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Orthopoxvirus targets for the development of new antiviral agents

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Abstract

Investments in the development of new drugs for orthopoxvirus infections have fostered new avenues of research, provided an improved understanding of orthopoxvirus biology and yielded new therapies that are currently progressing through clinical trials. These broad-based efforts have also resulted in the identification of new inhibitors of orthopoxvirus replication that target many different stages of viral replication cycle. This review will discuss progress in the development of new anti-poxvirus drugs and the identification of new molecular targets that can be exploited for the development of new inhibitors. The prototype of the orthopoxvirus group is vaccinia virus and its replication cycle will be discussed in detail noting specific viral functions and their associated gene products that have the potential to serve as new targets for drug development. Progress that has been achieved in recent years should yield new drugs for the treatment of these infections and might also reveal new approaches for antiviral drug development with other viruses.

Keywords

Orthopoxvirus; Nucleoside analog; CMX001; ST-246; Cidofovir; Drug targets

1. Background and rationale

The orthopoxviruses infect a variety of animal species and cause disease in many, including humans. The most important viruses from a public health standpoint are variola virus, the causative agent of smallpox, monkeypox virus, vaccinia virus, buffalopox virus, and cowpox virus. It has been more than three decades since the eradication of smallpox disease from the world's population; vaccination programs have been halted and immunity has waned. There has been little interest in the development of a drug for this disease that no longer exists in the population and few resources have been utilized to further combat it (Pennington, 2003). In recent years, however, there have been increasing concerns that variola virus or monkeypox virus might be used as agents of bioterrorism. Importantly, monkeypox virus is endemic in Western Africa (Breman and Henderson, 1998; Heymann et al., 1998; Hutin et al., 2001), and has resulted in an outbreak in the United States following the importation of infected rodents (Melnick et al., 2003; Reed et al., 2004). Other zoonotic infections also warrant attention including vaccinia virus in Brazil, cowpox virus in Europe, and buffalopox virus in India (Moussatche et al., 2008). All of these viruses are occasionally transmitted to human hosts. The potential release of variola virus as an act of bioterrorism or the progressive shift of zoonotic viruses from endemic reservoirs to an epidemic in man has

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stimulated renewed interest in the discovery and development of new antiviral agents that could be used to help mitigate consequences of future outbreaks (LeDuc et al., 2002; LeDuc and Jahrling, 2001). While vaccination would continue to be critical in the control of such outbreaks, the availability of antiviral therapies would be important for individuals for whom the vaccine is contraindicated as well as those that do become infected (Bray, 2003). Additionally, in the event of widespread vaccination, antiviral therapies would be required to treat, adverse events associated with inadvertent vaccination.

A few laboratories have continued to work in this arena and have identified several compounds that were effective against orthopoxvirus infections in vitro and in vivo including methisazone, ribavirin, idoxuridine, interferon, adenine arabinoside, cytosine arabinoside, S2242, cidofovir (CDV) and other phosphonate nucleotide analogs (De Clercq, 2001; De Clercq et al., 1976; Kern, 2003; Kern et al., 2002; Naesens et al., 1997; Nettleton et al., 2000; Neyts and De Clercq, 2001, 2003; Sidwell et al., 1972; Woodson and Joklik, 1965). For a variety of reasons, most notably lack of interest, limited efficacy, or excessive toxicity, few of these agents progressed into clinical studies.

As part of an extensive study of the antiviral activity of a group of acyclic phosphonate nucleosides, De Clercq and colleagues first described the activity of cidofovir (CDV, HPMPC, vistide) against orthopoxvirus infections and its activity has been reviewed extensively (Andrei and Snoeck, 2010; De Clercq, 2007, 2010a) (Fig. 1). A number of other investigators have also described the effectiveness of CDV for the inhibition of replication of all the orthopoxviruses that have been tested including variola, monkeypox, ectromelia, vaccinia, rabbitpox, and cowpox viruses (Adams et al., 2007; Baker et al., 2003; Buller et al., 2004; Kern, 2003; Kern et al., 2002; Parker et al., 2008). Interest in this compound as a potential therapy for orthopoxvirus infections increased as it became apparent that it was highly effective in mice infected with vaccinia or cowpox viruses (Bray et al., 2000; De Clercq, 1989; Neyts and De Clercq, 1993; Quenelle et al., 2003,2004a; Smee et al., 2000, 2001, 2002).

Although CDV was highly effective when given parenterally or by aerosol, even as a single dose, it was poorly absorbed and thus inactive when administered orally against vaccinia or cowpox virus infections (Cundy et al., 1996). The lack of oral bioavailability does not preclude its use in the treatment of orthopoxvirus infections in humans, yet it presents significant logistical issues, particularly for its use in the field. Another major shortcoming of the drug is that long term administration may result in nephrotoxicity (Safrin et al., 1997). However, its use for treatment or prevention of smallpox would undoubtedly be short term and toxicity may not be a significant issue. These concerns notwithstanding, CDV was approved under an Investigational New Drug Application for the emergency treatment of smallpox or complications from vaccination (http://emergency.cdc.gov/agent/smallpox/vaccination/mgmtadv-reactions.asp).

More recently, Hostetler and colleagues synthesized a series of alkoxyalkyl derivatives of acyclic nucleoside phosphonates in an effort to improve the oral bioavailability of this class of compounds (Hostetler, 2009). One of these, hexadecyloxypropyl CDV (HDP-CDV, CMX001) was about 150-fold more active against vaccinia and cowpox viruses in cell culture than CDV (Kern et al., 2002) (Fig. 1). Similar levels of enhanced activity have also been reported for variola, monkeypox, and ectromelia viruses (Buller et al., 2004; Kern, 2003). A number of studies have now been completed that clearly indicate that the lipid conjugate, HDP-CDV, is bioavailable after oral administration (Ciesla et al., 2003), can persist in tissues for up to 1 week, and its altered tissue distribution precludes kidney toxicity (Quenelle et al., 2004b, 2010). In a series of animal studies, the therapeutic efficacy of HDP-CDV was compared with CDV in mice infected with either vaccinia or cowpox

viruses. In all studies, regardless of whether the drugs were given 1 week prior to or up to 5 days after infection, with either single or multiple treatments, HDP-CDV given orally was at least equivalent to CDV given parenterally (Quenelle et al., 2004b). In mice infected with ectromelia virus (Parker et al., 2008), or rabbits infected with rabbitpox virus (Adams et al., 2007), essentially complete protection was obtained with HDP-CDV. This compound, also known as CMX001 is currently in clinical development for the prophylaxis and treatment of double strand DNA viral infections and has been recently reviewed (Lanier et al., 2010). At some point it might be possible to approve this compound for the treatment of smallpox under the "Animal Rule" using animal efficacy and human safety data (Parker et al., 2010).

A high throughput screen against vaccinia virus and cowpox virus infected cells also led to the identification of ST-246 (Tecovirimat) that is a highly potent inhibitor of these viruses (Yang et al., 2005) (Fig. 1). It is also active against all of the orthopoxviruses that have been tested (Jordan et al., 2010). It was also highly effective in preventing mortality or disease in animals infected with vaccinia, cowpox, ectromelia or monkeypox viruses when given orally (Quenelle et al., 2007a; Yang et al., 2005). This compound has also proven to be well tolerated in Phase I/II clinical studies and its development has been reviewed recently (Jordan et al., 2010).

It is important to note that since ST-246 and CMX001 are likely to be the first two compounds available for use in an orthopoxvirus outbreak, it is reasonable to expect that they will be used in combination. It would be desirable to have multiple drugs available for treatment of orthopoxvirus infections that have differing mechanisms of action to improve efficacy, minimize toxicity and reduce the development of drug resistance. These two compounds have in fact been evaluated for their interaction when administered together and act synergistically in vitro against both vaccinia and cowpox viruses and in mice infected with cowpox virus (Chen et al., 2011; Quenelle et al., 2007b).

Recent efforts have made significant progress in the development of therapies for orthopoxvirus infections. CDV is available for emergency use in an orthopoxvirus outbreak and both CMX001 and ST-246 are very active in vitro and in animal studies and results from early clinical studies look promising. These successes notwithstanding, neither CMX001 nor ST-246 has been approved for use in humans and it is essential to continue the development of additional antiviral compounds that have unique mechanisms of action to provide critical backup compounds. Additional therapies with distinct mechanisms of action will be essential to help manage uncertainties associated the unknown effectiveness of therapies for the treatment of variola virus infections in humans. The availability of a set of potential drugs with distinct molecular targets will help to ensure that at least one would be effective in preventing mortality in infected individuals. Combinations of existing drugs together with new compounds with novel modes of action should also help to increase efficacy, reduce the required doses, and mitigate risks associated with the transmission of drug resistant viruses or engineered viruses.

This review summarizes the many promising targets that might be exploited in the discovery of additional agents for orthopoxvirus infections. It focuses on the key molecular details of orthopoxvirus replication with an emphasis on specific viral proteins that might be targeted in the development of new antiviral therapies. While it is certainly possible that inhibitors of cellular targets could interfere with the replication of the virus, this general approach will not be addressed in this review.

2. Overview of the orthopoxvirus replication cycle

The orthopoxviruses are classified as the genus *Orthopoxvirus* in the subfamily *Chordopoxvirinae* within the *Poxviridae* family. Orthopoxviruses are important human

pathogens and their natural history, pathogenesis, and replication characteristics have been studied extensively. The natural history and pathogenesis associated with these infections have been reviewed elsewhere (Esposito and Fenner, 2001). The complex biology of their replication cycle and interaction with the host has also been the subject of similar excellent reviews (Moss, 2007; Roberts and Smith, 2008).

A summary of the replication cycle of vaccinia virus, the prototype orthopoxvirus, illustrates the complex biology of the virus and provides a glimpse of critical events that might be targeted by antiviral agents. Infection can be initiated with intracellular mature virus (IMV) or with extracellular enveloped virus (EEV), which represent two forms of infectious virus particles (Fig. 2). Initially EEV particles bind to receptors on permissive cells in the host through interactions with viral proteins in the exterior membrane of the virion. A non-fusogenic mechanism releases a distinct particle that is essentially equivalent to IMV from the secondary membrane of the EEV. Subsequent membrane fusion events between IMV particles and the cell membrane are mediated by viral glycoproteins resulting in the release of virus cores into the cytoplasm. Constituent viral enzymes within the cores initiate the expression of early transcripts. Most early gene products are immunomodulatory proteins, but enzymes are also expressed that direct the replication of viral DNA, which promotes the expression of intermediate genes from nascent genomes. This set of viral products includes late transcription factors that drive the expression of late transcripts coding for structural proteins required for the assembly of virus particles. Virion morphogenesis occurs in the cytoplasm within electron dense regions called virus factories, which are exceedingly complex structures that support the efficient production of progeny virus. Here, large concatameric intermediates of newly synthesized DNA are resolved into unit length genomes and packaged into immature virions, which also contain early transcription factors destined to be packaged into maturing virions. Maturation of these particles occurs through a series of proteolytic events that lead to the formation of infectious IMVs that can be released following cell lysis. However, some of these infectious particles undergo a secondary envelopment where a second double membrane and additional glycoproteins are acquired to yield EEVs that are critical for the dissemination of the virus within the host.

3. Potential molecular targets in orthopoxvirus replication cycle

The complex nature of the vaccinia virus replication cycle provides numerous targets for the development of new therapies for these infections and has been reviewed previously (Prichard and Kern, 2005, 2009). To highlight these targets, each step in the replication process will be described in detail and viral proteins that are known to participate in these functions will be noted. Known inhibitors of these processes will be described and the molecular targets will be identified insofar as they are understood. A summary of all the inhibitors and their molecular targets is also provided as a quick reference (Table 1). Finally, viral proteins that have the potential to be exploited in new therapeutic strategies will be discussed at the end of each section, focusing on those that perform essential functions in viral replication. These potential targets are summarized in Table 2 and are organized with respect to the stage of the replication cycle where they are thought to be required.

3.1. Virion and genomic structure

The replication of the orthopoxviruses occurs exclusively in the cytoplasm of infected cells and all the proteins required for their replication are encoded on a single large linear DNA genome. Members of the *Orthopoxvirus* genus include variola, cowpox, monkeypox, camelpox, buffalopox, ectromelia, and rabbitpox viruses. The prototypic member of this genus is vaccinia virus. This review will focus on this virus since it is a good model for orthopoxvirus replication in cell culture and has been the most extensively studied. Virions of vaccinia virus are large (270 × 350 nm) and its constituent proteins include a full

complement of enzymes sufficient to direct the expression of early viral mRNAs (Moss, 2001). At least three morphologically distinct forms of infectious virions are produced in cell culture and include the abundant IMV, the EEV that has acquired a secondary envelope and additional glycoproteins, and the CEV. Cell bound CEV are EEV that are retained on the cell surface atop pedestals formed through the induction of actin tails and become EEV once they have been released from the host cell. The secondary envelope on EEV with its characteristic glycoproteins significantly impacts the biological properties of this particle and is of considerable importance in the therapy of orthopoxvirus infections. The IMV represents a stable form of the virus that is thought to be important in the transmission between hosts while the EEV is critical for pathogenesis and dissemination of the virus in vivo.

The linear vaccinia virus genome is double stranded DNA with inverted tandem repeats of variable length at both termini. At each terminus, a single strand of DNA turns back on itself in the form a hairpin, such that a completely denatured genome would yield a covalently closed circle of single stranded DNA. When considering potential targets for antiviral therapy in the orthopoxviruses, it is important to understand the sequence diversity among the various viruses as well as the sequence diversity within the same virus species (Qin et al., 2011). The complete genomes of 16 strains of vaccinia virus have been sequenced and are generally collinear, although their size varies between 160 kb to more than 200 kb in length (for a current listing, see http://www.poxvirus.org). This includes the MNR-76 strain of horsepox virus as well as the Ultrecht strain of rabbitpox virus that are both closely related to vaccinia virus (Li et al., 2005; Tulman et al., 2006). The prototypic Western Reserve strain of vaccinia virus has a genome consisting of 194,711 base pairs (NC(_)006998), while more highly attenuated strains such as Copenhagen, Lister, and MVA tend to have shorter genomes that reflect an accumulation of small deletions that arose during their extensive passage histories (Lefkowitz et al., 2005). Nine strains of monkeypox virus have also been sequenced and most were in the range of 196–201 kb (Lefkowitz et al., 2005). Sequence analysis of isolates obtained in the United States, Western and Central Africa have revealed two clades that suggest a genetic basis for differences in pathogenicity (Chen et al., 2005; Likos et al., 2005). An analysis of the coding sequence of 45 strains of variola virus identified three distinct clades for viruses isolated in West Africa, South America, and isolates with increased virulence from Asia together with African isolates that were not from West Africa (Esposito et al., 2006). The continued analysis of new isolates and deep sequencing studies will improve our understanding of evolutionary relationships among the members of this genus and help to identify loci that are associated with virulence or attenuation.

The genome of the highly attenuated Copenhagen strain of vaccinia virus was the first isolate to be sequenced; however the pro-totypic Western Reserve strain that was sequenced by Esposito and colleagues encodes a number of additional genes that were not present in the Copenhagen strain. Recent analyses suggest that there are likely 202–206 non-redundant genes encoded by vaccinia virus that includes all the viral proteins required for viral replication in vitro and additional genes that are likely required for in vivo replication (Da Silva and Upton, 2005; Upton et al., 2003). A genomic analysis of all orthopoxvirus genomes identified 49 genes that were conserved among the insect and vertebrate orthopoxviruses, as well as 90 genes that are conserved among all the chordate orthopoxviruses (Upton et al., 2003). The latter subset of genes is thought to represent a minimum complement of genes required for basic replication of orthopoxviruses in vertebrates and is likely to contain some of the best targets for the development of antiviral therapies.

3.2. Entry and uncoating

Mechanisms of entry for IMV and EEV particles are distinct although they both ultimately utilize fusion events with the cell membrane to release virus cores into the cytoplasm (Moss, 2006). The initial interaction of the EEV with the cell is thought to be mediated by five viral proteins in the outer membrane (Smith et al., 2002). Subsequent binding events involve a ligand-dependent nonfusogenic unwrapping of the outer viral membrane that releases the internal virus particle that is essentially an IMV (Law et al., 2006). The single bilamellar membrane of the IMV-like particle released from the EEV fuses with the cellular membrane and releases the virus core into the cytoplasm and the IMVs also appear to fuse in a similar manner. Both these particles interact with the cell surface, in a process that involves an interaction between H3L, A27L, and D8L with cellular glycosaminoglycans (Carter et al., 2005; Sieczkarski and Whittaker, 2005). The A26L envelope protein also interacts with laminin in the extracellular matrix suggesting that it may also be involved in this process (Chiu et al., 2007). Monoclonal antibodies specific for A17L and L1R have also been shown to inhibit viral infection at early stages. The B5R type I membrane protein is crucial for the formation of EEV and contains neutralizing epitopes recognized by vaccinia immune globulin (VIG) (Bell et al., 2004), that are similar to those of the B6R homolog in variola virus (Aldaz-Carroll et al., 2007).

Entry of DNA containing cores into the cytoplasm also appears to be a complex process involving several viral proteins including A16L, A21L, A28L, F9L, G3L, G9R, H2R, J5L and L5R (Brown et al., 2006; Ojeda et al., 2006; Senkevich et al., 2005). Once cores enter the cytoplasm they are uncoated, which increases their permeability and allows the release of nascent early transcripts (Pedersen et al., 2000). The viral genome is then released and transported by an undescribed mechanism to a location in close proximity to the membrane of the endoplasmic reticulum and establishes a replication site (Tolonen et al., 2001). Each core is sufficient to induce a single replicative site since a cell superinfected with two distinct recombinant viruses expressing cyan or yellow fluorescent protein results in the formation of separate virus factories that appear either cyan or yellow (Katsafanas and Moss, 2007).

Initial events in infection, such as attachment and membrane fusion are essential and can be effectively targeted by neutralizing antibodies. Indeed, VIG appears to inhibit viral infection predominantly by antibodies that are specific for B5R and results in the neutralization of EEV (Bell et al., 2004). More recently, murine and humanized monoclonal antibodies specific for H3L and B5R were shown to be effective in animal models of vaccinia virus infection (Tomimori et al., 2011). The essential function of A28L in the entry/fusion complex also makes this an interesting new target for neutralizing antibodies (Nelson et al., 2008). Fusion inhibitors have proven to be successful in the therapy of human immunodeficiency virus (HIV) infections and it is possible that a similar path could be applied to the development of fusion inhibitors of orthopoxvirus infections.

3.3. Transcriptional regulation and mRNA processing

The temporally regulated cascade of vaccinia virus gene expression is controlled predominantly at the level of transcription and was the subject of an excellent review (Broyles, 2003). All viral factors required for the transcription of early genes, including each of the subunits of the RNA polymerase complex are packaged in the virion and have been characterized in detail (Yoder et al., 2006). One subunit of this complex (Rap94) is packaged within virions as part of the viral transcription machinery that imparts specificity to early viral promoters, which are also responsive to A8L, D6R, and cellular transcription factors (Yang and Moss, 2009). Viral DNA synthesis also plays a critical role in the transcription of intermediate genes suggesting that either virion DNA or the nascent DNA

has been altered in some manner to regulate the expression of intermediate promoters. Intermediate transcription factors are encoded by the genes *E4L*, *A23R*, *A8R* as well as those for the viral capping enzyme (*D1R* and *D12L*), and all are important for the expression of these promoters (Broyles, 2003). The cellular factors G3BP, SP1, YY1, and TBP have also been shown to bind intermediate promoter elements and are also likely to be important in the assembly of complexes that promote early transcription. Late transcription factors expressed from the intermediate genes *A1L*, *A2L* and G8R are required for the transcription of late viral promoters (Keck et al., 1990). In addition, heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3 have also been shown to contribute to late transcription (Wright et al., 2001).

Vaccinia virus also expresses an array of enzymes involved in posttranscriptional processing events that catalyze the capping and polyadenylation of nascent viral transcripts in the cytoplasm. The biochemical activities of these enzymes are essential, distinct from those of the cellular counterpart, and have been well characterized by Shuman and colleagues (Schwer and Shuman, 2006). The heterodimeric capping enzyme is encoded by *D1R* and *D12L* and catalyzes the reactions required to place a 7-methylguanosine cap on the 5′ end of viral mRNAs (Zheng and Shuman, 2008). Interestingly, the viral D10R protein catalyzes the efficient removal of the 7-methylguanosine cap from all mRNAs (Parrish et al., 2007). This activity results in the rapid turnover of all mRNAs in infected cells, and is thought to confer an advantage to viral messages by reducing the abundance of stable cellular transcripts. An additional methylation of the penultimate 5′ nucleotide of the mRNA is also catalyzed by the mRNA (nucleoside-2′-)-methyltransferase encoded by the viral VP39 protein that interacts with the RNA polymerase (Mohamed et al., 2001), and effects the elongation of intermediate and late viral transcripts (Latner et al., 2002). VP39 also functions as the processivity factor for the viral poly(A) polymerase (VP55) encoded by *E1L* (Gershon and Moss, 1993). This heterodimeric enzyme catalyzes the discontinuous but processive addition of approximately 30 nucleotide poly(A) tails on viral mRNAs (Yoshizawa et al., 2007), and the recently determined crystal structure of this enzyme suggests a structural basis for the processivity induced by VP39 (Moure et al., 2006).

Enzymes encoded by the virus that catalyze the transcription of viral genes as well as the polyadenylation and capping of viral transcripts are distinct from those of the cell and thus it is possible that specific inhibitors of these processes can be developed. The nucleoside analog, adenosine N_1-oxide has been reported to be a selective inhibitor of vaccinia virus replication and interferes with the translation of viral mRNA without affecting the translation of cellular messages (Kane and Shuman, 1995). The antibiotic, nigericin, has also been shown to inhibit early viral transcription as well as other steps in the replication cycle of vaccinia virus (Myskiw et al., 2010). Although the molecular targets of these compounds have not been defined, it possible that they specifically inhibit viral proteins involved in transcription and underscores the importance of this critical step in the viral replication cycle.

3.4. Transcriptional elongation and termination

Orthopoxviruses have developed specific mechanisms to regulate the elongation and termination of viral transcripts and this field has been reviewed previously (Broyles, 2003; Condit and Niles, 2002). Early transcripts possess homogeneous 3′ termini that are generated by a premature termination mechanism, which requires cis-acting sequences near the 3′ end of early transcriptional units, the capping enzyme, and a DNA dependent ATPase encoded by *D11L*. By contrast, the viral RNA polymerase reads through the termination signals in intermediate and late genes and produces long transcripts with heterogeneous 3′ ends. The essential DNA helicase encoded by the *A18R* gene is thought to be involved since null mutants yield aberrantly long transcripts, suggesting that it is an important transcript

release factor (Lackner and Condit, 2000). The product of the *G2R* gene is also important in the function of this process since viruses with mutations in this gene produce intermediate and late transcripts that are terminated prematurely and are uncharacteristically homogeneous (Black and Condit, 1996).

Thiosemicarbazones have been reported to exhibit good antiviral activity against vaccinia virus replication both in vitro and in vivo, but there has been limited clinical experience in humans with methisazone (marboran) (McLean, 2006), and the mechanism of action of these compounds is complex and incompletely understood (Bray, 2003). Nevertheless, the mechanism of methisazone is thought to involve transcription since drug resistant mutations map to the 132 kilodalton (kDa) subunit of the RNA polymerase encoded by *A24R* as well as additional subunits encoded by *J3R* and *H5R* (Cresawn and Condit, 2007). The compound can also complement recombinants with mutations in the essential *G2R* and *J3R* genes to minimize early termination (Latner et al., 2000; Meis and Condit, 1991). While the antiviral activity of methisazone suggests that this aspect of replication can be targeted for the development of antiviral therapies, this compound was not effective in preventing the mortality of mice infected with cowpox virus (Quenelle et al., 2006). Recently, a pyridopyrimidinone inhibitor of vaccinia virus replication was described that inhibits viral protein synthesis (Dower et al., 2011). Resistance to this compound maps to the large subunit of the RNA polymerase encoded by the *J6R* gene. Ethacrynic acid and aurintricarboxylic acid have also been reported to inhibit vaccinia virus transcription (Myskiw et al., 2007; Spisakova et al., 2009), but neither has been shown to be effective in vivo (Smee et al., 2010). It remains possible that new compounds might be identified that inhibit the transcriptional machinery in cells infected with vaccinia virus, and some of the existing agents might also prove to be useful in this regard, particularly if more effective analogs are identified (Pirrung et al., 2005).

3.5. Viral DNA synthesis and the DNA polymerase

The E9L DNA polymerase performs an essential role in the replication of viral DNA and its susceptibility to nucleoside and nucleotide analogs has made it the dominant target for the development of antiviral drugs (De Clercq, 2010b; Kern, 2003; Neyts and De Clercq, 2003). Genomes from virus cores give rise to replication centers where DNA synthesis takes place (Katsafanas and Moss, 2007). Vaccinia virus does not have an origin of replication that resembles those seen in other viruses, but rather has cis-acting sequences near the telomeres that are thought to initiate DNA synthesis through the nicking of extrahelical bases of the terminal hairpins (reviewed in (Traktman and Boyle, 2004)). Three viral proteins, I1L, I6L, and K4L have been shown to interact with the termini containing extrahelical bases and are important in this process (DeMasi et al., 2001). Once initiated, processive DNA synthesis requires viral genes from five complementation groups. These include the E9L DNA polymerase (Traktman et al., 1984), D5R nucleoside triphosphatase (Evans and Traktman, 1992), B1R protein kinase (Rempel and Traktman, 1992), D4R uracil DNA glycosylase (Stuart et al., 1993), and the A20R polymerase processivity factor (McDonald and Traktman, 1994). The E9L polymerase is a 1006 amino acid protein that possesses both DNA polymerase and proofreading activity within a single catalytically active polypeptide, that is also capable of processing recombination intermediates (Hamilton et al., 2007). The A20R processivity factor interacts with both the DNA polymerase and the D4R uracil DNA glycosylase. Together these three proteins comprise the holoenzyme (Klemperer et al., 2001; Stanitsa et al., 2006). Although D4R can excise uracil from viral DNA this does not appear to be its essential function (De Silva and Moss, 2003). Its critical function in DNA synthesis is more likely related to its role in the tethering of the holoenzyme to the DNA substrate to promote processive DNA synthesis (Boyle et al., 2011). The A20R processivity factor also plays a critical role in conferring processivity to the holoenzymes and appears to be

important in the recruitment of other molecules to the replication complex including H5R and D5R (Ishii and Moss, 2002). The function of the B1 protein kinase in DNA replication is incompletely understood, however it has been shown to phosphorylate the cellular BAF protein to help it overcome this innate defense to viral infection (Wiebe and Traktman, 2007). The function of D5R in DNA synthesis is as yet unclear, but its fast stop phenotype might be related to its ability to multimerize and hydrolyze ATP (Boyle et al., 2007).

A host of compounds are known to target viral DNA synthesis and are good inhibitors of viral replication. While most are nucleoside analogs and their metabolites inhibit the viral DNA polymerase directly, a few have been shown to target other proteins in the DNA replication complex. Inhibitors of D5R activity include both siRNAs (Vigne et al., 2008), as well as small peptide aptamers (Saccucci et al., 2009). Inhibitors that disrupt the interaction between D4R and A20R have also been identified and have been reported to inhibit viral replication (Schormann et al., 2011). Phosphonoacetic acid was also shown to be effective in an animal model of vaccinia virus infection and presumable inhibits the DNA polymerase directly (Smee et al., 2011).

Nucleoside analogs generally require an initial phosphorylation step catalyzed by the vaccinia virus thymidine kinase (TK) homolog and includes 4′-thioidoxuridine (Prichard and Kern, 2010) (Fig. 1), and 5 substituted thymidine analogs (Fan et al., 2006). Another nucleoside analog, N-methanocarbathymidine (N-MCT), is also a good inhibitor of vaccinia virus replication both in vitro and in vivo and is an excellent inhibitor of viral DNA synthesis (Prichard et al., 2006; Smee et al., 2007a). Although this compound is clearly phosphorylated by the vaccinia virus TK, only the monophosphate metabolite is detected and its ultimate molecular target is unknown.

Acyclic nucleoside phosphonate analogs do not require an initial phosphorylation by viral kinases but their metabolites are still excellent inhibitors of the DNA polymerase and this field has been reviewed previously (De Clercq and Neyts, 2004; Kern, 2003). Examples include cidofovir (De Clercq, 2003) and its prodrug CMX001 (Hostetler, 2009; Lanier et al., 2010), acyclic nucleotide analogs such as the 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines (De Clercq et al., 2005), alkoxyalkyl derivatives of 9-(S)-(3-hydroxy-2-phosphonomethoxypropyl)adenine (Beadle et al., 2006), and [(R)-2,4-diamino-6-[3-hydroxy-2-(phosphonomethoxy)propoxy]pyrimidine (HPMPO-DAPy) (Balzarini et al., 2004; De Clercq et al., 2005; Stittelaar et al., 2006). Similarly, cycloSaligenylmonophosphate (cycloSal-MP) derivatives of acyclovir (ACV), penciclovir and brivudin (BVDU) are also active and are not dependent on the viral TK (Sauerbrei et al., 2005).

Resistance to CDV has been characterized to help understand its potential significance during therapy and the mutations map to the DNA polymerase ORF (E9L). CDV diphosphate is incorporated into viral DNA and alters its conformation such that it reduces the efficiency of subsequent DNA synthesis and is completely resistant to excision by the 3′-to-5′ exonuclease activity of the polymerase (Julien et al., 2011; Magee et al., 2005). Its incorporation in the template strand also interferes with further chain elongation (Magee et al., 2008). Mutations in either the exonuclease domain (A314T,V, deletion of K174), or the polymerase catalytic domain (A684 V) are sufficient to confer resistance to the drug, as well as related nucleoside phosphonate analogs (Andrei et al., 2006; Becker et al., 2008). Additional mutations including S851Y also impart resistance to CDV and related analogs in a complex pattern of resistance (Gammon et al., 2008). Importantly, viruses containing these mutations exhibit reduced virulence in mice and suggest that they may compromise the replicative fitness in vivo (Andrei et al., 2006; Gammon et al., 2008; Kornbluth et al., 2006). Most significantly, CDV retained some ability to protect animals from mortality from the

resistant strains and the data taken together suggest that resistance to these agents may not be a significant problem during therapy.

3.6. Enzymes involved in the replication cycle

There are other viral enzymes in addition to the DNA polymerase that have well defined biochemical activities. These enzymes have been reported to provide nucleotides for viral DNA synthesis, modify genomic DNA, mRNA, or protein substrates. Many of these enzymes perform essential functions and have the potential to be exploited as targets for antiviral chemotherapy. Since common biochemical approaches can be used in the identification of new inhibitors, they warrant discussion as a group.

3.6.1. Nucleoside kinases—Vaccinia virus encodes two homologs of nucleoside kinases that are presumed to provide precursors for viral DNA synthesis. Unlike acyclic nucleoside phosphonates, nucleoside analogs generally require phosphorylation to the level of the triphosphate for their antiviral activity. This requirement for phosphorylation can be circumvented by prodrug strategies that deliver monophosphate metabolites of acyclovir, penciclovir or brivudin to cells that are further phosphorylated and are good inhibitors of viral replication (Sauerbrei et al., 2005; Sauerbrei et al., 2006). But many analogs require phosphorylation by vaccinia virus kinases such that an understanding of the substrate specificity of these enzymes is critical to the development of this class of nucleoside analog inhibitors.

Two viral kinases possess nucleoside or nucleotide kinase activity. The nonessential J2R TK is a type II enzyme that is homologous to the cellular cytosolic TK1 (Black and Hruby, 1992). Members of this class are active as homotetramers (Hruby and Ball, 1982), allosterically controlled by both dTTP and dTDP (Black and Hruby, 1992), and exhibit a narrow substrate specificity limited to thymidine and a few closely related analogs. The crystal structure of this enzyme revealed differences in the binding site of the enzyme indicating that its substrate specificity may be distinct from that of the cellular homolog (El Omari et al., 2006). Indeed studies with thymidine analogs also suggest that the enzyme can phosphorylate a wider array of nucleoside analogs than the cellular enzyme and thus may be used to help identify selective inhibitors of viral replication (Prichard et al., 2007; Prichard and Kern, 2010). The phosphorylation of thymidine monophosphate to the level of the diphosphate is catalyzed by the A48R thymidylate kinase. This enzyme is catalytically active and shares many characteristics of the cellular TMP kinase including the phosphorylation of TMP and dUMP (Topalis et al., 2005). It is unusual in that it also phosphorylates dGMP as well as 5′ halogenated deoxyuridine monophosphate analogs (Auvynet et al., 2009).

Strategies that utilize the substrate specificity of these viral kinases are currently being investigated and it may be possible to develop highly potent compounds that are only activated in infected cells. Idoxuridine has been shown to reduce mortality of mice infected with vaccinia virus and its phosphorylation by J2R is clearly important in its mechanism of action (Neyts et al., 2002). A related analog, 5-iodo-4′-thio-2′-deoxyuridine (4′-thioIDU) is also phosphorylated by this enzyme and is active against vaccinia and cowpox viruses in vitro and in vivo (Kern et al., 2009). This molecule is further phosphorylated to the level of the triphosphate and incorporated into viral DNA by the viral DNA polymerase within virus factories (Prichard and Kern, 2010). A number of novel 5-substituted thymidine analogs have also been shown to be phosphorylated by J2R, but not the cellular kinase and are potent inhibitors of orthopoxvirus replication in vitro (Fan et al., 2006; Prichard et al., 2007). Another compound, N-MCT, is also selectively phosphorylated by J2R (Prichard et al., 2006), and exhibits good activity against vaccinia virus both in vitro and in vivo (Prichard et

al., 2006; Smee et al., 2007a,b). Further studies should result in the identification of additional molecules that acquire specificity through the specific phosphorylation by these viral kinases.

3.6.2. Ribonucleotide reductase—The heterodimeric ribonucleotide reductase encoded by F4L and I4L ORFs catalyzes the conversion of ribonucleotides to the corresponding deoxyribonucleotides at the level of the diphosphate and is a critical step in the synthesis of deoxyribonucleoside triphosphates to support DNA replication in the cytoplasm. The large subunit of this enzyme, F4L, is not required for viral replication in cell culture although its deletion impairs replication in vivo (Child et al., 1990). The small subunit was recently shown to interact directly with the large subunit of the host ribonucleotide reductase presumably to modify the catalytic properties of the enzyme to support viral replication (Gammon et al., 2010). Deletion of the small subunit also renders the virus hypersensitive to hydroxyurea and CDV and is consistent with its role in a catalytically active enzyme. The inhibition of viral replication by hydroxyurea also suggests that ribonucleotide reductase activity is important for efficient viral replication in vitro and indicates that targeting this enzyme might be a viable strategy, particularly if more selective and less toxic inhibitors could be identified.

3.6.3. Protein kinases and phosphatases—Vaccinia virus encodes two protein kinases that are required for viral replication. The activity of the B1R kinase (VPK1) has kinase activity that has been well characterized (Rempel and Traktman, 1992), and the H5R product is one of the many natural substrates of this enzyme (Brown et al., 2000). Both H5R and B1R co-localize as punctate sites in the cytoplasm that are precursors to sites of viral DNA synthesis (Domi and Beaud, 2000). Cellular proteins are also phosphorylated by this kinase including p53, which results in the ubiquitination and degradation of p53 (Santos et al., 2004), and other cellular signaling pathways related to cellular stress (Santos et al., 2006). The B1R kinase also inhibits CD1d-mediated antigen presentation and antagonizes an important innate immune response (Webb et al., 2006). It is not yet clear which, if any, of these activities are responsible for its essential role in viral DNA synthesis.

The F10L protein kinase (VPK2) is required for viral replication and is the major kinase encapsidated in virions (Lin and Broyles, 1994). This serine/threonine kinase predominantly phosphorylates serine residues, but also appears to be involved in the phosphorylation of tyrosine residues on the A17L protein, which is one of the natural substrates for this kinase (Derrien et al., 1999). F10L is required for early stages of morphogenesis where it associates with intracellular membranes and is involved with the formation of immature virions (Punjabi and Traktman, 2005). The F10L dependent phosphorylation of A30L and G7L has been described and provides a reasonable explanation why all three proteins are required for the early stages of morphogenesis (Mercer and Traktman, 2005).

The H1L protein phosphatase dephosphorylates phosphotyrosines and phosphothreonines and homologs of this phosphatase are present in each of the orthopoxviruses (Guan et al., 1992; Hakes et al., 1993; Rosel et al., 1986). Its function is thought to be essential because recombinant viruses with repressible expression of H1L replicate poorly (Liu et al., 1995). Dimers of H1L induce the dephosphorylation of STAT1 that promotes the inhibition of interferon-gamma signal transduction and inhibits viral replication (Koksal and Cingolani, 2011; Mann et al., 2008).

Specific inhibitors of B1R and F10L have the potential to inhibit viral replication. Although such a strategy has proven to be effective against human cytomegalovirus, no specific inhibitors for the vaccinia virus kinases have been reported. However, one inhibitor of cellular tyrosine kinases, imatinib mesylate, has been reported to impair the release of cell-

associated enveloped virions by inhibiting Abl- and Src-family tyrosine kinases (Reeves et al., 2005). This strategy has also been reported to be effective against the virus in a murine model of vaccinia virus infection (Reeves et al., 2011). Aurintricarboxylic acid has been reported to inhibit the replication of vaccinia virus and it also inhibits the enzymatic activity of the H1L phosphatase (Myskiw et al., 2007). It is unclear if these two activities are related, but the activity of this compound did not translate into animal models of infection (Smee et al., 2010). The inhibition of B1R and G7L expression by siRNA has also been reported that results in significant reductions in the replication of vaccinia virus (Vigne et al., 2009).

3.7. Resolution and packaging of genomic DNA

Orthopoxviruses synthesize considerable quantities of genomic DNA as a mass of intertwined and branched concatemers that are subsequently resolved into unit length genomes in a process that requires at least five viral gene products. Holliday junctions are resolved by the product of the A22R gene that yields linear duplex products (Garcia and Moss, 2001). This gene also resolves a number of related intermediates including Y junctions and single stranded DNA flaps (Culyba et al., 2007). These structures are also affected by K4L, but this activity is not required for viral infection (Eckert et al., 2005). The A50R DNA ligase is also required for the resolution of replication intermediates and serves to recruit the host topoisomerase II from the nucleus to virus factories in the cytoplasm. The viral topoisomerase might also be expected to participate in this process (Tian and Shuman, 2007), but a potentially more important function is to promote early gene transcription within the confines of virus cores (Da Fonseca and Moss, 2003). Three viral proteins I1L, I6L, and K4L have been identified that bind to the telomeric region of the genome (DeMasi et al., 2001). A temperature sensitive mutant for one of these (I6L) replicates DNA normally and correctly processed viral genomes, however, DNA failed to enter immature virions and proteolytic cleavage of the structural proteins was defective, suggesting that it is required for the encapsidation of viral DNA (Grubisha and Traktman, 2003).

Viral proteins involved in the maturation and packaging of viral genomes provide excellent targets for the development of small molecule inhibitors and such strategies have proven to be highly effective for the herpesviruses. Although the virus topoisomerase is not strictly required for vaccinia virus replication its function appears to be important since some quinolone analogs have been reported to inhibit its enzymatic activity (Kamau and Grove, 2004), and related analogs have been shown to inhibit viral replication (Sekiguchi and Shuman, 1997b). Additional specific inhibitors of the viral topoisomerase enzymatic activity have been identified but their antiviral activity has not been reported (Bond et al., 2006; Fujimoto et al., 2006; Yakovleva et al., 2004). The antineoplastic agent, mitoxantrone, was also reported to inhibit the replication process at the stage of virion assembly and mutations mapped to the DNA ligase (Deng et al., 2007). Although this compound was not shown to be effective in vivo, it suggests that inhibitors of the ligase warrant further investigation.

3.8. Virion assembly

Orthopoxvirus virions are extraordinarily complex structures and their assembly was the subject of excellent reviews (Condit, 2007; Roberts and Smith, 2008). Virions form within virus factories in a process that initiates with the formation of crescents consisting of trimers of the D13L scaffold protein on the convex surface that is thought to stabilize the lipid membrane on the concave surface (Szajner et al., 2005). The source of the membrane is uncertain, but its formation requires L2R (Maruri-Avidal et al., 2011). The F10L protein is also part of an assembly complex containing A10L, A11R, A15L, A30L, A32L, D2L, D3R, G7L, and J1R, which are among the most abundant of the approximately 70 constituent core proteins (Resch et al., 2005; Szajner et al., 2003, Szajner et al., 2004). The scaffold and

membrane complex proceeds to enclose aggregates of core proteins to yield immature virions into which genomic DNA is packaged.

Immature virions subsequently undergo maturational events that lead to the condensation of virus cores and involves proteolysis, the loss of the scaffold, and the formation of new disulfide linkages catalyzed by the viral redox system (Senkevich et al., 2002). Core proteins I1L, F17R, A4L, A3R, and A13L are required for this critical transition, yet their precise functions have not yet been elucidated. Proteolytic events catalyzed by I7L protease (Byrd et al., 2002), and the G1L metalloprotease (Ansarah-Sobrinho and Moss, 2004), are both essential for the formation of mature virions and their substrates have been reviewed previously (Byrd and Hruby, 2006).

The complexity of virion morphogenesis presents a host of interesting targets, and specific inhibitors for some of these proteins have been reported. Rifampicin inhibits the formation of crescents and drug resistant mutations map to D13L (Charity et al., 2007). Infected cells treated with CDV have also been reported to exhibit an abnormal condensation of nascent DNA that results in packaging defects yet these appear to be a consequence of defects in the DNA rather than the specific inhibition of viral proteins involved in this process (Jesus et al., 2009). The viral proteases are obvious targets for the development of specific inhibitors of vaccinia virus and experience with HIV illustrates the tremendous potential of protease inhibitors. Specific inhibitors of I7L activity, such as TTP-6171, have been described and continued efforts will likely yield a number of candidate compounds that have potential for the treatment of orthopoxvirus infections (Byrd et al., 2004; Katritch et al., 2007).

3.9. Secondary envelopment and egress

Following maturation, IMVs are actively transported out of the virus factory by a mechanism that requires A27L, which is also required for the stage of secondary envelopment (Sanderson et al., 2000). A subset of IMVs are enwrapped in a secondary double-layer membrane via mechanisms that have been reviewed previously (Roberts and Smith, 2008; Smith et al., 2002). The membrane surrounding intracellular enveloped virions (IEV) is likely derived from late endosomes (Chen et al., 2009). Required for this process are the viral membrane protein A27L (Sanderson et al., 2000), B5R (Wolffe et al., 1993), E2L (Domi et al., 2008), and most notably, F13L (Blasco and Moss, 1991). The secondary membranes that surround the resulting IEVs contain additional viral envelope proteins F13L, A33R, A34R, and B5R that are not found in IMVs and confer critical biological properties to EEVs (Perdiguero et al., 2008).

Both IMVs and IEVs are actively transportedto the cell surface by processes that have been reviewed previously (Smith and Law, 2004). Release of most IMVs occurs following the lysis of infected cells, but they can also be released by budding from the cellular membrane (Meiser et al., 2003). Active transport of IEV on microtubules requires the F12L protein which has kinesin motifs required for active transport (Morgan et al., 2010). Once an IEV reaches the cell surface, its outermost membrane fuses to the plasma membrane by exocytosis and results in the formation of a cell-associated enveloped virion (CEV). Clathrin mediated endocytosis is then used by the virus to recycle B5R and F13L (Husain and Moss, 2003). At the cell surface, the B5R membrane protein on CEVs activates Src, which in turns phosphorylates A36L and promotes the formation of actin tails at the base of the virions (Newsome et al., 2004). The formation of actin tails at the cell surface helps to promote cell to cell spread of the virus and is important for the formation of large plaques in cell culture (Smith et al., 2002). The ratio of CEV to EEV particles is virus strain specific with the IHD-J strain releasing a much higher proportion of CEV than does the Western Reserve strain (Blasco et al., 1993).

The formation of EEVs involves many viral genes and presents opportunities for the development of specific inhibitors. Inhibition of secondary envelopment was initially noted with N1-isonicotinoyl-N2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) that resulted in impaired EEV formation and an accumulation of IMV in the cytoplasm (Hiller et al., 1981). Mutations that confer resistance to this drug map to the major EEV envelope protein F13L (p37) and confirmed that this protein is important in viral replication (Schmutz et al., 1991). F13L is a palmitoylated membrane protein (Grosenbach et al., 1997) that associates with post-Golgi vesicles and recruits B5R and A36R to this location (Husain and Moss, 2002). The essential activities of F13L require palmitoylation sites, a catalytically active phospholipase D motif, as well as a YPPL motif (Honeychurch et al., 2007). More recently, ST-246 was described as a specific inhibitor of F13L and an excellent inhibitor of viral replication (Yang et al., 2005). The mechanism of action of ST-246 appears to be similar to IMCBH in that drug resistant mutations mapped to F13L, however ST-246 was very active in animal models of vaccinia, cowpox, ectromelia, monkeypox, and variola virus infections (Jordan et al., 2010). This mechanism of action confirmed that the formation of EEV was important in the pathogenesis of orthopoxviruses and is consistent with genetic data that indicated that the inhibition of EEV production was sufficient to limit the spread of infection in vivo and prevent mortality (Jordan et al., 2010; Payne and Kristensson, 1985).

Egress and cell to cell spread might also be targeted through the inhibition of Src and related kinases which are required for actin based motility at the cell surface. The inhibitor PP1 (Frischknecht et al., 1999), terameprocol (Pollara et al., 2010), and the Src kinase inhibitor, Gleevec (imatinib), have all been shown to interfere with actin dependent motility (Reeves et al., 2005). The activity of imatinib has also been reported to be effective in mice infected with vaccinia virus (Reeves et al., 2011).

4. Summary

Recent advances have improved significantly our understanding of the mechanisms involved in the replication of orthopoxviruses. An appreciation of the function of viral proteins and the molecular details of their activity highlights opportunities to identify new inhibitors. This review summarized each of the stages of viral replication, noted the viral proteins that perform critical functions, and identified specific inhibitors that have been reported to affect these processes. While a large number of compounds covered in this review have been shown to specifically inhibit viral enzymes or viral replication in cell culture, it is critical at this stage to distinguish among existing molecules that are inhibitors of viral replication, those that warrant further development, and those that have sufficient activity in vivo and toxicity profiles to warrant further development in clinical studies.

The mechanism of action of existing inhibitors has been discussed and the molecular targets for many of these compounds have been ascribed to viral targets by genetic and biochemical means (Table 1). It is possible that some inhibitors could be developed further as antiviral drugs, particularly those that have been approved for other indications and have already been shown to exhibit some degree of efficacy in animal models (imatinib, rifampicin). However, their minimal efficacy and toxicity in vivo suggests that more potent and less toxic analogs need to be developed and their activity requires confirmation against variola virus and monkeypox virus. Nevertheless, many of the compounds hold promise and should be pursued further, particularly if their mechanism of action is novel.

A large number of inhibitors are listed in Table 1, but at this time, CMX001 and ST-246 appear to have the greatest potential to be approved for the treatment of orthopoxvirus infections and their development has been recently reviewed (Jordan et al., 2009; Lanier et al., 2010). Both of these compounds offer significant advantages over CDV, which has

already been approved for the emergency treatment of smallpox infections. Chief among them, are their superior safety profile, activity against all the orthopoxviruses, good oral bioavailability, and efficacy in animal models. The fact that these drugs target different steps in the viral replication cycle is also important and suggests that a combination of these drugs might offer improved efficacy that has already been documented in cell culture and in animal models of infection (Chen et al., 2011; Quenelle et al., 2007b). It is critical to consider that while these new drugs hold promise, neither CMX001, nor ST-246 has been approved for use in humans. The further development of backup drugs with novel mechanisms will help ensure that effective therapies are available in the event of an outbreak. This can be accomplished by continuing the development of new drugs specifically targeting the orthopoxviruses, and also identifying drugs with activity against other viruses that also exhibit activity against the orthopoxviruses. Indeed the development of broad spectrum agents against the DNA viruses has the potential to provide additional therapies in a cost effective manner. Both these strategies demand the continued assessment of antiviral activity against the orthopoxviruses.

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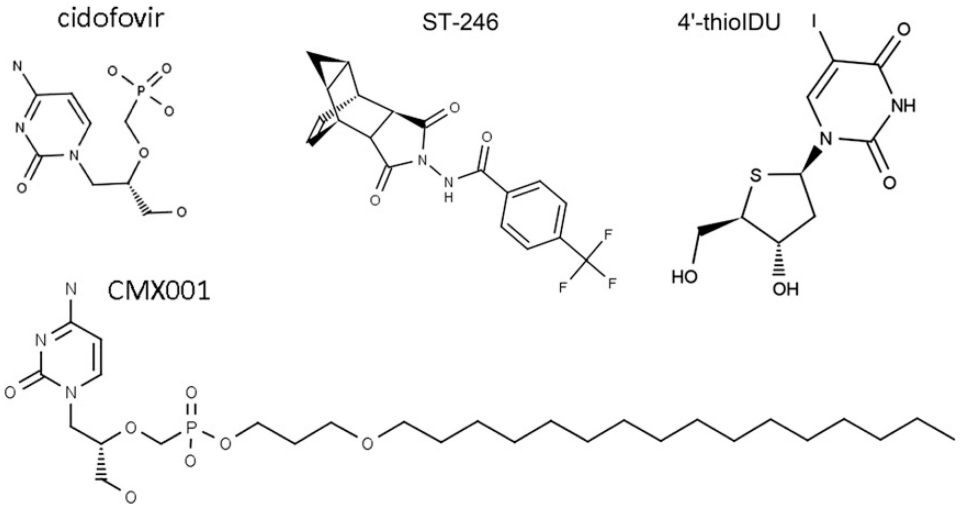

Fig. 1.
Chemical structure of selected inhibitors of orthopoxvirus replication.

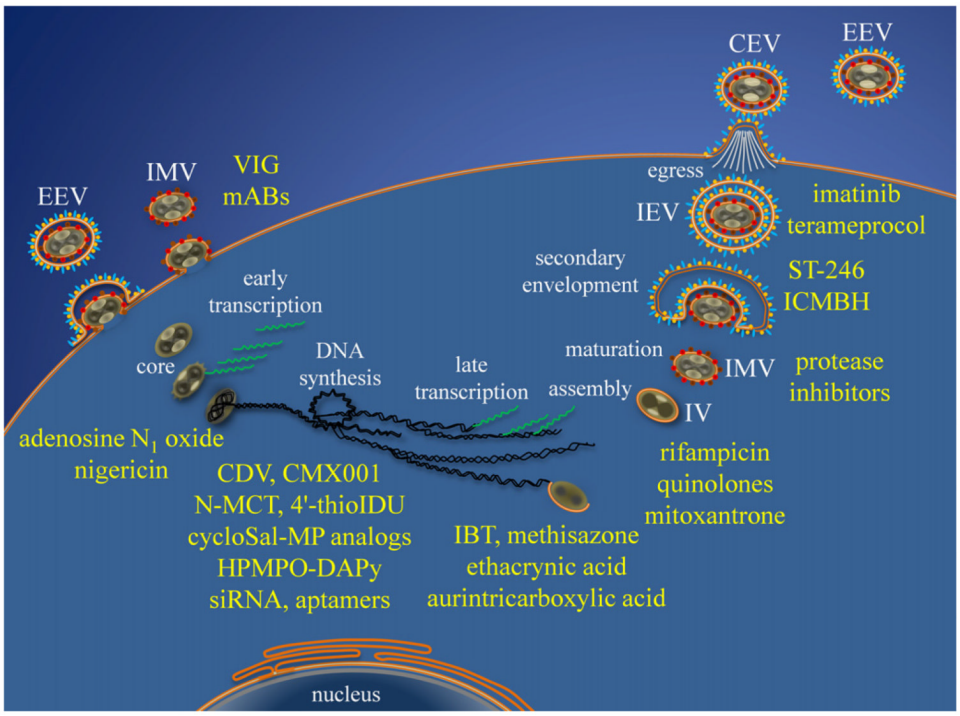

Fig. 2.

Selected inhibitors of orthopoxvirus replication. The major stages of vaccinia virus replication are represented as indicated by the white text. Representative compounds that interfere with each of the steps are shown in yellow below the stage of replication that they inhibit. Extracellular enveloped virions (EEV), intracellular mature virions (IMV), immature virions (IV), intracellular enveloped virions (IEV), and cell associated enveloped virions (CEV) are also depicted.

Table 1

Inhibitors with defined targets in vaccinia virus.

Inhibitor	Target ORFs	Protein ID	Function	References
Hydroxyurea	F4L (VACWR043)	AAO89322.1	Ribonucleotide reductase subunits	Slabaugh and Mathews (1986)
	I4L (VACWR073)[a,c]	AAO89352.1		
CMX001	E9L (VACWR065)[a]	AAO89344.1	DNA polymerase	Gammon et al. (2008), Kornbluth et al. (2006)
Cidofovir (S)-HPMPDAP				
Mitoxantrone	A50R (VACWR176)[a]	AAO89455.1	DNA ligase	Deng et al. (2007)
Hydroxyurea	F4L (VACWR043)	AAO89322.1	Ribonucleotide reductase subunits	Slabaugh and Mathews (1986)
	I4L (VACWR073)[a,c]	AAO89352.1		
IBT, methisazone[a,a]	G2R (VACWR080)[a]	AAO89359.1	Late transcription factor	Condit et al. (1991), Cresawn et al. (2007); Latner et al. (2000), Meis and Condit (1991), Dower et al. (2011)
	J3R (VACWR095)[a,b]	AAO89374.1	Poly(A) polymerase Subunit	
	J6R (VACWR095)[a]	YP_232980		
	A24R (VACWR144)[a]	AAO89423.1	Largest RNA polymerase subunit	
			RNA polymerase subunit	
Rifampicin	D13L (VACWR118)[a]	AAO89397.1	Virus assembly, scaffold protein	Charity et al. (2007)
ST-246 IMCBH	F13L (VACWR052)[a]	AAO89331.1	Secondary envelopment and EEV formation	Schmutz et al. (1991), Yang et al. (2005)
VIG	B5R (VACWR187)[a]	AAO89466.1	EEV type-I membrane glycoprotein;	Bell et al. (2004)
Aurintricarboxylic acid	H1L (VACWR099)	YP_232981.1	Tyr/ser phosphatase	Myskiw et al. (2007)
Ofloxacin	H6R (VACWR104)[c]	AAO89383.1	Topoisomerase I	Bond et al. (2006), Kamau and Grove (2004)
Novobiocin (other small molecules)				
TTP-6171	I7L (VACWR076)[c]	AAO89355.1	Protease	Byrd et al. (2004)
siRNA	B1R (VACWR183)[e]	AAA48194	Protein kinase	Vigne et al. (2009)
siRNA	D5R (VACWR110)[e]	AAA48102	NTPase	Vigne et al. (2008)
(N) MCT	J2R (VACWR094)[d]	AAO89373.1	Thymidine kinase	Kern et al. (2009), Prichard et al. (2007), Prichard et al. (2006), Smee et al. (2008)
4′-thioIDU 5-substituted deoxyuridine analogs				

[a]Mapped by drug resistance.

[b] Drug dependence.

[c] Identified in enzymatic assay.

[d] TK dependence, enyme assays, final targets undefined, possibly E9L DNA polymerase.

[e] Specific reduction of target mRNA levels.

Table 2

Potential targets for the development of drugs to treat orthopoxvirus infections.

Stage of replication	ORF	Function	References
DNA processing and packaging	(A18R)	DNA helicase	Lackner and Condit (2000)
	(A22R, K4L)	Holliday junction resolvase	Garcia et al. (2006), Culyba (2007) #3819
	(A50R)	DNA ligase	Sekiguchi and Shuman (1997a)
	(H6R)	Topoisomerase type IB	Da Fonseca and Moss (2003), Tian and Shuman (2007)
	(I1L, I6L, K4L)	Telomere binding proteins	DeMasi et al. (2001)
DNA replication	(A20R, D4R)	Polymerase processivity factor; Uracil DNA glycosylase	Boyle et al. (2011), Stanitsa et al. (2006)
	(D5R)	NTPase interacts with A20R	Boyle et al. (2007)
	(E9L)	DNA polymerase	Traktman and Boyle (2004)
	(H5R)	Substrate of B1R kinase;	Santos et al. (2006)
	(I3L)	ssDNA-binding phosphoprotein	Domi and Beaud (2000)
Enzymatic targets	(A48R)	Thymidylate kinase	Caillat et al. (2008)
	(B1R)	ser/thr kinase	Santos et al. (2006)
	(F4L, I4L)	Ribonucleotide reductase subunits	Gammon et al. (2010)
	(F10L)	Essential ser/thr kinase	Punjabi and Traktman (2005)
	(H1L)	tyr/ser protein phosphatase	Koksal and Cingolani (2011), Mann et al. (2008)
	(I7L,G1L)	Essential viral proteinases	Ansarah-Sobrinho and Moss (2004), Byrd and Hruby (2006)
	(J2R)	Thymidine kinase	Black and Hruby (1992), El Omari et al. (2006)
Entry and uncoating	(A16L, A17L, A21L, A26L, A27L, A28L, B5R, D8L, F9L, G3L, G9R, H2R, H3L, J5L, L1R, and L5R	Structural and membrane proteins	Brown et al. (2006), Ojeda et al. (2006), Senkevich et al. (2005)
Morphogenesis	(A10L, A15L, A30L, D2L, D3R, F10L, F13L, G7L, J1R)	Assembly complex	Condit et al. (2006), Szajner et al. (2004)
	(A11R;A32L)	Nonstructural proteins	Cassetti et al. (1998), Resch et al. (2005)
	(D13L)	Rifampicin resistance protein	Charity et al. (2007), Zhang and Moss (1992)
	(H3L)	IMV maturation	da Fonseca et al. (2000)
	(L1R)	IMV membrane protein	Brown et al. (2006)
Transcription and mRNA processing	(A24R, A29L, A5R, D7R, E4L, G5.5R, H4L, J4R, J6R)	RNA polymerase subunits	Broyles (2003), Yoder et al. (2006)
	(D11L)	NPH-I	Deng and Shuman (1998)
	(D12L, D1R)	mRNA capping enzyme	Schwer and Shuman (2006)
	(E1L, J3L)	Poly(A) polymerase VP55 and V39	Moure et al. (2006)
	(G2R)	Late transcription elongation factor	Cresawn and Condit (2007)
	(H4L)	RNA polymerase associated protein (RAP94)	Mohamed et al. (2001), Yang and Moss (2009)

Stage of replication	ORF	Function	References
Formation of EEV	(A27L)	IMV surface protein	Lawrence et al. (2007), Sanderson et al. (2000)
	(A36R)	IEV transmembrane protein	Newsome et al. (2004), Ward et al. (2003)
	(B5R)	EEV membrane glycoprotein	Aldaz-Carroll et al. (2007)
	(A33R)	Actin tail formation	Perdiguero and Blasco (2006)
	(F13L)	EEV formation, target of ST-246	Honeychurch et al. (2007)
	(A34R)	Actin tail formation	Law et al. (2006)