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Review Article

Orthopoxvirus: biology, pathology and therapy

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Abstract

Smallpox, once the scourge of mankind, has been eradicated through a coordinated vaccination campaign orchestrated by the World Health Organization (WHO). Despite this success, members of the *Orthopoxvirus* genus, including monkeypox, vaccinia and cowpox viruses, remain a public health concern and continue to cause sporadic disease in humans. Even variola virus, the etiological agent of smallpox, is of significant concern due to its potential use as a bioterrorism agent. This review describes the biology and pathogenesis of *Orthopoxvirus* infections in humans and discusses current information regarding potential therapeutics for the treatment of infections caused by these viruses.

Introduction

There are close to 100 species of poxviruses found in nature, ranging from those that infect invertebrate hosts (Entomopoxvirinae subfamily) to those that infect vertebrate hosts (Chordopoxvirinae subfamily). The most well known of these may be variola virus, the causative agent of smallpox, and a member of the *Orthopoxvirus* genus. Other human pathogens of the *Orthopoxvirus* genus include monkeypox, vaccinia and cowpox viruses. While smallpox was successfully eradicated as a source of natural infection as a result of a World Health Organization (WHO)-sponsored global vaccination campaign, poxviruses remain a public health concern due to their potential for use as bioterrorist weapons, as well as through natural infection caused by endemic viruses such as monkeypox virus, which continues to cause disease in humans. Moreover, termination of routine vaccination in the 1970s has left a population that is immunologically naïve and susceptible to infection. Fortunately, recent developments in virology, immunology and drug development are providing therapeutic options for poxvirus infections.

Biology

The life cycle of *Orthopoxvirus* has been extensively studied using vaccinia virus, the prototypic virus, which is a biosafety level 2 (BSL-2) agent and conducive to laboratory study. Poxviruses are unique among DNA viruses in that they replicate exclusively in the cytoplasm of infected cells, and thus encode an array of replication enzymes and associated machinery necessary for replication. Poxviruses are among the largest animal viruses containing a linear double-stranded DNA (dsDNA) genome of approximately 200 kb that temporally expresses ~200 genes necessary for replication and spread of the virus in the host. There are four types of infectious poxvirus particles that differ in their lipid membranes, location and relative quantity: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). These different forms of the infectious virus play distinct roles in pathogenesis. IMV is the most abundant virion, contains a single membrane (1) and remains within the cell until cell lysis, whereupon it infects neighboring cells. IMV is environmentally stable and is responsible for the spread of infection between host organisms (2). A fraction of the IMVs become enwrapped in two additional layers of membrane to form IEV. IEV is transported to the cell periphery via microtubules, where its outer membrane fuses with the plasma membrane to form CEV, which remains associated with the cell surface. A portion of the CEV particles are actively released from the cell

through a process involving actin tail formation, to become EEV, which is the least abundant virion. EEV is responsible for the long-range dissemination of the virus and systemic disease (2).

The vaccinia virus replication cycle begins with attachment and entry into a host cell by either IMV particles or EEV after shedding the outer envelope (for a review on poxvirus entry and fusion, see the recent article by Moss [1]). Entry is mediated by direct fusion of the viral envelope to the plasma membrane and through receptor-mediated endocytosis. Multiple routes of entry may help explain the broad host range of vaccinia virus.

After entering the host cell, the virion undergoes a series of uncoating steps with concurrent gene expression (3). To facilitate temporal expression of its gene products, the poxvirus genome contains transcriptional promoters specific to each gene. First, the outer viral envelope is removed, activating the viral RNA polymerase and accessory enzymes, including the capping enzyme and poly(A) polymerase, which allows the synthesis of early mRNA in the virion core (4). Following initial uncoating, the early mRNA is released from the core, is translated by cellular machinery and its products are involved in the evasion of the host immune system and viral DNA replication (5, 6). The translation of the early mRNA triggers virion core disassembly and produces DNA replication enzymes and intermediate gene transcription factors (5, 7-9).

To carry out cytoplasmic replication, poxviruses encode a plethora of enzymes, including but not limited to thymidine kinase (10-12), thymidylate kinase (13), ribonucleotide reductase (14, 15), dUTPase (6), DNA helicase (16) and a DNA polymerase (17, 18). The cytoplasmic site of viral DNA replication is termed a virus factory or virosome, and this becomes the site of further viral maturation. Following DNA replication, intermediate gene expression begins (19-21). The prerequisite for DNA replication before the initiation of intermediate gene expression has not been well characterized. The intermediate genes encode transcriptional promoters for late gene expression (22) and enzymes that interact with viral DNA and RNA (23). Late gene expression lasts longer than early and intermediate gene expression, producing a continuous supply of late mRNA for about 48 h (24, 25). The late genes encode major virion components and transcription factors for early gene expression that are packaged within the progeny virion cores. Post-translational processing of several late gene products is significant in the vaccinia virus replication cycle, and the proteases responsible for this cleavage represent an attractive target for antiviral drug development (see Byrd et al. [26] for a review on vaccinia virus proteolysis).

The first sign of virion formation is the appearance of crescent-shaped membrane structures in the virosome (27). The crescents grow until they become oval-shaped immature virions (IVs). The origin of the membranes acquired in the formation of the IVs is unknown. They are hypothesized to be derived from either the intermediate compartment, located between the endoplasmic reticulum and the Golgi network, or synthesized de novo (28). The viral core proteins, specifically p4a, p4b and p25k, are then proteolytically processed by a viral cysteine proteinase, I7L, to facilitate core condensation (29). Core condensation into the characteristic biconcave phenotype marks the acquisition of infectivity and the formation of an IMV. As stated above, most IMV particles remain in the cell until lysis occurs, but some travel away from the virosome on microtubules to become IEVs. IMV particles mature into IEV particles by acquisition of a double membrane derived from either the trans-Golgi or the endosomal cisternae (2). The IEV particles then continue to travel along microtubules, powered by conventional kinesin, to the cell surface (30). Once an IEV particle reaches the cell surface, it fuses with and buds through the plasma membrane and is exposed to the extracellular environment as a CEV. A small fraction of CEV particles are released from the plasma membrane to form EEVs in a process mediated by actin tail formation (31). Virus maturation and egress are regulated in part by the F13L protein, which encodes a viral late assembly domain (32). In the absence of F13L activity, vaccinia virulence is significantly reduced by inhibition of EEV formation, revealing F13L as a useful drug target.

An effective antiviral drug will target one of the essential viral processes described above, including those whose function involves entry and uncoating, transcription and mRNA processing, DNA replication, DNA processing and packaging, viral enzymes and morphogenesis, without introducing toxicity to the host. The unique specificity imparted to virally encoded enzymes required for cytoplasmic replication provides opportunities for selective inhibition of viral replication by antiviral drugs. An overview of the vaccinia virus life cycle is shown in Figure 1, highlighting the target of several compounds that are currently being developed for the treatment of *Orthopoxvirus* infections and several drugs that have antipoxvirus activity that were developed for other indications. An overview of the mechanism of action, target specificity and stage of development of these drug candidates is presented in Table I.

Pathogenesis and natural history of human *Orthopoxvirus* infections

Prior to the eradication of smallpox, the most common *Orthopoxvirus* infection in humans was caused by variola virus. While no other *Orthopoxvirus* has caused such widespread devastation, there are many documented cases of human infections caused by monkeypox, vaccinia and cowpox viruses. These viruses are endemic in various parts of the world, with rodents, domestic pets and cattle being major reservoirs of virus. Zoonotic transmission to humans can cause a spectrum of diseases. In general, disease progression is dependent upon the species of poxvirus infecting the host, the amount of virus in the inoculum, the portal of entry and the host response to infection. These factors influence the extent of infection and spectrum of disease, ranging from a self-limiting,

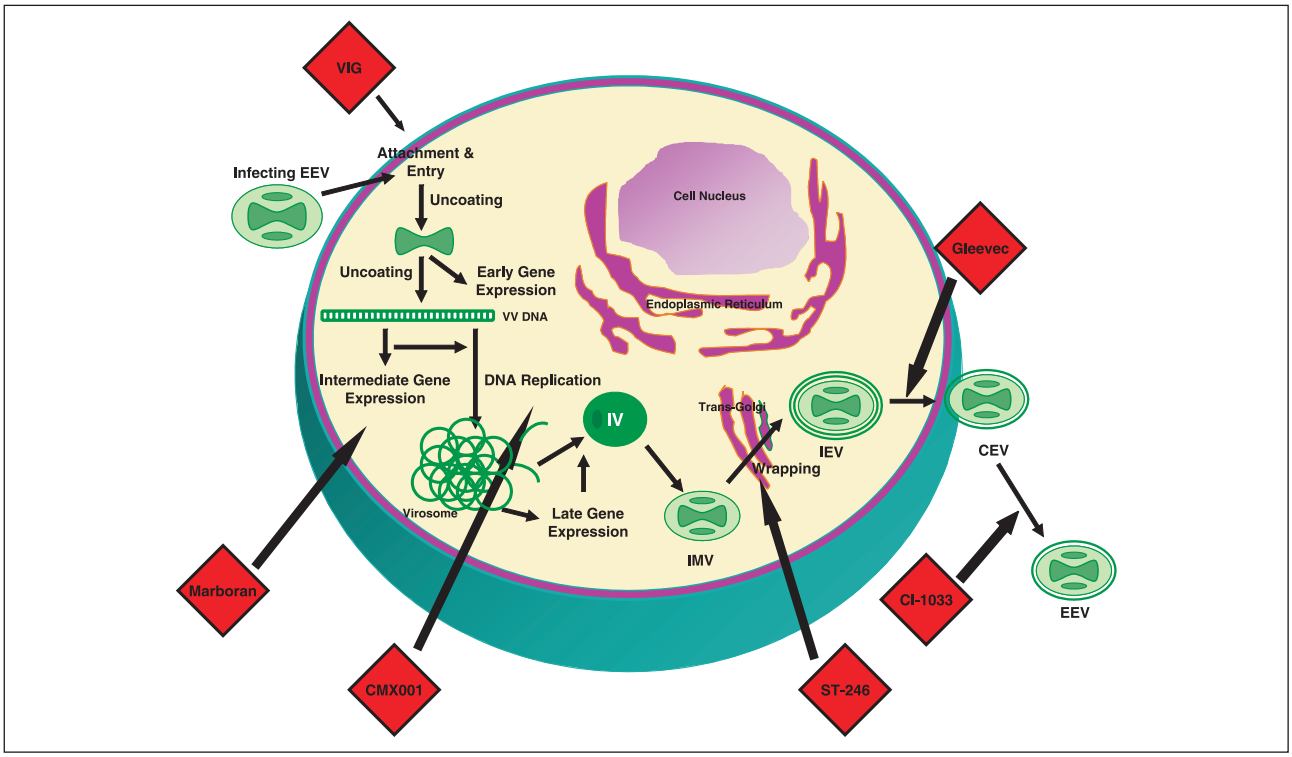


Fig. 1. Vaccinia virus life cycle highlighting antiviral drug targets. VIG, vaccinia immune globulin; EEV, extracellular enveloped virus; VV, vaccinia virus; IV, immature virion; IMV, intracellular mature virion; IEV, extracellular enveloped virus; CEV, cell-associated enveloped virus.

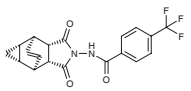
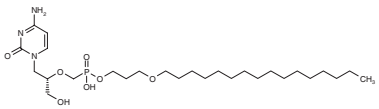
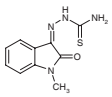
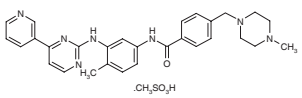
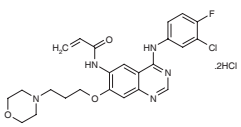
localized infection at the site of inoculation to a systemic, often fatal infection characterized by extensive skin lesions. Recent outbreaks of fatal cowpox virus infections in nonhuman primates, coupled with the observation that host range genes and virulence factors are undergoing positive selection, suggest that poxviruses are evolving, leading to increased zoonotic transmission and variants with altered virulence (33, 34). While smallpox is no longer a disease found in humans, the possibility exists that new variants of circulating poxvirus may emerge to cause more common disease in humans.

Smallpox

1. History

Smallpox is one of the oldest documented infectious diseases, with the earliest descriptions dating back more than 2,000 years (35). Variola virus, the etiological agent of smallpox, has only one known host, humans, with infection spread via aerosol or direct contact. Smallpox exists in two forms: variola major, which produces a more serious disease with a mortality rate of 30-35%, and a milder form, variola minor, with a mortality rate of ~1%. The impact of smallpox on human health should not be understated. Smallpox is estimated to have killed more of the human population than all other infectious diseases combined. Early attempts at protection from smallpox included the practice of variolation where dried smallpox scab material or pustular fluid from infected individuals was administered to immunologically naïve individuals. This resulted in disease that was generally milder, with case fatality rates 10-fold lower (36). However, since the material used in variolation contained active variola virus, a small percentage of those treated developed full-blown disease, and even mild cases of disease in those treated could still lead to severe and fatal cases of smallpox in close contacts. At the end of the 18th century and during the early 19th century, variolation gave way to vaccination, a technique introduced by Edward Jenner, where naïve individuals were administered a related *Orthopoxvirus* derived from scab material of cows infected with cowpox virus as a means of preventing smallpox. By the mid-20th century, vaccination against smallpox was used in many industrialized countries, although the virus component had changed to vaccinia virus rather than cowpox virus. The origin of vaccinia virus and the reasons for the change in virus used in the vaccine remain a mystery. In the mid-1960s, the WHO launched a global smallpox eradication campaign based on mass vaccination, surveillance and containment. By 1979, the world was claimed to be free of endemic smallpox (37). Although no longer found in the natural environment, stocks of variola virus were maintained in various laboratories, including the Centers for Disease Control and Prevention (CDC) in Atlanta and the Research Institute for Viral Preparations (VECTOR) in Moscow (38). Questions have been raised, however, about whether these official stocks are the only ones remaining.

Table I: Leading antipoxvirus drug candidates. The common names, structure, mechanism of action and/or target and stage of development of various antiviral drugs shown to have activity against poxviruses.

Drug	Structure	Mechanism/target	Stage of development
ST-246		IMV wrapping/IEV formation, inhibits egress	Clinical development for poxvirus treatment
CMX001 (HDP-cidofovir)		Acyclic nucleoside phosphonate, inhibits viral DNA polymerase	Clinical development for poxvirus treatment
Methisazone (Marboran)		Unclear, may act at transcription against the viral RNA polymerase	Limited clinical experience in humans during WHO vaccination campaign
Imatinib mesilate (STI-571, Gleevec)		Inhibits ABL tyrosine kinase, inhibits egress	Licensed for treatment of chronic myeloid leukemia – approved for use in humans
Canertinib (CI-1033)		Inhibits pan-ErbB tyrosine kinase, inhibits egress	Clinical development for cancer treatment

2. Clinical features

The clinical case definition of smallpox includes an acute onset of fever, followed by a rash that is characterized by firm vesicles or pustules that are in the same state of development without other apparent cause. The rash generally persists for 2 weeks, progressing through the macular, papular, vesicular and pustular stages before finally scabbing over. Variola major can cause several types of clinical disease which are defined by the nature and evolution of the virus-induced lesion and by the severity of disease symptoms. Ordinary smallpox is the most frequent presentation and is characterized by raised pustular skin lesions that are either discrete, semiconfluent or confluent. Variola sine eruptione is distinguished as infection with fever but no rash, with symptoms usually of short duration. Modified smallpox is characterized by relatively few, superficial lesions, which occur most often in previously vaccinated individuals. Flat-type smallpox is characterized by severe disease and confluent flat pustules, with a mortality rate of 97%. Hemorrhagic-type smallpox is uncommon but a very severe form of the disease that is almost always fatal. It is distinguished by extensive bleeding into the skin, mucous membranes and gastrointestinal tract, with death usually occurring within the first week of illness, with only a few maculopapular lesions (36, 39, 40). Mortality is attributed to toxemia, a poorly defined clinical syndrome that is thought to be related to a hyperinflammatory immune response to systemic infection.

3. Host response to infection

The severity of smallpox disease is determined by the host response to variola infection, as well as by the ability of the virus to counteract the host response through immune evasion mechanisms. The host response serves to limit viral replication and spread early in infection, but also contributes to the immunopathology associated with later stages of infection. Both humoral and cell-mediated immunity are believed to play a role in limiting the replication and spread of the virus. Since variola virus is no longer endemic and has no natural host other than humans, the study of its pathogenesis must be inferred from similar *Orthopoxvirus* infections, including vaccinia, ectromelia, cowpox and monkeypox. Smallpox pathogenesis has been primarily studied in mice infected with ectromelia virus. The genetic similarity, along with the common features of disease that this model shares with variola infection of humans, and the laboratory convenience of mouse studies, make the ectromelia model an effective model of *Orthopoxvirus* pathogenesis.

Variola virus infection begins with inhalation of viral particles, which subsequently inoculate the mucous membranes of the mouth, nasal cavity, oropharynx, nasopharynx and the alveoli of the lungs (41). From animal studies it was deduced that, during the early stages of infection, cell-associated and free virus migrate to and replicate in the lymph nodes (42, 43). Virus then travels to blood, bone marrow, spleen and liver to establish a primary viremia. The phagocytic cells of the spleen and liver

are infected first, followed by widespread replication in the parenchymal cells. Both the spleen and liver show semiconfluent necrosis consistent with extensive virus replication in these tissues (43). Virus released into the bloodstream following replication in the reticuloendothelial system produces a secondary viremia and invasion of the dermal layer of blood vessels. The secondary viremia correlates with the onset of fever and other symptoms, and marks the end of the incubation period and the beginning of the prodromal phase of infection. In mice infected with ectromelia virus, both viral antigen and infectious virus are detected in the bone marrow, nasal mucosa, intestine, kidneys, skin and other organs for approximately 13 days postinfection (44). An interval of extensive viral replication in the epidermis, hair follicles and sweat glands ensues. When approximately 10^7 virus particles (vp)/g have accumulated, early rash papules are visible (45). Virus replication in the epidermis results in the formation of papules that mature and ulcerate within a few days, resulting in severe rash with infectious virus contained in the pustules.

In response to infection, proinflammatory mediators are released from infected cells which have profound effects on virus replication and spread. Intradermal inoculation of mice with ectromelia virus results in the release of interleukins IL-1β, pro-IL-1β and pro-IL-18 from damaged keratinocytes. These cytokines are involved in the production of growth factors and other keratinocyte-specific stimulatory factors (46-48). The activated dermal layer provides a highly permissive environment for viral replication. Release of proinflammatory molecules also results in vasodilatation, allowing increased flow of lymphocytic cells to the sites of infection, as well as access of the virus to the bloodstream. The increased blood flow causes erythema and edema around the pustules, creating more severe and pronounced lesions, but is necessary for cell-mediated clearance of viral infection (49-53).

Disease severity is determined by the strength of the host immune response. The ability of the immune system to block extensive viral spread during secondary viremia is of prime importance. Once the virus has disseminated, symptoms of advanced disease can manifest in the form of hypotension and coagulopathy due to the pronounced inflammatory response. At this stage of disease, the inflammatory response begins to work against the host, facilitating viral replication and advanced illness. Without sufficient viral clearance, the release of proinflammatory molecules can cause vascular dysfunction, coagulopathy and multiorgan failure, leading to irreversible shock syndrome (36, 54-59). This fatally hemorrhagic disease form is an extreme case, but exemplifies the host response working against itself and thus contributing to disease lethality.

Poxviruses have evolved a variety of immune evasion mechanisms to counteract the innate and humoral immune response to infection. A number of poxvirus genes identified as virulence factors have been shown to modulate the immune response to infection and deletion of these genes attenuates virus replication in animal hosts. Several of these genes share sequence homology with variola virus genes, suggesting that they function to downregulate the immune response during infection (60). Phylogenic analysis of poxvirus genomes indicates that many of the immunomodulatory genes are undergoing positive selection, suggesting that they are coevolving with their respective hosts (34). A detailed review of poxvirus immune evasion strategies was presented by Seet et al. (50).

Monkeypox

Monkeypox virus was identified in 1958 as a disease of captive monkeys when it was isolated from the lesions of infected primates at the State Serum Institute in Copenhagen. Monkeypox virus has a broad host range that includes many common laboratory animals, as well as humans. The first human case of monkeypox was identified in Zaire in 1970. Human monkeypox occurs primarily in remote villages of central and western Africa, and is transmitted to humans from direct contact with infected rodents, pets and primates, as well as occasional cases of person-to-person transmission via respiratory droplets. Comprehensive knowledge of the clinical manifestations and progression of human monkeypox in adults was fairly limited due to limited access to medical care in remote villages and many cases being diagnosed retrospectively. However, in 2003, an outbreak of monkeypox in the United States was reported in residents in the Midwest after contact with infected pet prairie dogs (61), which increased our working knowledge of the infection. While the disease symptoms were found to be generally less severe than those typically seen in Africa, the route of exposure was found to influence the severity of the disease symptoms (62), with the predominant symptoms being rash, fever, chills, adenopathy, headache and myalgia (63).

Clinically, human monkeypox closely resembles ordinary smallpox, being a systemic disease with a generalized pustular rash. One obvious clinical difference that occurs with monkeypox, both in infected humans and monkeys, however, is pronounced lymph node enlargement (63). In Africa, human monkeypox has a case fatality rate of ~10% and occurs predominantly in children (64, 65). In the U.S. outbreak, 20% of the infected children experienced severe complications that could have resulted in death if not for the intensive medical intervention they received (63).

Vaccinia

Vaccinia virus (VV) is the prototypic member of the *Orthopoxvirus* genus, the origin of which is still a mystery and a matter of discussion, although it is the species of *Orthopoxvirus* that has been used as the primary component of the current smallpox vaccine. VV infection typically causes a localized lesion at the site of inoculation, although infection can result in more generalized disease which can be severe and even fatal. During vaccination,

vaccinia virus is introduced into the skin with a bifurcated needle. Four to five days following vaccination, a papule appears at the site of viral replication, which becomes pustular a few days later due to the infiltration of inflammatory cells (39). Clinical symptoms may include malaise, fever and enlarged lymph nodes (66). The pustule reaches its peak 10-12 days following vaccination, and then begins to dry from the center outward, with the shedding of the scab after about 3 weeks leaving a characteristic scar (39).

Complications from vaccination can result from escape of the virus from the inoculation site and include progressive vaccinia (vaccinia necrosum), eczema vaccinatum, generalized vaccinia, postvaccinal encephalitis or accidental inoculation of naïve individuals through contact with the vaccination site. Progressive vaccinia occurs in persons with immunological deficiencies and is characterized by gross enlargement of the primary lesion, spread of virus deep into the tissues and the appearance of other large lesions. Progressive vaccinia left untreated can lead to death weeks to months following vaccination (66). Eczema vaccinatum occurs in persons with atopic dermatitis (eczema) or Darier's disease (keratosis follicularis), with lesions following vaccination appearing on areas of active or previously active eczema, which can spread and become difficult to distinguish from generalized vaccinia. In untreated patients, the mortality rate can range from 7% to 30%, with severe scarring occurring in survivors (67). Generalized vaccinia can occur in vaccinated individuals and their close contacts and is not associated with immunodeficiency. Generalized vaccinia is due to hematogenous dissemination of the virus, which leads to lesions that can cover the whole body (68). Postvaccinal encephalitis is most common among infants less than 1 year old, exhibiting clinical symptoms that include cerebral or cerebellar dysfunction with headache, fever, vomiting, altered mental status, lethargy, seizures and coma (69). Accidental infection occurs when vaccinia virus is transferred from the vaccination site to another location on the body or to a close contact and is usually self-limited.

Vaccinia virus was generally believed to be a laboratory virus with no known natural reservoir. However, infection of domestic animals including cows, pigs, rabbits and buffaloes has been documented. In India, Egypt and Indonesia, a disease of water buffaloes termed buffalopox was found to be caused by vaccinia virus. This disease has persisted in India.

There have been numerous published accounts of vaccinia-like viruses being isolated from mice, as well as cattle and their human handlers, in Brazil, with genetic similarity to Brazilian vaccine strains of vaccinia virus used during the eradication campaign, suggesting that these viruses have now become endemic. One of these, BeAn 58058 virus (BAV), was isolated from the blood of a rodent in the rain forest in Brazil in 1963, with subsequent genetic analysis establishing its relationship to vaccinia virus (70). A similar virus, SPAn232 (SPAnv), was isolated from sentinel mice in the forest in Cotia, Brazil, and characterized as another vaccinia-like virus (71). In 1999, a newly discovered *Orthopoxvirus* was isolated from cows and their human handlers during an exanthema outbreak on farms in Rio de Janeiro, Brazil, and termed Cantagalo virus (CTGV). PCR and sequence analysis, restriction digest profiles and the morphology of the lesions from infected individuals led to the classification of Cantagalo as a strain of vaccinia virus (72). A similar cowpox-like disease outbreak affecting a dairy herd and their human handlers unveiled yet another vaccinia-like virus, designated Aracatuba virus (73). An increasing number of similar zoonotic outbreaks are being reported in different areas of Brazil, suggesting that the viruses may be derived from vaccine escape either from the original introduction of VV to the region during the slave trade in the early 19th century, the introduction of animal-cultivated vaccine at the end of the 19th century, or from the WHO smallpox eradication campaign in the 20th century. Trindade et al. analyzed molecular data from eight different Brazilian vaccinia isolates and suggested that, due to the genetic variation, ancestral Brazilian vaccinia-like viruses existed before the introduction of the smallpox eradication campaign and that these viruses have become endemic in the region (74).

Cowpox

Cowpox has been recognized in Europe for several hundred years as a skin disease of cattle which could be transmitted to humans through contact with lesions on infected udders. Edward Jenner is recognized as having noted that milkmaids who had been infected with cowpox were immune to smallpox, and thus he used cowpox virus to vaccinate humans to protect against smallpox. The reservoir hosts for cowpox virus are rodents, including bank voles and wood mice (75), which can transmit the virus to cows, humans, cats and zoo animals, including large cats and elephants. Currently, domestic cats are the most common host for cowpox virus, with transmission to humans occurring through skin lesions or mucosa of the eye. Infection is usually localized on the skin or mucosa and typically begins with a papule that turns into a painless vesicular lesion within 7-12 days, which is accompanied by regional lymphadenopathy (76). The lesions are pustular, similar to those caused by vaccinia virus. The time to complete recovery can range from 3 to 12 weeks. Secondary lesions are rare and appear to occur primarily in individuals with immunological deficiencies. Cowpox differs from other types of *Orthopoxvirus* that cause disease in humans in that it forms two types of cytoplasmic inclusion bodies in infected cells, the irregular B-type inclusions found in most poxvirus infections, as well as large acidophilic A-type inclusion bodies (39).

Species specificity

Poxviruses have species specificity that ranges from narrow, with variola being a human-specific pathogen, to broad, for example cowpox, which can infect a range of

Table II: Species specificity of types of Orthopoxvirus that infect humans.

Virus	Maintenance host	Naturally susceptible species	Human disease	Geographic distribution
Variola	Humans	Humans	Disseminated	Previously worldwide
Monkeypox	Rodents, squirrels	Humans, nonhuman primates	Disseminated	West and Central Africa
Vaccinia	Unknown	Humans, cattle, horses, rabbits	Localized	Worldwide
Cowpox	Rodents	Humans, cows, cats, foxes, zoo animals	Localized	Europe, Asia

animals from rodents to large zoo animals to humans (Table II). However, the exact mechanisms that mediate host tropism are still being studied. Complicating these studies is the fact that some poxviruses have a host cell specificity *in vitro* that is very different from their *in vivo* host range. Many types of poxviruses are able to infect a range of mammalian cells, but their ability to replicate and spread varies remarkably among cell types (for a recent review on poxvirus tropism, see McFadden [77]). The outbreak of human monkeypox in the United States in 2003 demonstrates how susceptible the human population is to the emergence or re-emergence of *Orthopoxvirus* pathogens.

Animal models of *Orthopoxvirus* disease and animal efficacy rule

Efforts to develop antiviral therapies for poxviruses take advantage of the genetic similarity between variola and other species of *Orthopoxvirus*. Since naturally occurring variola virus has been eradicated, with the last reported case of smallpox in 1977, detailed studies of the pathogenesis of human infection were limited. Due to the host range specificity, as well as the need for high containment to work with variola virus and limited access to these facilities at the CDC, studies designed to evaluate antiviral efficacy in animals are currently conducted with closely related species of *Orthopoxvirus.* Small animal models using vaccinia virus have been useful for evaluating promising antiviral compounds and data from these systems are often predictive of efficacy against variola virus. Nonhuman primate models have been developed to evaluate compounds against monkeypox and variola virus infection. While these models are desirable given the genetic similarity of nonhuman primates to humans, they require the use of select agents and specialized containment facilities.

A major regulatory challenge for licensure of smallpox antiviral products is the “Animal Efficacy Rule” (21 CFR Parts 314 and 601) that requires linking efficacy data in animal models of smallpox to clinical correlates predictive of human disease outcome. This rule was developed for drug products where conducting human efficacy studies is not feasible or ethical (78). However, since very few products have been approved using this mechanism, the exact requirements for approval are unclear. Data from a variety of animal models are often necessary to demonstrate sufficient efficacy, especially for smallpox, since no single model is completely predictive of human disease. Ideally, animal models would recapitulate the pathophysiology of human disease. Important features include establishment of disease with small amounts of virus, replication of the virus at the periphery prior to generalized dissemination, appearance of pocks and a high degree of lethality (for reviews, see Jordan and Hruby and Painter et al. [79, 80]). The most relevant animal models, highlighted in Table III, use host-adapted viruses that replicate at the periphery and spread systemically, similar to variola virus infection of humans. While all models result in significant mortality, only models in nonhuman primates infected with monkeypox virus or variola virus produce a lesional disease that resembles human smallpox. These models use non-host-adapted virus and require large amounts of virus in the inoculum delivered by unnatural routes to establish disease. However, the pathogenesis of infection and timing of mortality are similar to human disease (Fig. 2).

Murine models

Much of what is thought to occur in smallpox virus pathogenesis in humans is inferred from ectromelia virus studies in mice, where virus–host relationships have been extensively studied. Mice can be infected with ectromelia virus by footpad scarification, similar to the natural route which is through abrasions in the skin, or intranasal delivery. The virus first replicates locally at the site of infection and then migrates through the bloodstream and lymphatic system to internal organs, especially the spleen and liver, generating a secondary viremia within a week (81). Viral replication in the internal organs leads to death by day 6-10 postinoculation (79). Antiviral efficacy is measured by a reduction in viral titer, inhibition of weight loss and decreased mortality.

Murine models of vaccinia virus and cowpox virus infection have also been developed to study the efficacy of antiviral compounds. These models differ from natural host infections, such as ectromelia in mice, in that pathogenesis is dependent upon the route of infection (79). Intranasal inoculation of mice with vaccinia virus results in replication of the virus in the nasal tissue and lungs, followed by spread of the virus and viral replication in the reticuloendothelial system, liver, spleen, lung and kidney to cause systemic disease (82). Weight loss correlates with severe disease and is an easily measured marker of disease progression. Mortality occurs by day 10 and infection is almost 100% fatal.

While murine models are convenient, allowing for easy evaluation of potential smallpox antiviral products in animals, they are limited in that pathogenesis can be influenced by strain of virus, genetic background of the murine host and level of virus adaptation to the host.

Table III: Relevant animal models of Orthopoxvirus disease.

Animal	Virus	Natural host	Animal strain	Route of infection	Approximate innoculum	Endpoints	Lethality
Mouse	VV	No	BALB/c	Intranasal	10^4-10^6 pfu	Mortality; viral load in lung, liver, spleen, kidney; weight loss/gain	100% in 6-8 days
	ECTV	Yes	A/NCR	Intranasal, footpad	50-100 pfu	Mortality; viral load in lung, liver, lymphoid tissue; weight loss/gain	100% in 6-12 days
	CPX	No	BALB/c	Intranasal	10^4-10^6 pfu	Mortality; viral load in lung, liver, spleen, kidney; weight loss/gain	100% in 8-10 days
Rabbit	RPV	Yes	New Zealand White	Aerosol or intradermal	100-1000 pfu	Mortality; viral load in respiratory tract; weight loss/gain; ocular/nasal secretions	100% in 7 days
Ground squirrel	MPX	Yes	GGS	Subcutaneous	100 pfu	Mortality; viral load in blood, liver, lung, spleen	100% in 6-9 days
Monkey	MPX	Yes	Macaque	Intravenous	~10^7 pfu	Mortality; lesion count; viral load in blood, liver, spleen, lymphoid tissue	100% in 7-14 days
	Variola	No	Macaque	Intravenous	~10^8 pfu	Mortality; lesion count; viral load in blood, lung, lymphoid tissue	33% in 8-11 days

VV, vaccinia virus; ECTV, ectromelia virus; CPX, cowpox virus; RPV, rabbitpox virus; MPX, monkeypox virus.

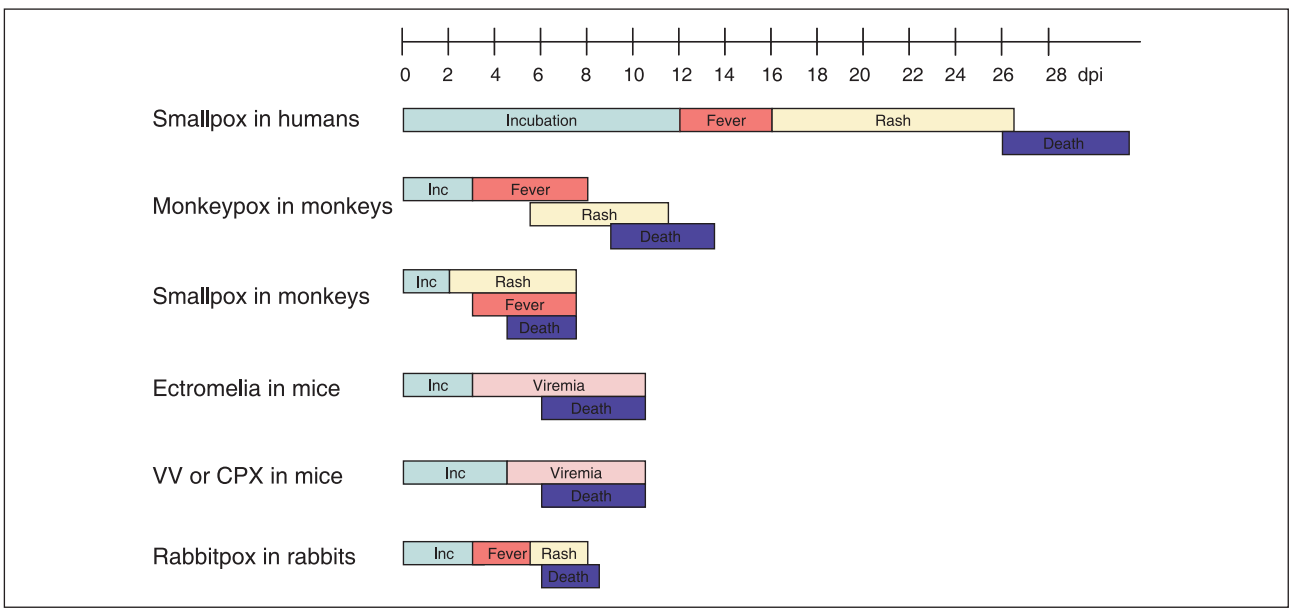


Fig. 2. The pathogenesis of *Orthopoxvirus* disease in various animal models. VV, vaccinia virus; CPX, cowpox virus.

Rabbit model

Rabbits infected with rabbitpox virus develop a disease similar to that seen in humans infected with smallpox. Virus can be administered via aerosol spray to the nasal cavity or by intradermal injection of the footpad. Following inoculation, there is a 2-4-day incubation period during which virus replicates in the mucosa or local lymph tissue, followed by the onset of clinical disease characterized by fever, weight loss, discharge from the nose and eyes, and a generalized skin rash (83). Viral load peaks between days 5 and 8 in the lung and spleen (84). Animals experience severe respiratory distress and death is preceded by a drop in body temperature and typically occurs before scabs can form over the lesions (83). Rabbitpox can be transmitted to uninfected cage mates

via aerosol, which is a unique feature to this model system. Antiviral efficacy is measured by a reduction in viral load, anorexia and mortality, and improvement of hematological scores.

Ground squirrel and prairie dog models

Monkeypox virus has a broad host range that includes nonhuman primates, rabbits, many common laboratory animals, wild rodents including prairie dogs, ground squirrels, Gambian rats and mice, as well as humans.

Ground squirrels (*Spermophilus tridecemlineatus*) native to North America are susceptible to monkeypox virus infection producing a lethal disease from intranasal, intraperitoneal (i.p.) or subcutaneous (s.c.) inoculation with as little as 1 plaque-forming unit (pfu) of virus (85, 86). Animals develop a fulminant systemic disease, with death occurring 6-9 days postinoculation. Necropsy examinations demonstrate the appearance of necrotic lesions in the liver and spleen, and interstitial inflammation in the lungs. Virus can be cultured from the blood and from the oropharynx several days before death. Antiviral efficacy in this model can be measured by reduction in mortality, change in body weight, viral load, blood chemistry, histopathology and immunohistochemistry.

During the outbreak of monkeypox virus infection in the U.S. in 2003, most of the patients had a history of contact with sick pet prairie dogs. Like ground squirrels, prairie dogs (*Cynomys ludovicianus)* are susceptible to monkeypox virus infection and lethal disease results from intranasal infection, although the time to death is slightly longer than in ground squirrels at 11 days postinoculation (87, 88). Necropsy of infected animals showed hepatic and splenic necrosis with some inflammatory changes in the lungs. However, with i.p. inoculation, mortality is not uniform (~60%), and with both routes the degree of pathology observed was less than that seen in ground squirrels, suggesting that monkeypox infection is more severe in the ground squirrel model (85).

Nonhuman primate models

Monkeypox virus infection of nonhuman primates produces a range of disease severity depending on viral dose in the inoculum, with administration of ~10^7 pfu of virus to either cynomolgus macaques or rhesus macaques via intratracheal inoculation or i.v. injection producing a lethal disease characteristic of the lesional disease observed with monkeypox or smallpox infection of humans (89, 90). Administration of less virus, ~10^6 pfu, results in nonlethal but severe monkeypox, with a rash that progresses to disseminated exanthema, while delivery of more virus, ~10^8 pfu, results in hemorrhagic disease that is rapidly fatal before development of a generalized exanthema can occur (90). After challenge with ~10^7 pfu of virus, the monkeys develop a generalized vesiculopustular rash, with other characteristics of disease including fever, elevated white blood cell count, lymphadenopathy, splenomegaly and pulmonary edema, and death occurring between 7 and 15 days postinfection (90). Antiviral efficacy is measured by decreased viral load, lesion count and mortality, as well as improvement of selected hematological parameters.

In the past, various attempts have been made to induce clinical disease in animal models infected with variola virus without much success (36). Variola virus has a narrow host range and is highly adapted to replicate in humans. Cynomolgus monkeys can be infected with variola virus, but in order to produce a lethal disease resembling smallpox, large quantities of virus administered by i.v. injection must be used (91). Injection of 10^9 pfu of variola virus results in a fulminant hemorrhagic disease with elevated white blood cell count, fever, anorexia, cough and skin lesions similar to those observed in humans infected with smallpox, with death occurring between 3 and 7 days postinfection (92). Injection of less virus results in a disseminated infection with lesion formation and reduced mortality. Antiviral efficacy is measured by decreased viral load, lesion count and mortality, as well as improvement of selected hematological parameters. However, due to i.v. administration of virus, the disease course bypasses the incubation and prodromal phase typical of human smallpox and establishes instant viremia. Thus, the opportunity for effective intervention is short and there is some question as to how antiviral efficacy in this model would correlate with human disease.

Therapy

For FDA approval, a drug must be designated for a specific indication, which for an antiviral agent can be: 1) a preexposure prophylactic, given before exposure to the virus to prevent disease; 2) a postexposure prophylactic, given immediately after exposure to the virus in order to prevent disease; or 3) a therapeutic, given after exposure to reduce disease severity. Although no drug is currently approved for the treatment of smallpox, there are several compounds that have shown varying degrees of efficacy at preventing or ameliorating disease in both animal models of disease and in humans (Table IV).

Methisazone

Methisazone, *N*-methylisatin-β-thiosemicarbazone, known under the trade name Marboran, is an antiviral agent that was used in the 1960s to treat complications of smallpox vaccination or for prophylaxis against variola infection. Previously produced by Burroughs Wellcome, it was the first antiviral drug for the treatment of smallpox to be used in humans, although it is no longer produced and not available for clinical use. While the exact mechanism of action is unknown, in vitro studies showed that methisazone inhibited the synthesis of a late structural vaccinia virus protein, thereby inhibiting viral assembly (93). More specifically, the mechanism of action has been suggested to be at the level of viral transcriptional termination (94). Methisazone was stated to be protective against smallpox if administered within 3 days after expo-

Table IV: Protective efficacy of antivirals against Orthopoxvirus.

Animal	Virus	Methisazone	Vaccinia immune globulin	Imatinib mesilate	Canertinib	Cidofovir	CMX001	ST-246
Mouse	VV	10 mg/kg i.p. o.d. x 5 days; 100% survival (94)	5 mg i.p. at 24 h postinfection; 25% survival (131)	100 mg/kg[4] continuously by s.c. pump x 15 days; 100% survival[5] (106)	50 mg/kg[6] i.p. o.d. x 8 days; 100% survival (102)	100 mg/kg i.p.; 100% survival (123)	5 mg/kg by oral gavage o.d. x 5 days; 86% survival (122)	100 mg/kg by oral gavage o.d. x 14 days; 100% survival (123)
	ECTV	12.5 mg/kg s.c. b.i.d. x 5 days; 33% survival (132)	ND	ND	ND	100 mgkg i.p.; 100% survival (123)	3 mg/kg by oral gavage o.d. x 5 days; 28% survival (121)	50 mg/kg by oral gavage b.i.d. x 14 days; 100% survival (123)
	CPX	30 mg/kg i.p. o.d. x 5 days; 0% survival (94)	6 mg/kg i.p. at 24 h postinfection; 0% survival (84)	ND	ND	15 mg/kg i.p. o.d. x 5 days; 100% survival (124)	3 mg/kg by oral gavage o.d. x 5 days; 100% survival (125)	100 mg/kg by oral gavage o.d. x 14 days; 100% survival (124)
Rabbit	RPV	ND	ND	ND	ND	ND	5 mg/kg by oral gavage b.i.d. x 5 days; 100% survival (80, 83)	100 mg/kg by oral gavage o.d. x 14 days; 100% survival
Ground squirrel	MPX	ND	ND	ND	ND	ND	ND	100 mg/kg by oral gavage o.d. x 14 days; 100% survival (126)
Monkey	MPX	50 mg/kg[1] every 6 h x 5 days orally[2]; 67% survival[3] (133, 134)	ND	ND	ND	5 mg/kg i.p. every other day x 14 days; 83% survival (89)	0% survival (80)	3 mg/kg by oral gavage o.d. x 14 days; 100% survival
	Variola	ND	ND	ND	ND	ND	ND	300 mg/kg by oral gavage o.d. x 14 days; 100% survival (127)

VV, vaccinia virus; ECTV, ectromelia virus; CPX, cowpox virus; RPV, rabbitpox virus; MPX, monkeypox virus; ND, no published data found. [1]200 mg/kg 8 h postinfection, 100 mg/kg 0 h postinfection and +4 h postinfection, and then 50 mg/kg every 6 h. [2]Orally by gastric feeding tube. [3]Only 2 of the 3 control monkeys died; there was no difference in clinical features between treated and untreated monkeys. [4]Treatment initiated 24 h prior to infection. [5]The infectious virus dose was close to the LD_{50}, so not all control mice died. [6]Treatment initiated 6 h prior to infection.

sure to the virus (93). Side effects of the drug included nausea and vomiting in 10-65% of the drug recipients (95), making accurate drug dosing complicated. Previous efficacy studies in animal models and human clinical trials produced variable results, prompting Quenelle et al. to reevaluate the effect of methisazone against vaccinia and cowpox virus infections in vitro and in vivo (94). They found that methisazone was effective in reducing viral replication in vitro and in protecting mice systemically infected with VV by intranasal inoculation, but was not effective at protecting mice infected with cowpox or mice infected cutaneously with either VV or cowpox.

Vaccinia immune globulin

Vaccinia immune globulin (VIG) is a solution of immunoglobin (IgG) manufactured from the plasma of individuals who have been previously immunized with vaccinia virus that confers passive immunity to the recipient. VIG has been used in two injectable forms, either intramuscular (i.m.) or intravenous (i.v.), and has been approved by the FDA (the i.v. form is currently approved; the i.m. form was previously approved but has returned to IND status [96]) for the treatment of severe complications due to vaccinia virus. Side effects are generally mild and can include back pain, chills, headache, muscle pain, joint pain, itching, weakness, fever, nausea, vomiting, abdominal cramps, changes in blood pressure, dizziness, shortness of breath and wheezing, and it may also cause allergic reactions that can be serious to life-threatening (97). There have been numerous reports of the use of VIG for the treatment of vaccine complications, several reports for the prophylaxis of vaccine complications among at-risk individuals, or for the prevention of smallpox among exposed individuals. However, these studies did not include a placebo control so a direct conclusion on efficacy is difficult (98).

Canertinib and imatinib

Typically, antiviral drug development focuses on targeting virus-specific proteins or functions, although host cellular pathways that are essential for viral replication can also be targeted for antiviral development. Targeting a host pathway would be less likely to lead to the development of antiviral drug resistance, although issues of toxicity could be a concern. Two anticancer drugs –canertinib (CI-1033) and imatinib (Gleevec)– that are tyrosine kinase inhibitors have recently been shown to block the release of vaccinia virus from infected cells and to promote survival of vaccinia-infected mice.

Canertinib, a 4-anilinoquinazoline, is a protein tyrosine kinase inhibitor that selectively targets the ErbB receptor family that mediates physiological growth factor signaling (99). Poxviruses encode epidermal growth factor (EGF)-like growth factors to facilitate viral replication, a property that may explain the major skin manifestations observed with most *Orthopoxvirus* infections (100). Variola virus encodes the smallpox growth factor (SPGF), which binds to ErbB-1 to stimulate host cells, thereby increasing viral replication (101). The viral activation of ErbB-1 also results in the subsequent phosphorylation of c-Src, which then goes on to phosphorylate the viral membrane protein A36, triggering actin tail formation and viral egress (102-105). Canertinib selectively binds ErbB-1, effectively blocking the activity of SPGF. In vitro, canertinib was able to reduce the size and comet formation but not the number of variola virus plaques, suggesting that the drug is inhibiting the release of EEV particles. In vivo, mice challenged intranasally with close to the LD_{50} of VV and treated with canertinib prior to infection showed an increase in survival and a slight decrease in viral load in the lung (102).

Imatinib a 2-phenylaminopyrimidine derivative and tyrosine kinase inhibitor marketed by Novartis as Gleevec, is licensed for use in chronic myeloid leukemia (CML) and has also recently been shown to inhibit the release of EEV particles from vaccinia virus-infected cells. In vivo, mice given imatinib continuously via an s.c. pump and challenged with i.p. inoculation of vaccinia virus 1 day after the start of drug treatment showed a 5-log decrease in viral load in the ovaries at 4 days postinfection. In mice challenged intranasally with close to the LD_{50} of VV and treated with imatinib via s.c. pump prior to infection, an increase in survival was observed (106).

Cidofovir and CMX001

Cidofovir is a nucleoside analogue whose 5'-diphosphorylated metabolite is recognized by viral DNA polymerases and terminates DNA chain elongation (107). Cidofovir (Vistide™) is licensed for the treatment of cytomegalovirus (CMV) retinitis in HIV-infected patients, but it and its analogue, hexadecyloxypropyl (HDP)-cidofovir, also known as CMX001, have shown strong inhibition of poxvirus infections both in vitro and in vivo (108-116). Cidofovir has been shown to be effective in treating mice infected with cowpox virus (82, 114, 117) or vaccinia virus (82, 118), and monkeys infected with monkeypox virus (89). In fact, postexposure treatment with cidofovir was shown to be more protective against lethal monkeypox challenge than postexposure vaccination (89). An investigational new drug (IND) application was filed for cidofovir in 2003 which allows for its emergency use in the event of an outbreak; however, the therapeutic effectiveness of cidofovir is limited by its poor oral bioavailability, restriction to i.v. use and nephrotoxicity in vivo (119). In an effort to eliminate these shortcomings, several ether lipid esters of cidofovir were synthesized (115), which showed enhanced bioavailability due to their similarity to a natural compound, lysophosphatidylcholine, which is primarily absorbed intact in the small intestine (120). CMX001 was shown to be active in vitro against vaccinia and cowpox viruses and in vivo in murine (121, 122) and rabbit models (83) of *Orthopoxvirus* disease, and is currently in clinical development (115, 121).

ST-246

ST-246 is a novel, orally bioavailable, low-molecular-weight compound developed by SIGA Technologies that can be given prophylactically, postexposure prophylactically and therapeutically to prevent or treat *Orthopoxvirus* infection in numerous animal models, including mice infected with either vaccinia virus, cowpox virus or ectromelia virus (123-125), ground squirrels infected with monkeypox virus (126) and monkeys infected with monkeypox virus or variola virus (127), as well as rabbits infected with rabbitpox virus. ST-246 targets the *VO61* gene in cowpox, a homologue of the vaccinia virus *F13L* gene, to prevent the production of extracellular enveloped virus, thus preventing egress and spread of the virus

(123). This unique mechanism of action provides protection from infection without interfering with the development of a protective immune response, suggesting that ST-246 may also be useful in combination with the smallpox vaccine to improve safety and reduce vaccine-related complications (128). In a phase I clinical trial, ST-246 was found to be readily absorbed after oral administration, well tolerated with no severe adverse events and to provide blood exposure levels predicted to be sufficient for inhibiting *Orthopoxvirus* disease (129).

Combination therapy

Drug resistance is a common shortcoming of antiviral therapy. The likelihood of treatment failure caused by the development of resistance is low for DNA viruses, especially for an acute disease like smallpox, since DNA-containing viruses have lower mutation rates compared to RNA-containing viruses and the pool of naturally occurring resistant variants is low (1 in 10^6) for any given population of virus. In order to minimize the risk of treatment failure caused by drug resistance, combination therapy using two antiviral drugs with different mechanisms of action will be necessary. Preliminary studies have shown that a combination of CMX001 and ST-246 produced a synergistic antiviral effect against poxvirus infection both in vitro and in vivo (125). Thus, using two drugs that act by different mechanisms may provide adequate protection against human *Orthopoxvirus* infections. This strategy was used recently in the case of a 28-month-old child with atopic dermatitis who developed severe eczema vaccinatum after exposure to vaccinia virus from his father, a military recruit, who was recently vaccinated. Upon admission, the child was listed in critical condition and was administered VIG daily beginning on day 6 postadmission, one dose of cidofovir (5 mg/kg) with probenecid on day 8 postadmission and increasing doses of ST-246 from 5 mg/kg to 10 mg/kg via nasogastric tube on days 9-22 postadmission. The child's condition improved upon administration of ST-246 and the child was discharged from the hospital 48 days after admission (130).

Summary

The global eradication campaign has not eliminated smallpox as a threat to human health. Coupled with the potential threat of an engineered poxvirus and the emergence or re-emergence of other types of poxviruses, there is clearly a need for the development of effective antiviral therapies. Development of antiviral therapeutics for smallpox poses unique regulatory challenges since smallpox is no longer endemic in the human population. Development of these antiviral therapeutics will require the use of a combination of animal models that can link antiviral efficacy to human disease outcome. Two clinical candidates, CMX001 and ST-246, show promising results in early studies and continued development may lead to viable therapeutic options for the treatment of pathogenic *Orthopoxvirus* infections.

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