultures, i.e., AL18 (Loregian and Coen, 2006), was found to also block the interaction between the PB1 and PA polymerase subunits of influenza A virus (Table 2). Furthermore, AL18 effectively inhibited influenza A virus polymerase activity and the overall replication of influenza A and B viruses (Muratore et al., 2012b). The discovery of these compounds provided a proof-of-principle that the PA/PB1 interaction can be disrupted by a small-molecular-weight compound.

Along the same line, a similar, but more restricted approach was undertaken by Fukuoka and colleagues (Fukuoka et al., 2012). By performing a docking simulation using a drug database including ~4000 compounds, they selected candidate compounds targeting the interface of PA interacting with PB1. Among these, benzbromarone, diclazuril, and trenbolone acetate (Table 2) exhibited anti-influenza A virus activities. Furthermore, benzbromarone and diclazuril were confirmed to bind the PA subunit and could decrease the transcriptional activity of the viral RNA polymerase. Overall, the compounds targeting the PA/PB1 binding interface could provide the basis for the development of a new generation of therapeutic agents against influenza A and B viruses.

The possibility of targeting other interaction sites in the polymerase complex, e.g., those between PB1 and PB2 subunit (Reuther et al., 2011), recently emerged with the publication of the crystal structure of the PB1/PB2 binding interface (Sugiyama et al., 2009). The structure showed that only a short region of PB1 (residues 678–757) and of PB2 (residues 1–37) are required for tight binding. As the PB1/PB2 interface is very small, yet has a crucial function in regulating the polymerase complex, and it is highly conserved among avian and human influenza viruses, the PB1/PB2 interaction appears as a promising target for novel anti-influenza drugs of use against all strains of influenza A virus. A synthetic peptide corresponding to residues 1–37 of PB2 (Table 2) was shown to inhibit the PB1/PB2 interaction *in vitro* with an IC_{50} of 375 nM (Chase et al., 2011), thus demonstrating the feasibility of such an approach. However, to date no small molecule targeting this PPI has been yet reported.

Very recently, Li and colleagues reported that a peptide derived from amino acids 731–757 of PB1 (Table 2) can disrupt the interaction between the C-terminal part of PB1 (corresponding to $PB1_{676–757}$) and the N-terminal part of PB2 (corresponding to $PB2_{1–40}$) (Li et al., 2013). The $PB1_{731–757}$ peptide was also capable of inhibiting viral polymerase activity and virus replication. Interestingly, the authors showed that the $PB1_{731–757}$ peptide interacts with PB1 rather than PB2. The inhibitory binding appears to be mainly mediated by hydrophobic interactions between the C-terminus of PB1 and $PB1_{731–757}$. Furthermore, mutational analyses and computational modelling suggested that $PB1_{731–757}$ acts as a competitor of PB2 with respect to binding to PB1. Thus, the inhibitory mechanism of the $PB1_{731–757}$ peptide is likely different from that of the interfacial peptides $PB1_{1–25}$ and $PB2_{1–37}$, which inhibit complex assembly by binding to its interaction partner PA or PB1, respectively, and could suggest new avenues for antiviral discovery.

The inhibitors that act against subunit interactions of the influenza virus RNA polymerase might have a number of advantages compared to other classes of anti-influenza compounds. First, since these antiviral agents have a different mode of action to the current anti-influenza drugs, they are unlikely to suffer from cross-resistance. Second, since the amino acids of PB1, PB2, and PA that are essential for polymerase subunit interaction are highly conserved among influenza A virus strains (Ghanem et al., 2007; Liu and Yao, 2010; Sugiyama et al., 2009), inhibitory molecules will likely have broad efficacy against influenza A viruses of both human and animal origin, including the pandemic swine-originated influenza virus and highly pathogenic avian H5N1 strains. In addition, the identification of compounds that inhibit the replication of both influenza A and influenza B viruses by inhibiting the PA/PB1 interaction (compound 1 and AL18; (Muratore et al., 2012a,b)) suggests the possibility of developing drugs that are active against both major subtypes of human orthomyxoviruses. Finally, the possibility of targeting different interaction sites in the polymerase complex, i.e., those between PA and PB1 along with those between PB1 and PB2, may allow the generation of an antiviral "cocktail" that could reduce the probability of generating escape mutants.

4. Conclusions

The importance and specificity of protein associations between the subunits of viral polymerases make such PPIs very attractive targets for therapeutic intervention. Although the development of PPI inhibitors is often associated with several drawbacks (Wells and McClendon, 2007), the identification of some small molecules with potent and specific antiviral activity suggest that the interactions between the subunits of viral polymerases are suitable targets for this strategy. However, the inhibitors described here are still very far from reaching the market. Although some of them obey Lipinski's rule-of-five (Lipinski et al., 2001), not all possess drug-like properties, and many preclinical and clinical studies will be necessary to determine if any of them can be used in human therapy. Nevertheless, it is our hope that the promising results obtained so far will encourage further efforts with this antiviral strategy.

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