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MINIREVIEW

Drug Development against Smallpox: Present and Future

Déborah Delaune,[a] Frédéric Iseni[a]

[a]Unité de Virologie, Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France

ABSTRACT Forty years after the last endemic smallpox case, variola virus (VARV) is still considered a major threat to humans due to its possible use as a bioterrorism agent. For many years, the risk of disease reemergence was thought to solely be through deliberate misuse of VARV strains kept in clandestine laboratories. However, recent experiments using synthetic biology have proven the feasibility of recreating a poxvirus *de novo*, implying that VARV could, in theory, be resurrected. Because of this new perspective, the WHO Advisory Committee on VARV Research released new recommendations concerning research on poxviruses that strongly encourages pursuing the development of new antiviral drugs against orthopoxviruses. In 2018, the U.S. FDA advised in favor of two molecules for smallpox treatment, tecovirimat and brincidofovir. This review highlights the difficulties to develop new drugs targeting an eradicated disease, especially as it requires working under the FDA "animal efficacy rule" with the few, and imperfect, animal models available.

KEYWORDS FDA animal rule, antiviral, smallpox

The world's last case of endemic smallpox occurred in 1977 in Somalia (1). A year later, a lab-acquired infection was reported at the Birmingham University Medical School (2). Since then, there has been no other report of smallpox anywhere in the world, and the World Health Organization (WHO) officially declared the disease to be eradicated in 1980 (3). As a result, by 1984, all countries had ceased vaccinating the general population against smallpox. Additionally, all stocks of variola virus (VARV; genus *Orthopoxvirus*), the causative agent of smallpox, present in research laboratories had to be destroyed. The remaining VARV strains were only kept in two repositories, the State Research Center for Virology and Biotechnology Vector, Koltsovo, Russian Federation, and the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. In September 2001, terrorist attacks, followed by envelopes contaminated with *Bacillus anthracis* spores, drew attention to possible bioterrorist risks. VARV was considered a potential biological weapon candidate and was classified as a category A agent by the CDC because of its high mortality rate, person-to-person transmissibility, impact on public perception, and the need for special preparedness (4). The weak vaccination coverage of the population (in particular, people under 30 years old) was another reason to fear such a misuse. In 2002, the World Health Assembly encouraged research on VARV diagnosis and medical countermeasures. Thus, many countries developed research on new vaccines or treatments and prepared plans to respond to a potential bioterrorist attack. In June 2015, the Independent Advisory Group on Public Health Implications of Synthetic Biology Technology Related to Smallpox provided a report to the WHO underlying the risk of recreation of a VARV strain using synthetic biology (5). To prevent this risk, the WHO decided to limit access to VARV DNA to ≤20% of the whole genome for research laboratories except for the two WHO Collaborating Centers. The year 2018 was a turning point in the debate concerning VARV and its potential use as a biological weapon. First, Noyce et al. demonstrated the feasibility of creating an extinct poxvirus *de novo* using DNA synthesis technologies (6), implying that the risk of smallpox recurring can never be excluded. Second, the U.S. Food and Drug Adminis-

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Address correspondence to Déborah Delaune, deborah.delaune@intradef.gouv.fr.

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tration (FDA) advised in favor of the following two molecules for smallpox treatment: tecovirimat was approved for treatment of the disease in adults and pediatric patients, and brincidofovir (BCV) received the Orphan Drug Designation. These major events are the trigger of this review, which aims to expose the difficulties of developing new treatments against an eradicated disease (for which animal models are few and imperfect), the current pharmacopeia, and the most promising molecules.

HOW TO DEVELOP A TREATMENT AGAINST SMALLPOX?

An ideal treatment to be used in a bioterrorist response context should be (i) orally administered, (ii) safe for special populations (i.e., children, immunocompromised individuals, etc.), (iii) inexpensive to allow for large stockpiles, and (iv) stable over long periods even under adverse conditions (7). The development of drugs against VARV is scarce because smallpox was eradicated; thus, clinical trials cannot be fully completed. Furthermore, work with live virus is only authorized in the two repositories where the virus is kept. Consequently, drug discovery must rely on surrogate viruses and validated animal models. The FDA animal efficacy rule defines recommendations on drugs and biological product development when human efficacy studies are not ethical or feasible (8, 9). In addition to demonstrating safety in humans, the rule requires validation of the following criteria: the agent's pathophysiological mechanisms of action and the way the drug inhibits them have to be well understood; the treatment must show efficacy in at least two animal models; the criteria for validation in the animal models should be close to the expected benefit in humans; and an effective dose in humans should be correctly determined with available pharmacokinetic and pharmacodynamic data in humans and animal models (10). Since humans are the only natural hosts of VARV and no animal presents a disease similar to smallpox, the establishment of a relevant animal model requires the determination of infection conditions with VARV or another orthopoxvirus responsible for a disease similar to smallpox. Thus, the development of such models requires a strong knowledge of the human disease.

Smallpox, the disease. Interhuman transmission of VARV occurs via respiratory droplets, cutaneous lesions, infected body fluids, or fomites. Airborne transmission, however, is the most important route of transmission. A recent meta-analysis estimates an R_0 (average number of secondary cases generated by a single primary case) for VARV of between 1.5 and 10, with a mean of 4.5 (11). After infection, the incubation period lasts about 12 days (range, 7 to 17 days [12]). An early transient viremia is followed by virus replication in regional lymph nodes and later in the reticuloendothelial system (spleen and bone marrow). A second viremia occurs simultaneously with the first clinical symptoms of fever and pains. Three to 4 days later, the eruptive stage begins with a centrifugal generalized macular rash. An enanthem is observed in the mucous membranes, especially on the tongue and palate. Viruses excreted from these oropharyngeal lesions are responsible for the pulmonary transmissibility. Skin lesions progress from macules to papules and then vesicles and pustules. Eruption is denser on the face and the extremities of the limbs. Pustules become umbilicated and change to scabs. When scabs desquamate, they can cause depigmented areas and scars, especially on the face, where pustules are numerous due to the viral tropism for sebaceous glands. Complications can happen and are due to secondary bacterial infections, including pneumonia or skin bacterial infection, swelling eyelids, keratitis, corneal ulceration, arthritis, and demyelinating encephalitis (13). In fatal cases, death occurs between days 10 and 16. Main sequelae are keratitis, corneal ulcers, and scars. The classical form of smallpox, also known as variola major, is responsible for 30% to 40% mortality. Two other clinical forms of smallpox have been described. The malignant or hemorrhagic form (more frequent in pregnant women) is associated with near 100% mortality and characterized by massive hemorrhages. In contrast, variola minor is a less severe disease with fewer cutaneous lesions and a mortality rate of about 2% (13).

The exact cause of death in smallpox infection is not well understood. In 1904, autopsies of 54 patients who died from different clinical forms of smallpox were performed (14). An epithelial degeneration was observed with a serous exudation that

resulted in vesicle formation. Mucous membranes had different characteristics, with limited exudation at the beginning, and then edema, erosions, or submucosal hemorrhages. Lungs presented bronchopneumonia, atelectasis, edema, abscesses, ecchymosis, or hemorrhages. Enlargement of the liver with larger and more granular cells was reported with, in some cases, liver degeneration or necrosis. Nephritis and glomerulonephritis were noted, but no modification was observed for the pancreas. Spleen and lymph node changes were relatively moderate, mainly consisting of enlargement due to edema. These observations, while unable to determine the precise cause(s) of death, are essential information for developing an animal model.

Animal models. Animal model development has to take into account all the previous observations when selecting relevant animal-virus pairings. Of note, establishing a relevant animal model with airborne infection proved difficult. Indeed, initial experiments realized in Asian macaques infected with aerosolized VARV resulted in only mild clinical consequences (15, 16). In contrast, intravenous inoculation of VARV induced a classic or hemorrhagic type of smallpox disease depending on the administered dose (17). Thus, alternative routes of inoculation (i.e., intraperitoneal and intravenous) were often used even if they require the use of high infectious doses and bypass the first viremia, resulting in a shorter disease course (17).

Nonhuman primates (NHP) infected with monkeypox virus (MPXV) were also assessed as smallpox model. MPXV, which is not genetically the closest known virus to VARV (18), is responsible for a rare zoonotic disease similar to human smallpox but with milder symptoms and lower lethality (case fatality is between 1% and 10%) (19). It is mostly transmitted to people from different wild animals such as rodents and primates. Marmosets challenged intravenously with high doses of MPXV developed a disease fairly similar to human hemorrhagic smallpox after 3 days of incubation. The absence of primary viremia and typical cutaneous lesions, such as macules or vesicles, are limitations of this model however. Reducing the administered viral dose delayed the incubation period to 9 days, similar to the human disease, but the cutaneous lesions remained atypical (20).

Cowpox virus (CPXV) is responsible for cutaneous lesions in humans after contact with infected wild rodents or domestic animals (21, 22). The genomic variability of CPXV is large, and several viral clades can be distinguished (23). Surprisingly, NHP were shown to develop a disease similar to smallpox when infected with some strains of CPXV; calpox virus, responsible for a lethal infection in New World monkeys, gives clinical features similar to those of smallpox in marmosets when administered intranasally. The absences of serological markers for very low infectious doses and systematic lethality in infected animals are some limitations of this model (24). Intrabronchial inoculation with CPXV (Brighton Red strain) in cynomolgus macaques resulted in an infection with only a few differences from what is seen with VARV infection in humans. There were fewer cutaneous lesions, and pulmonary symptoms were more severe. Nevertheless, target organs, viremia, histopathology results, and development of neutralizing antibodies were similar to those in VARV or MPXV infections (25). These models are a significant step forward for the development of medical countermeasures, especially because of their biosafety level 2 (BSL-2) experimental conditions.

As part of the investigation for a relevant animal model, mice were also evaluated as a potential model of smallpox. Inoculation of VARV in immunocompetent (ICR) or immunodeficient (SCID) mice mimics the first step of smallpox with similar primary target cells and morphopathological changes in the respiratory tract. However, the following steps of infection diverge from those of smallpox. Thus, this model is more suitable for prophylactic treatment evaluation (26). CAST/EiJ mice were shown to be highly sensitive to an intranasal lethal infection with MPXV, displaying high viral loads in the lungs (27). This mouse strain was evaluated more recently for intranasal VARV inoculation, in which virus was detected in oral secretions, as shown for MPXV, but only mild clinical symptoms were reported (28). Other models that were developed include intranasal or aerosol infection route of BALB/c mice with CPXV strain Brighton Red (29),

intranasal inoculation of BALB/c mice with vaccinia virus (VACV), the virus used as a vaccine for smallpox eradication (30, 31), and cutaneous inoculation of VACV in hairless mice (SHK-1 strain; as a model for progressive vaccinia virus infection in immunocompetent patients) and hairless SCID mice (SHO strain; mimicking infection in immunocompromised patients) (32). Ectromelia virus (ECTV), the causative agent of mousepox, is a good surrogate for smallpox study. This virus is a mouse-specific pathogen responsible for a severe disease that resembles human smallpox in certain mouse species (A/Ncr, BALB/c, DBA/2, and C3H/He) and subclinical infection in other species (C57/BL6, SKH1, and AKR) (33). The natural route of infection with ECTV is thought to be through cutaneous contact with an infected animal. Consequently, the major experimental inoculation route for this virus is the footpad. Other routes of inoculation in sensitive mice species are intranasal, intravenous, intraperitoneal, and subcutaneous. Some species resistant to infection through the footpad route are sensitive to the intranasal route (C57BL/6) (34). In this case, the pathogenesis is similar to that with smallpox, with two steps of viremia. Major limitations in using ECTV as a surrogate virus are a shorter course of disease and the major lesions in the liver and spleen that are not observed in human smallpox.

Rabbitpox virus (RPXV) was first isolated in 1932 when an epidemic of highly lethal airborne infection occurred in laboratory rabbits in New York (35). Although closely related to VACV (36), it causes a severe disease in its natural host, with pox-like skin lesions and a high rate of mortality. Recently, rabbits infected with RPXV were shown to be an interesting animal model because the virus could be propagated between rabbits through aerosol or direct contact (37). Clinical features depend on the infectious dose; when the viral load is low, the disease resembles the human smallpox classical form, whereas a higher dose leads to a clinical presentation similar to the hemorrhagic form (38). The major limits of this model are the uncontrollability of animal breathing during virus exposure and the fluctuating RPXV stability when aerosolized (39). In contrast, intradermal inoculation of RPXV seems to be a more reproducible model exhibiting biomarkers useful for evaluating therapeutic interventions (40). Due to the difficulties of developing a relevant animal model, candidate antiviral molecules are usually evaluated in multiple models. This was the case for the two recently FDA-approved molecules.

FDA-APPROVED MOLECULES FOR THE TREATMENT OF SMALLPOX

Tecovirimat. Tecovirimat (TPOXX), first named ST-246, is a small synthetic molecule selected from a high-throughput screening of more than 350,000 chemical compounds tested *in vitro* against VACV (41). The molecule was shown to inhibit VARV, VACV, CPXV, MPXV, ECTV, and camelpox virus (CMLV) at submicromolar concentration (41–43), and cellular cytotoxicity was $>50\ \mu$M in several cell lines, including human lines (42). Tecovirimat inhibits the production of extracellular viruses by interacting with the *F13L* gene product, which encodes a phospholipase involved in the formation of a protein complex that catalyzes the envelopment of intracellular mature virus particles (44). Resistant viruses mutated in *F13L* were selected in culture (45) and displayed small plaque size *in vitro*. There was no information obtained concerning virulence or fitness *in vivo*, however. Tecovirimat has occasionally been used for treatment of eczema vaccinatum (46) or accidental vaccination of an immunocompromised patient (47). Of note, a resistant virus strain was described in a patient with progressive vaccinia and treated with immunoglobulins and antivirals, including tecovirimat at suboptimal concentrations at the beginning of the treatment (48). Tecovirimat showed potent efficacy in multiple animal models. Oral administration of the drug protected BALB/c and A/NCr mice that received intranasal inoculation of VACV and ECTV, respectively. Naval Medical Research Institute mice inoculated via the tail vein with VACV were also protected (41). Tecovirimat efficacy was shown in NHP infected with VARV (49) or MPXV (50) and for postexposure prophylaxis in rabbits inoculated with aerosolized RPXV (51).

The drug can be administered orally and was shown to be well tolerated and safe in phase I clinical trial studies (52). Extrapolating pharmacokinetic and pharmacody-

namic studies in animals, a regimen treatment (single oral dose of 400 mg or 600 mg per day during 14 days) was proposed for humans and validated for tolerability (53). Recently, a large study was conducted to assess tecovirimat efficacy in smallpox treatment according to the FDA animal rule; efficacy and pharmacokinetics were studied in NHP (infected with MPXV) and rabbit (infected with RPXV) models. Treatment showed great efficacy when administered up to 5 days after exposure, at the onset of symptoms. Moreover, a phase I study realized in adults aged between 18 and 79 years old allowed the validation of a dose regimen of 600 mg twice daily with a good safety profile (54). More recently, Russo et al. reported 100% survival in macaques infected with a lethal dose of aerosolized MPVX when tecovirimat was administered up to 5 days after challenge and 50% when administered 8 days after challenge (55). In July 2018, the FDA approved tecovirimat for the treatment of smallpox disease in adults and pediatric patients weighing $\geq$13 kg (56, 57).

Brincidofovir. Brincidofovir (BCV) (hexadecyloxypropyl-cidofovir [HDP-CDV]), originally named CMX001, is a lipid conjugate of cidofovir (CDV) (58). CDV, an acyclic nucleoside phosphonate discovered in 1986, has a broad antiviral activity against several DNA viruses (59). The drug, which is approved for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients, requires intravenous administration because of poor oral bioavailability. The compound had also been developed into a topical formulation to treat immunocompromised patients with extensive molluscum contagiosum (60). Its efficacy has been shown in several mouse (61–65), NHP (66–68), and rabbit (69) models. Despite limitations due to intravenous administration and nephrotoxicity, CDV was the first antiviral drug integrated in national stockpiles for facing a possible VARV resurgence. However, industrial synthesis of CDV was stopped in 2013 because of production issues, decreased prevalence of retinitis in AIDS patients, and the existence of alternative therapies.

The efficacy of BCV was shown to be higher than that of CDV due to increased cellular uptake and better conversion to the active form by intracellular enzymes (70). BCV enters into cells using the endogenous lipid uptake pathways. CDV is then released by cleavage and converted to CDV diphosphate (CDVpp) by intracellular kinases. The incorporation of CDVpp in a growing DNA strand results in slower incorporation of the next nucleotide before a complete block of DNA synthesis (71). The DNA synthesis inhibition by CDVpp was also shown to impair genome encapsidation and virion assembly (72). Viral strains resistant to CDV were isolated and characterized. All of the mutations were located in the *E9L* gene encoding the viral DNA polymerase (73) and were associated with a decrease in virulence.

BCV displayed better antiviral activity than did CDV against VARV, VACV, MPXV, and CPXV *in vitro* (70, 74). Tested against several VACV strains, the efficacy of BCV was found to be at least 25-fold higher than that of CDV. While cellular toxicity was higher for BCV, its better efficacy led to a greater selective index (75), allowing further drug development.

BCV has proven its efficacy in two animal models that follow the U.S. FDA animal efficacy rule criteria for the treatment of smallpox. An aerosol challenge of ECTV in mice was successfully treated with BCV up to 5 days after inoculation (34, 76). BCV also protected New Zealand White rabbits from mortality following intradermal infection (77) or aerosolized challenge (78) with RPXV. Efficacy was demonstrated when treatment began after the first cutaneous lesions or onset of fever (38). Efficacy was also shown with a treatment delay of 48 h in the same rabbit/RPXV model (79).

The advantages of BCV are its oral bioavailability and the absence of nephrotoxicity, as demonstrated by a pharmacokinetic study in a mouse model (80). Indeed, no accumulation of BCV was observed in the kidneys. Because of its efficacy against several double-stranded DNA viruses, BCV has been approved for the treatment of immunocompromised patients with or at risk of infection with CMV or adenovirus (ADV), in particular, hematopoietic cell transplant recipients. A phase I study showed mild adverse effects, such as gastrointestinal events, and low transient elevation of transami-

nases. Phase II and III studies performed in immunocompromised adults and children with or at risk of infection with CMV or ADV showed the same type of adverse events, as well as good safety (81). These results are in favor of the use of BCV at a dose regimen of 200 mg/week during 3 weeks in adults for the treatment of smallpox. Furthermore, safety and tolerability profiles allow its use in immunocompromised patients and children. Today, BCV is available as oral capsules, and an intravenous formulation is in development. In October 2016, the European Drug Agency gave favorable opinion for BCV to obtain the status of an orphan treatment for smallpox. BCV received the Orphan Drug Designation from the FDA for the same reasons in June 2018.

OTHER PROMISING ANTIPOXVIRUS COMPOUNDS EVALUATED *IN VIVO*

Numerous compounds have shown *in vitro* antiviral activity against poxviruses. However, few of them have been tested *in vivo*. Given the importance of evaluating new drugs in animal models (according to the FDA animal rule), we present here the most promising molecules whose efficacy was evaluated *in vivo*.

Nucleoside analogue inhibitors have been extensively studied for their activity against poxviruses. Several of their prodrugs or derivatives were tested *in vitro,* with contrasting results (82–86); however, three molecules showed antiviral activity in animal models. *N*-Methanocarbathymidine [(*N*)-MCT], a thymidine analog, was first described for its activity against herpesviruses. Its antiviral activity is mediated by its triphosphate metabolite, whose formation is dependent on a viral thymidine kinase (TK). (*N*)-MCT showed efficacy against VACV in a mouse model of respiratory infection with a treatment regimen of 100 to 500 mg/kg of body weight twice a day, depending on the VACV strain used (87). Efficacy was also observed in BALB/c mice infected intranasally with CPXV (strain Brighton Red) (88). The second molecule that showed efficacy against VACV and CPXV *in vitro* is the 4′-thio derivative of idoxuridine (4′-thioIDU). This molecule retained its antiviral activity against viral strains resistant to CDV or tecovirimat (89). 4′-thioIDU is likely phosphorylated by the viral thymidine kinase (89). The drug was highly effective in preventing mortality in mice inoculated intranasally with VACV or CPXV and treated intraperitoneally (87% survival for a dose of 1.5 mg/kg twice a day beginning 3 days postinfection) (90). More recently, KAY-2-41 (1′-carbon-substituted 4′-thiothymidine derivative) was shown to be efficient against VACV, CPXV, and CMLV *in vitro* (91). Its efficacy was greater than that of CDV but lower than that of BCV or tecovirimat. KAY-2-41 also retained activity on viral strains resistant to CDV. Selection of viruses resistant to KAY-2-41 identified mutations in the viral TK which were responsible for the resistance. However, only a mild resistance level was observed. *In vivo* studies showed protection (100% survival and no morbidity) in mice inoculated intranasally with VACV at a dose of 50 mg/kg once per day intraperitoneally (91).

NIOCH-14 (a derivative of tricyclodicarboxylic acid and a precursor of tecovirimat) is also effective *in vitro* against VARV, MPXV, and ECTV (92). No significant difference with tecovirimat was found when administered in an ICR mouse model infected intranasally with ECTV. One hundred percent of the animals were protected when the treatment was administered up to 2 days postexposure; however, a significant difference in survival rate was observed for later administration (60%, 6 days postinfection). After drug treatment, viral load was significantly reduced in target organs such as the lungs and nose. Similar effects were obtained in ICR mice infected with VARV or MPXV (92). Finally, NIOCH-14 was evaluated in a marmot model used for MPXV treatment evaluation. Its efficacy was similar to that of tecovirimat when used at 40 mg/kg once per day (92). Owing to its potent activity against several orthopoxvirus infections and considering the fact that it is easier to produce than is tecovirimat, NIOCH-14 remains a relevant antiviral candidate for the future and retains the WHO's attention (93).

CONCLUSION

Despite smallpox eradication after a long global vaccination campaign, VARV remains one of the most relevant viruses in the context of bioterrorism. This is particularly

true since the possibility of recreating a poxvirus using synthetic biology was demonstrated in 2018, even if it constitutes a technical challenge that could only be realized by teams that are highly knowledgeable in molecular biology and advanced technologies. Thus, improving preparedness by increasing the number of laboratories with diagnostic capacity, developing point-of-care diagnostic tests, and raising clinicians' awareness for smallpox diagnosis (in particular, by distinguishing it from chickenpox) are some of the WHO recommendations (94). For prophylactic or therapeutic aspects, the development of third-generation vaccines (minimizing potential adverse effects) that can be administered to immunocompromised people is a major step forward. Nonspecific treatment, such as vectored interferon, could also be an option for first responders and medical personnel in the event an orthopoxvirus is used as a bioterrorist weapon (95).

The development of new drugs is difficult, time-consuming, and expensive. In the case of an eradicated disease (like smallpox), the situation is even more complex and does not appear worthy of sustained interest by pharmaceutical companies due to a poor cost-benefit. Thus, the recent FDA approval of tecovirimat and brincidofovir to treat a potential smallpox reemergence is a key achievement on this front. Nevertheless, efforts to develop new antivirals are necessary to anticipate the appearance of resistant viral strains. It is possible to deal with this issue by preparing strategies based on multiple therapies, or combining drugs with different targets and mechanisms of actions. The WHO Advisory Committee on Variola Virus Research recommended continuing research on drugs with promising antiviral activities using surrogate orthopoxviruses in relevant animal models. Compounds with good efficacy and low toxicity should be considered for completing the current pharmacopoeia. As mentioned above, NIOCH-14 and some nucleoside analogue inhibitors are in advanced development and may be future candidates for the treatment of poxvirus infections.

Apart from the threat of smallpox reemergence due to intentional release of VARV, the emergence of orthopoxviruses is still possible, particularly in the current human population with very low vaccine coverage. MPXV is a zoonotic virus responsible for a disease resembling smallpox with frequent complications and sequelae in naive populations. Since its first description in 1972 in the Democratic Republic of the Congo (96), MPXV infections have been frequently reported in East Africa (97) and West Africa (98). Risk due to MPXV emergence was heightened in 2003 when an outbreak occurred in the United States (99). There, wild rodents imported from Ghana contaminated prairie dogs, which then became a reservoir for human infection. Other orthopoxviruses, such as CPXV and VACV, are responsible for cutaneous lesions, and recent outbreaks due to VACV were reported in South America (100, 101). Finally, CMLV is responsible for outbreaks of a smallpox-like disease in camels in the Middle East, Asia, and Africa. Cases of human infections with CMLV have also been described (102). These biological risks constitute a supplementary argument to pursue the development of new antipoxvirus molecules.

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