DDR

Research Overview

Viral Proteinases: Targets of Opportunity

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ABSTRACT   During antiviral drug development, any essential stage of the viral life cycle can serve as a potential drug target. Since most viruses encode specific proteases whose cleavage activity is required for viral replication, and whose structure and activity are unique to the virus and not the host cell, these enzymes make excellent targets for drug development. Success using this approach has been demonstrated with the plethora of protease inhibitors approved for use against HIV. This discussion is designed to review the field of antiviral drug development, focusing on the search for protease inhibitors, while highlighting some of the challenges encountered along the way. Protease inhibitor drug discovery efforts highlighting progress made with HIV, HCV, HRV, and vaccinia virus as a model system are included. Drug Dev. Res. 67:501–510, 2006.   © 2006 Wiley-Liss, Inc.

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INTRODUCTION

Traditional antiviral strategies have relied on the use of vaccines to prevent viral infection or the use of rest and supportive therapy when a person did become infected since specific antiviral agents were not available. However, there are many virus infections for which a vaccine is not available or for which vaccine development may be problematic due to the virus having many serotypes, so the discovery of antiviral drugs has become a high priority. Initially, the discovery of antiviral drugs was based on screening large chemical libraries for compounds that would inhibit viral replication, and this effort met with relatively little success. The development of new antiviral agents is now making rapid progress due in large part to the recent advances in research on specific virus families. These advances include the advent of sequencing of entire viral genomes, discovery of permissive cell lines, replicon systems, and pseudotype viruses, as well as improvements in rational drug design and combinatorial chemistry. Until the 1980s, amantadine was the only approved antiviral drug, authorized by the FDA for the treatment of influenza A in 1966 (http://www.fda.gov/cder/drug). However, the past 20 years have seen the development of new antiviral agents targeting various stages of the viral life cycle for a number of viruses including human immunodeficiency virus (HIV), hepatitis B and C viruses, herpes simplex virus (HSV), cytomegalovirus (CMV), varicella-zoster virus (VZV), influenza A and B viruses, and respiratory syncytial virus (RSV) (Table 1). Additional antiviral drug examples include the use of interferon for human papilloma virus (HPV) [Cantell, 1995].

Antivirals are most commonly used against active viral disease. However, prophylactic use is becoming

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TABLE 1. Antiviral Agents Approved for Use in the United States

Stage of virus life cycle	Antiviral agent	Approved	Target viruses
Entry and uncoating	Enfuvirtide	2003	HIV
	Amantadine	1966	Influenza A
	Rimantadine	1993	Influenza A
Replication	Azidothymidine (AZT)	1987	HIV
	Didanosine	1991	HIV
	Zalcitabine	1992	HIV
	Stavudine	1994	HIV
	Lamivudine	1995	HIV. HBV
	Nevirapine	1996	HIV
	Delavirdine	1997	HIV
	Abacavir	1998	HIV
	Efavirenz	1998	HIV
	Tenofovir	2001	HIV
	Adefovirn dipivoxil	2002	HBV
	Emtricitabine	2003	HIV
	Acyclovir	1997	HSV, VZV
	Foscarnet	2005	CMV, HSV, VZV
	Ganciclovir	2003	HSV, CMV
	Penciclovir	1996	HSV
	Trifluridine	1995	HSV
	Valacyclovir	1995	HSV, VZV
	Cidofovir	1996	CMV
	Ribavirin	1980	RSV, HCV, Lassa
Protease inhibitors	Saquinavir	1995	HIV
	Ritonavir	1996	HIV
	Indinavir	1996	HIV
	Nelfinavir	1997	HIV
	Amprenavir	1999	HIV
	Lopinavir	2000	HIV
	Atazanavir	2003	HIV
	Tipranavir	2005	HIV
Release	Oseltamivir	1999	Influenza A, B
	Zanamivir	1999	Influenza A, B

increasingly recognized as a viable strategy especially for those at risk of contracting infection such as immunocompromised individuals, organ transplant recipients, elderly patients, those in a "hot zone" near an outbreak, military personnel, and travelers to areas where the virus may be endemic.

One of the main challenges to the development of a successful antiviral drug is specificity, to identify a target that is specific to the virus and not to the host cell. Common stages of the virus life cycle to target are attachment and entry, replication, assembly, and release. Regardless of the stage of the life cycle targeted, the drug must be more toxic to the virus than to the host cell. To date, the majority of the approved antiviral agents are nucleoside analogs that inhibit viral DNA synthesis. While many of these drugs are highly effective, their continued use leads to the

emergence of drug-resistant viral strains, so the development of new antiviral agents with a different virus-specific target is necessary. Entry inhibitors are a promising class of antivirals with several drugs approved for use in humans, such as enfuvirtide for HIV [Burton, 2003; Robertson, 2003] and amantadine for influenza A [Nahata, 1987]. However, susceptibility and the development of resistance may still be a significant hurdle to overcome. For example, in the case of HIV, Env is the most variable HIV protein [Weiss et al., 1986; Yasunaga et al., 1986], and the susceptibility of each virus strain to specific entry inhibitors may vary. It is possible that the development of mutations leading to resistance to some entry inhibitors may affect viral tropism and pathogenicity. Viral enzymes that are essential for the production of infectious progeny virus are an attractive target and recent advances in molecular biology, structural biology, computational biology, and biochemistry are making protease inhibitors an attractive viral target. While there is usually some variability in the coding region of proteases, the active-site region is usually very highly conserved, making it likely that a drug that targets the active site of a protease will inhibit all serotypes, while a drug targeting a less highly conserved viral protein would have more variability. This has been demonstrated with the success of rupintrivir, a novel inhibitor of the 3C protease of human rhinovirus (HRV), in being effective against each of the picornaviruses that were tested, including almost 100 HRV and human enterovirus (HEV) serotypes [Binford et al., 2005].

PROTEASES

Studies during the last 20 years have shown that viral proteases, enzymes that selectively cleave polypeptide bonds, are absolutely essential during the life cycle of many viruses [Dougherty and Semler, 1993; Kay and Dunn, 1990; Krausslich and Wimmer, 1988]. Proteases can be either peptidases, which cleave single amino acids from the end of a peptide chain, or proteinases, which cleave peptide bonds within a substrate [Polgar, 1989]. Viral proteinases function either to cleave high-molecular-weight viral polypeptides into functional protein products during formative proteolysis or to cleave structural proteins necessary for assembly during morphogenic proteolysis. Proteinases can be further divided into four separate categories (serine, cysteine, aspartic, and metalloproteinases) based on the identity of their catalytic residues, the mechanism of catalysis, and their substrate specificity. For a general review of each type of proteinase, see Dougherty and Semler [1993] and Barrett et al. [2004]. Serine proteinases have a catalytic triad composed of

serine, histidine, and aspartic acid and usually have an accompanying oxyanion hole intermediate. The active serine hydroxyl carries out a nucleophilic attack of the carbonyl group of the amide bond. Serine proteases can be categorized based on substrate specificity as being trypsin-like, chymotrypsin-like, or elastase-like. Cysteine proteinases are similar in amide bond hydrolysis to serine proteases, but have a catalytic triad composed of cysteine, histidine, and either asparagine or aspartic acid. Cysteine proteinases have been categorized as papain-like, interleukin-1 beta-converting enzyme (ICE)-like, or picornaviral (which are similar to serine proteases but with a cysteine instead of serine). Aspartic proteinases contain an active site composed of two aspartic acid residues and generally bind 6–10 amino acids of their substrate [Leung et al., 2000]. Catalysis is through an acid-base mechanism where a deprotonated catalytic aspartic acid residue activates a water molecule, which then carries out a nucleophilic attack on the scissile bond. Finally, metalloproteinases use a zinc atom to effect amid bond hydrolysis. Serine, cysteine, and aspartic proteinases have been well characterized in viral systems [Hellen and Wimmer, 1992; Kay and Dunn, 1990]. However, although examples of viral proteinases that coordinate a zinc atom during catalysis have been described [Love et al., 1996], there are currently no known viral examples of true metalloproteinases. One possible exception to this may be the G1L protein of vaccinia virus, which has been predicted, although not yet proven, to be a metalloproteinase [Ansarah-Sobrinho and Moss, 2004b; Byrd et al., 2004b; Hedengren-Olcott et al., 2004; Whitehead and Hruby, 1994].

Protease inhibitors work by binding either to the active site of the enzyme or to the substrate-binding groove to inhibit the ability of the enzyme to either recognize its substrate or to cleave it. Inhibition can be either direct (by directly competing with the substrate) or indirect (by competing with a non-catalytic cofactor).

History of protease inhibitor discovery

Drug discovery and development efforts have changed a great deal over the past few decades to become more target specific and less toxic. Originally, drug discovery was largely centered on screening available compound libraries or natural products for compounds that would inhibit viral replication in tissue culture, without the exact mode of viral inhibition being known. As more information became available about the roles of specific essential enzymes in the viral life cycle, there was a shift in focus to look for specific inhibitors. With protease inhibitors, drug design began with looking at substrate-derived products and peptides normally cleaved by the protease. Peptides can serve as inhibitors in several ways, one of which is by replacing the scissile bond in the natural substrate peptide with a non-cleavable bond as has been demonstrated for HCV [Ingallinella et al., 2000], or through product inhibition since many of the products of protease-catalyzed reactions naturally serve as inhibitors when present in the reaction mixture such as that observed with the HCV NS3 protease [Steinkuhler et al., 1998]. By introducing structural modifications to shorten the peptide, make it non-cleavable, and increase potency, these peptides could be optimized as inhibitors. While natural peptide substrates have the benefit of providing information about molecular interactions with proteases and can, therefore, provide clues to inhibitor design, there are some significant drawbacks to their use as antivirals. Unfortunately, the use of peptides as a drug is often limited by issues of instability and poor pharmacokinetic profiles. Peptides can be susceptible to degradation and fast metabolism, low membrane permeability, low oral bioavailability, and quick elimination from plasma [Ghosn et al., 2004; Hostetler et al., 1994; Kempf et al., 1991; Matsumoto et al., 2001]. Peptide drugs also have the potential to induce an immunogenic response, which may lead to a loss of drug efficacy or adverse events in the recipient. However, PEGylation, the addition of one or more polyethylene glycol (PEG) chains, can reduce immunogenicity and degradation of the peptide drug [Veronese and Pasut, 2005]. In addition to specificity, bioavailability, and membrane permeability, for a drug to be successful it should have acceptable toxicity, absorption, distribution, metabolism, and excretion profiles.

Fortunately, with the availability of the three-dimensional structure of many proteinases determined by X-ray crystallography and NMR, drug discovery has progressed into mechanism-based drug design. By using computer-assisted structure-based design, small molecule compounds could be queried for their ability to fit into the active site pocket of the proteinase in silico. Small molecule inhibitors have the advantage of being more orally bioavailable, permeable, selective, often work in the micromolar or sub-micromolar range, have few or no hydrolysable bonds, can be easily modified through combinatorial chemistry, and can have reduced production costs over peptide inhibitors. This rational drug design approach has the benefit of looking at many parameters of the molecule/protease interaction to improve the inhibitory activity and improve the pharmacokinetic (PK) profile of the compound. Small molecule compounds identified through in silico screening are then screened in vitro to look for their ability to inhibit the proteinase target and viral replication in general. Combinatorial chem-

istry can then be used to optimize the compound into a lead drug.

SPECIFIC EXAMPLES

HIV provides an excellent example of the success of antiviral drug treatment as the virus was isolated in 1983 and the first treatment (AZT) was approved for use only 4 years later in 1987. The first HIV antivirals approved for use were nucleoside analogs, which target reverse transcriptase (RT) to inhibit viral replication. However, their monotherapeutic use had only moderate clinical efficacy, was limited by adverse side effects, and rapidly generated highly resistant drug variants making the search for new inhibitors necessary. Enfuvirtide, a novel 36-amino-acid synthetic peptide entry inhibitor, has shown promise in clinical trials and has been approved for use, but remains very expensive to manufacture and has to be administered by frequent injections [Chen et al., 2002; Hanna, 1999; Lalezari et al., 2003; Robertson, 2003; Steinbrook, 2003]. The HIV protease, an aspartic protease, has become an extremely attractive target for antiviral drug development with great success. The crystal structure has been solved and several of the protease-inhibitor crystal structures are available at www.rcsb.org/pdb. Several low molecular weight inhibitors have been approved for use in humans and are among the first successful examples of structure-based drug design that target the active site substrate-binding groove of the enzyme (Table 1). Beginning with saquinavir, which was approved in 1995, protease inhibitors for HIV have been developed with improved oral bioavailability, plasma concentrations, and increased half-life, which allow for less frequent administration of the drug and therefore less adverse side effects. Saquinavir suffered from low oral bioavailability, but was quickly supplemented with ritonavir in 1996, which had a high oral bioavailability [Vella, 1995]. The success of these protease inhibitors was followed by the development of indinavir, nelfinavir, aprenavir, lopinavir, atazanavir,

and tipranavir. Indinavir required precise dosing every 8 h. However, as improvements were made in protease inhibitors, dosing requirements decreased, with aprenavir allowing twice a day dosing, and atazanavir being the first HIV protease inhibitor to allow once a day dosing. Ritonavir is unique amongst the approved HIV protease inhibitors in that it inhibits the host liver enzyme cytochrome P450-3A4 (CYP3A4) [Kumar et al., 1996], which normally metabolizes other protease inhibitors. Ritonavir is, therefore, mainly used in combination with other protease inhibitors since it decreases their metabolism. The rest of the HIV protease inhibitors target the active site substrate-binding groove of the enzyme. Nelfinavir and tipranavir are the only two non-peptidic HIV protease inhibitors. Table 2 highlights some of the major drug-resistant mutations in the protease gene of HIV-1 [Johnson et al., 2005] developed in response to the various protease inhibitors, pointing out where cross-resistance between drugs is likely. Not indicated in Table 2 are many of the minor mutations that appear in drug-resistant isolates that by themselves do not cause drug resistance but may improve fitness of viruses containing another major mutation. Newly emerging drug-resistant strains of HIV intensify the need for more antiviral drugs. Recently, a new morphogenesis inhibitor, PA 457, which appears to act on the substrate of the proteinase instead of the enzyme active site, is in phase II clinical trials and has been granted fast-track status by the FDA [Reeves and Piefer, 2005].

Most aspartic protease inhibitors developed to date have been found through either screening compound libraries or through rational drug design and bind to the protease through non-covalent interactions, making them reversible inhibitors that must show greater affinity for the protease than the protease for its natural substrate. This high affinity is achieved through rational drug design by increasing the number of interactions between the inhibitor and enzyme. There are currently 8 different HIV protease inhibitors

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TABLE 2. Major Drug-Resistant Mutations in the Protease Gene of HIV-I

	D30N	V32I	L33F	M46I/L	I47V/A	G48V	150L/V	V82A/F/T/ V/S/L	184V	N88S	L90M
Saquinavir						X					X
Ritonavir								X	X		
Indinavir				X				X	X		
Nelfinavir	X										X
Amprenavir							X		X		
Lopinavir/ritonavir		X			X			X			
Atazanavir							X		X	X	
Tipranavir/ritonavir			X					X	X		

on the market that can be used in combination therapy to increase antiviral potency and decrease the chances of the development of resistance.

Although the only currently approved protease inhibitors are for HIV, there are several very promising protease inhibitors currently in clinical trials for a variety of other viruses including inhibitors of the NS3 serine protease of HCV. First identified in 1989, HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Since the virus establishes a chronic infection, and long-term treatment may be necessary, a combination drug approach such as that used for HIV may be required to avoid problems of resistance. Until recently, drug discovery efforts for HCV were hindered by factors such as the persistence of the virus in the host, which can lead to genetic diversity, development of drug resistance, and lack of good cellular and in vivo models with which to screen antiviral compounds [Magden et al., 2005]. Initial efforts to overcome these problems were benefited by the development of a replicon system that relies on stable replication of subgenomic RNAs in human hepatoma cells [Lohmann et al., 1999] and the creation of pseudotype viruses expressing HCV glycoproteins [Bartosch et al., 2003; Hsu et al., 2003; Lagging et al., 1998]. While useful for studying and targeting specific viral proteins, these systems still lacked the production of infectious virus. The recent identification of cell lines that produce infectious HCV virus [Cai et al., 2005; Kanda et al., 2006; Valli et al., 2006; Yi et al., 2006] will allow significant improvements in the ability of researchers to study the virus life cycle and look for antiviral drugs. BILN 2061, an orally bioavailable small molecule inhibitor, showed initial promise in human trials with a reduction of HCV RNA in the plasma, and established the proof-of-concept in humans that a HCV protease inhibitor can be effective [Lamarre et al., 2003]. Unfortunately, due to studies that showed that BILN 2061 may cause heart damage in animal models, further progress with this particular drug was halted [Hinrichsen et al., 2004]. However, the FDA has granted Fast Track development status to two other HCV protease inhibitors, Schering-Plough's oral protease inhibitor SCH 503034 and Vertex's VX-950, which are both in Phase II clinical trials. The current treatment for HCV is a combination of ribavirin with PEG-interferon alpha and, while efficient, it does cause significant toxicity, and many patients do not respond to treatment, prompting the search for other antivirals.

Significant progress has also been made in the search for protease inhibitors against the human rhinovirus (HRV) 3C cysteine protease. The HRV protease is a 20-kDa picornavirus protease with homology to trypsin-like serine proteases. The HRV 3C protease selectively catalyzes the peptide bond after the Gln residue in the Val/Thr-X-X-Gln-Gly-Pro consensus sequence [Long et al., 1989]. With the determination of the 3D structure of the HRV 3C proteinase [Matthews et al., 1994], progress in rational drug design against this target has been made. Structure-assisted design resulted in the discovery of rupintrivir, formerly AG7088, which has shown good selectivity and activity against all the serotypes tested [Binford et al., 2005]. Rupintrivir binds irreversibly to the active site cysteine and has shown promise in phase II clinical trials [Hayden et al., 2003]. Another orally bioavailable small molecule inhibitor of the HRV protease is being developed by Pfizer and has shown efficacy in cell-based assays and safety in human trials [Patick et al., 2005]. Like HIV, the specificity of protease inhibitors may prove to be critical for HRV inhibitors. The small molecule inhibitor pleconaril, which inhibits the viral uncoating process, has recently shown efficacy in reducing the symptoms associated with upper respiratory tract disease [Pevear et al., 2005]. However, in two clinical trials 13% of isolates were not susceptible to the drug, demonstrating a limit in the spectrum of activity of some entry inhibitors [Fleischer and Laessig, 2003]. The FDA did not grant permission to use pleconaril to treat the common cold due to some evidence that it interfered with the action of contraceptives (www.fda.gov/ohrms/dockets/ac/02/briefing/ 3847b1_02_FDA.pdf).

CHALLENGES TO DEVELOPMENT

The development of a successful antiviral drug has many challenges to overcome before being approved for use in humans. The first and often the most difficult challenge can be assay development, designing a suitable system to study the effects of an antiviral both in vitro and in vivo. Some viruses have not been adapted to grow in tissue culture cells or due to their genetic makeup are difficult to engineer or manipulate. Creative solutions to these issues are exemplified by research around HCV, which led to the use of replicon systems and pseudotype viruses to be able to study specific aspects of the virus life cycle.

Once a suitable assay is developed to screen specific inhibitors, the challenge can then be with rational drug design. If the crystal structure of a protease is not available, homology modeling must be relied on to approximate the three-dimensional structure of the enzyme. However, this method can still be reasonably reliable, especially if there is significant similarity in the active site region of the protein being targeted and a homolog with a known 3D-structure. As molecules are being screened for their ability to interact with this active site pocket (or a secondary

allosteric binding site), specificity again becomes an issue. Reversible inhibitors, which make hydrogen bonds, ionic, and van der Waal's interactions, are generally preferred over irreversible inhibitors, which usually form a covalent bond between enzyme and inhibitor since irreversible inhibitors could be expected to covalently bind with many proteins before encountering the target protein and thus result in more toxic side effects.

After compound inhibitors are screened in a suitable in vitro assay, whether biochemical or cell based, the compound should then be tested against a variety of viruses for specificity and against a number of cell lines for toxicity. Those compounds that show good activity and specificity with a lack of toxicity need to be tested in an appropriate animal model to determine both the pharmacokinetic profile of the drug as well as its effectiveness in preventing or treating disease. Determining an appropriate animal that approximates the human disease can prove quite challenging, especially for viruses that normally only infect humans such as HIV and those that are rare or naturally nonexistent such as variola, which still pose a bioterror threat. Even if an animal model is found that is predictive of the disease in healthy adults, it may not approximate the disease in immunocompromised individuals, infants, the elderly, or pregnant women.

In addition to the challenges surrounding drug development, there is a need for more rapid and specific diagnostics in order to use antiviral drugs. By the time most acute viral infections become evident, the symptoms are generally side-effects of the immune response and viral titers are already dropping, thus a drug would only be useful if you knew you were previously exposed. Also, many different viruses can cause the same disease. For example, the common cold can be caused by rhinoviruses, adenoviruses, coronaviruses, and so on. Each virus has a different genome and different drug targets, so knowing exactly which virus is causing the infection is necessary to prescribe a specific antiviral drug.

VACCINIA VIRUS AS A MODEL SYSTEM

Ongoing studies with vaccinia virus (VV) demonstrate the process of antiviral drug discovery efforts. Though smallpox was eradicated in 1980, there has been concern in recent years that variola virus (the causative agent of smallpox), a related orthopoxvirus such as monkeypox virus, or a genetically engineered poxvirus may pose a threat as a bioterrorism agent. With the discontinuation of vaccination against smallpox, the subsequent 30 years have produced a population that is immunologically naive and susceptible to infection. With the potential adverse events associated with widespread use of the currently available vaccine, which can include myocarditis, pericarditis, generalized vaccinia, eczema vaccinatum, encephalitis, and neurologic illness [Casey et al., 2005; Cohen et al., 2006; Fulginiti et al., 2003; Sejvar et al., 2005], antiviral drugs against orthopoxviruses are actively being sought. An antiviral would be useful both with co-administration with the vaccine to limit adverse effects as well as a prophylactic or therapeutic for exposure to poxviruses. Since smallpox is a biosafety level 4 agent with the only available stocks in the United States at CDC, much of the antiviral research is done using a highly related virus, vaccinia.

VV is a large DNA virus with a cytoplasmic site of replication. The life cycle begins with attachment and entry of the virus through an as yet unknown mechanism, followed by uncoating to release the viral core that contains the viral genome and the viral transcriptional apparatus. Gene expression occurs in an orderly cascade with the products of early gene expression necessary for DNA replication, intermediate gene expression, and late gene expression. After the late genes are expressed, which encode many of the structural proteins and enzymes, assembly of the virus occurs in areas of the cytoplasm that are termed virosomes or virus factories. During assembly of immature viral particles, viral-encoded proteinases cleave the major core protein precursors during morphogenic proteolysis to lead to the first of four infectious forms of virus, intracellular mature virus (IMV). A portion of the IMV particles acquire an additional membrane(s) from the trans Golgi network to become intracellular enveloped virus (IEV). Whether IMV acquire one or two membranes during this transition is a well-debated issue. Regardless, the IEV then travel to the cell surface via actin tails to become either cell-associated enveloped virus (CEV) or are released to form extracellular enveloped virus (EEV). Figure 1 depicts the events that occur during the VV life cycle along with potential targets for antiviral drug development. Initially, the search for an effective orthopoxvirus antiviral focused on looking at compounds that are already approved for use against other indications. While these efforts have identified compounds that have proven useful for studying the replication of the virus in vitro, they have not provided antivirals that are effective in vivo either due to toxicity or lack of effectiveness against pox viruses. Stages of the life cycle that have been targeted include DNA synthesis, transcription, morphogenesis, and exit of IMV and EEV from the cell. Examples of some of these are listed in Table 3. The DNA synthesis inhibitor cytosine arabinoside (AraC), which is used to treat some leukemia, inhibits VV replication [Herrmann,
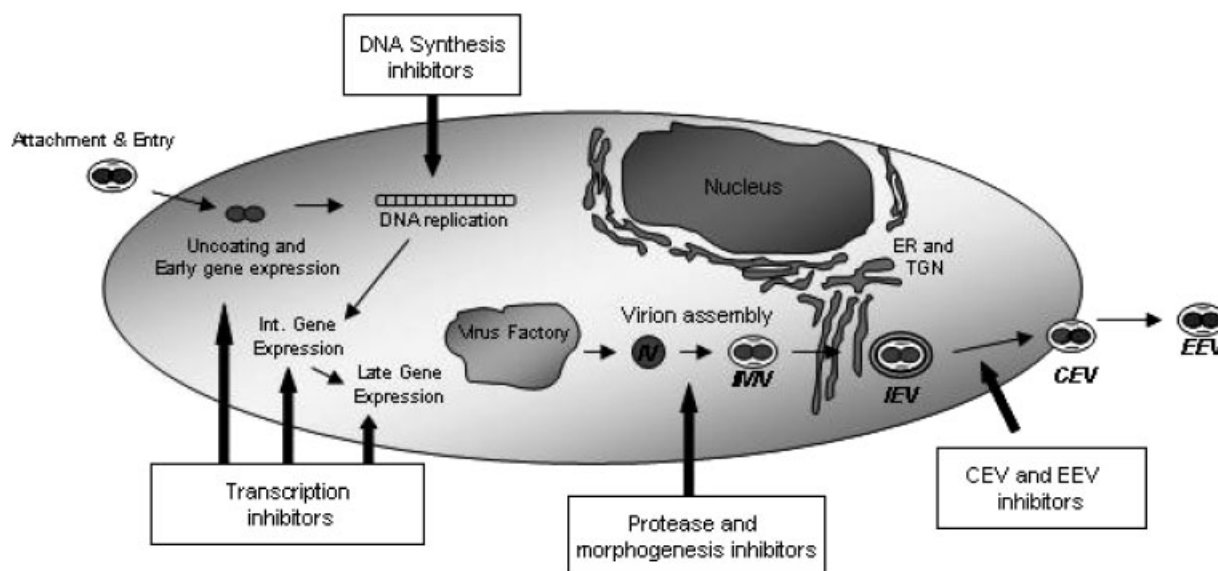
Fig. 1. Vaccinia virus life cycle. Stages of the life cycle essential for replication that make good targets for drug development are indicated and include DNA synthesis, transcription, morphogenesis, and exit from the cell.

TABLE 3. Compounds That Inhibit Vaccinia Virus Replication In Vitro[a]

Stage of V V life cycle targeted	Drug	Used for	Adverse effects
DNA synthesis	AraC	Leukemia	Bone marrow suppression, cerebellar toxicity
	Hydroxyurea	Hematological malignancies	Bone marrow toxicity
	Cidofovir	CMV retinitis in AIDS	Lack of oral bioavailability
	Ribavirin	RSV, HCV, Lassa	Not effective against pox in vivo
Transcription	Distamycin	Antibiotic	Thrombophlebitis, bone marrow toxicity
Morphogenesis	Rifampicin	Mycobacterium	Hepatotoxicity
	Novobiocin	Staphylococcus epidermidis	Hepatotoxicity and blood dyscrasias
	IMCBH	Pox viruses	Not effective in animal models
	TTP-6171	Novel compound	Not effective in animal models
CEV and EEV	ST-246	Novel compound	No adverse effects→in clinical trials

[a]Stage of V V life cycle targeted, what the compound is commonly used for, and some adverse effects that may limit its use are indicated. AraC, cytosine arabinoside; IMCBH, N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine.

1968] but causes bone marrow suppression, leucopenia, and cerebellar toxicity in humans [Barista, 2000] so its use is mainly limited to cancer chemotherapy. Hydroxyurea, which is used for hematological malignancies [Johnson et al., 1992] also acts as an antiviral by inhibiting ribonucleotide reductase and inhibits vaccinia virus at an early stage [Rosenkranz et al., 1966]. However, it can cause bone marrow toxicity, hair loss, and skin changes [De Benedittis et al., 2004].

The other approach to orthopoxvirus antiviral drug discovery is to screen new chemical libraries for specific inhibitors of various stages of the virus life cycle. Recently, the small molecule compound ST-246 has shown to be effective in inhibiting EEV formation and is protective in several animal models [Yang et al., 2005]. ST-246 was discovered through a high-throughput screening assay looking at compounds that inhibited virus-induced cytopathic effects. Drug-resistant virus variants were created and used to map the location of resistance to the F13L gene in vaccinia virus, which encodes a protein necessary for the production of extracellular virus. This compound is currently in human clinical trials and shows promise for development as an antiviral drug.

Another very promising target is the recently discovered V V I7L proteinase, which is the viral enzyme responsible for cleavage of the major core protein precursors as well as membrane proteins and is absolutely essential for viral replication [Ansarah-Sobrinho and Moss, 2004a; Byrd and Hruby, 2005a].

While other proteinases, either cellular or viral, may be involved in the virus life cycle, to date I7L is the only characterized proteinase involved in the virus life cycle. I7L is a 47-kDa cysteine proteinase that cleaves its substrate proteins at a conserved Ala-Gly-Xaa motif [Byrd et al., 2002]. Mutagenesis of conserved residues within I7L and around the catalytic triad (H241, D258, C328) have helped define functional regions of the enzyme [Byrd et al., 2003]. Although efforts to date to express and purify active enzyme in vitro have not been successful, an in vitro assay to screen for potential I7L inhibitors has been developed [Byrd and Hruby, 2005b]. Homology modeling of the I7L proteinase, based on similarity to the C-terminal domain of the ULP1 protease in yeast, has been successfully used for in silico screening of a large chemical library of small molecules for potential inhibitors [Byrd et al., 2004a]. One of these, TTP-6171, showed initial promise against I7L and was a potent inhibitor of viral replication in vitro [Byrd et al., 2004a]. However further studies in animals showed less efficacy, highlighting the need for additional pharmacokinetic analysis and combinatorial chemistry (data not shown). I7L shares regions of similarity with several other viral proteinases including the African Swine Fever virus protease and the Adenovirus protease. However, there are no cellular homologs of I7L, making it likely that a drug that inhibits it will be highly specific for the virus and not the host cell. I7L remains an attractive target and efforts are ongoing in the search for an effective antiviral drug against this enzyme.

Some of the difficulties with antiviral drug research are exemplified through research with vaccinia virus. Once an attractive target is discovered, such as the I7L core protein proteinase, biochemical and whole cell assays need to be set up and validated to screen inhibitory compounds. Compounds can be identified as potential inhibitors either through rational drug design, using homology modeling of the enzyme, since the crystal structure has not yet been solved, in combination with in silico screening of compound libraries, or through high-throughput screening of large chemical libraries. Once a compound has been identified as an inhibitor of the I7L enzyme through biochemical assays, its ability to inhibit viral replication needs to be validated in tissue culture both against vaccinia virus as well as other orthopoxviruses. Of particular interest to the development of poxvirus antivirals is the need to satisfy the animal efficacy rule set up by the FDA to evaluate new drug candidates. This can be a unique problem with smallpox antivirals since the disease is no longer endemic, and animal models of disease require the use of surrogate viruses, which do not closely mimic the human disease. A review addressing these concerns as well as how to select appropriate animal models has recently been published [Jordan and Hruby, 2006].

CONCLUSIONS

Modern molecular biology has provided the tools necessary to study the details of viral replication and has allowed the identification and development of many specific antiviral drugs. However, the problems of toxicity and resistance that occur with long-term use of many of these antiviral agents remain, not to mention that many of the approved antivirals have a narrow spectrum of activity and limited therapeutic usefulness. Combine this with the ongoing identification and characterization of newly emerging virus infections, and it can be seen that there are a very limited number of effective antiviral compounds against a small number of viruses. While significant progress in antiviral drug development has been made in the last several years, there is a clear gap in our arsenal of available antiviral agents, and the continued search for new compounds and strategies is essential.

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