REVIEW ARTICLE

Annals of Agricultural and Environmental Medicine 2013, Vol 20, No 1, 1-7
www.aaem.pl

Research on substances with activity against orthopoxviruses

Marcin Kołodziej[1], Justyna Joniec[1], Michał Bartoszcze[1], Romuald Gryko[1], Janusz Kocik[2], Józef Knap[3]

[1] *Biological Threats Identification and Countermeasure Center, Military Institute of Hygiene and Epidemiology, Puławy, Poland*
[2] *Military Institute of Hygiene and Epidemiology, Warsaw, Poland*
[3] *Department of Epidemiology, Medical University, Warsaw, Poland*

Kołodziej M, Joniec J, Bartoszcze M, Gryko R, Kocik J, Knap J. Research on substances with activity against orthopoxviruses. Ann Agric Environ Med. 2013; 20(1): 1-7.

Abstract

Although smallpox was eradicated over 30 years ago, the disease remains a major threat. High mortality, high infectivity and low resistance of the contemporary population make the smallpox virus very attractive to terrorists. The possible presence of illegal stocks of the virus or risk of deliberate genetic modifications cause serious concerns among experts. Hence, it is reasonable to seek effective drugs that could be used in case of smallpox outbreak. This paper reviews studies on compounds with proven *in vitro* or *in vivo* antipoxviruses potential, which show various mechanisms of action. Nucleoside analogues, such as cidofovir, can inhibit virus replication. Cidofovir derivatives are developed to improve the bioavailability of the drug. Among the nucleoside analogues under current investigation are: ANO (adenozine N_1-oxide) and its derivatives, N-methanocarbothymidine [(N)-MCT], or derivatitives of aciklovir, peninclovir and brivudin. Recently, ST-246 – which effectively inhibits infection by limiting release of progeny virions – has become an object of attention. It has been also been demonstrated that compounds such as: nigericin, aptamers and peptides may have antiviral potential. An interesting strategy to fight infections was presented in experiments aimed at defining the role of individual genes (E3L, K3L or C6L) in the pathogenesis, and looking for their potential blockers. Additionally, among substances considered to be effective in the treatment of smallpox cases, there are factors that can block viral inhibitors of the human complement system, epidermal growth factor inhibitors or immunomodulators. Further studies on compounds with activity against poxviruses are necessary in order to broaden the pool of available means that could be used in the case of a new outbreak of smallpox.

Key words

Orthopoxvirus, smallpox, antivirals

INTRODUCTION

The smallpox virus (VARV – variola virus) is dsDNA virus belonging to the *Poxviridae* family, genus *Orthopoxvirus*. This genus also contains other viruses: vaccinia virus (VACV), cowpox (CPXV), monkeypox (MPXV), camelpox (CMLV) and ectromelia virus (ECTV) [1, 2]. Humans are susceptible to the viruses: variola, vaccina, cowpox and monkeypox [3, 4].

Among all known infectious diseases, smallpox caused the greatest number of deaths worldwide [3]. The disease has two main forms: variola major, with mortality rate reaching 30%, and variola minor, with mortality rate below 5% [1, 3, 5]. Variola virus can spread by respiratory droplets or by direct contact. Smallpox is a disease characteristic of humans. There has been no reports on any animal or insect species that would serve as reservoirs or vectors of the virus [3].

In the past, vaccination was the basic method of prevention and control of smallpox. A mass vaccination campaign conducted under the auspices of the World Health Organization (WHO) in 1967-1977 made the world free of the disease. Eradication of smallpox was announced in 1980, abolishing the need for further vaccination [1]. The majority of existing smallpox virus stocks were destroyed, leaving the remainder only in the possession of two WHO laboratories: Centers for Disease Control and Prevention in Atlanta (USA) and (after a transfer in 1994) the Russian State Research Center of Virology and Biotechnology (the Vector Institute) in Novosibirsk [4, 5, 6].

The cessation of immunization has resulted in lack of immunity to smallpox virus in the present population. This situation raises concerns regarding the possibility of purposeful use of smallpox virus as a biological weapon in bioterrorist attacks [6, 7]. It is suspected that there are still illegal stocks of the virus that could be used in the studies on its genetic modifications, which could potentially lead to dangerous infections, even in vaccinated individuals [5, 6, 7].

In Poland, the last cases of smallpox occurred in Wrocław, Silesia, in 1963, when the disease was diagnosed in 99 people, causing the death of 7 patients. Extensive means of security, such as mass vaccination and restrictions on transport of people, were undertaken to prevent the spread of the epidemic [8].

In the USA in 2003, a small epidemic of monkeypox broke out, a disease that may be fatal to humans. This event has shown that monkeypox virus or other animal poxviruses are able to break the species barrier and become a human pathogen [4, 9].

Since none of the anti-smallpox vaccines are regarded entirely safe, seeking new antiviral agents that could protect people in the case of a natural outbreak of epidemic, or deliberate use of smallpox virus, becomes a priority. A pool of

Address for correspondence: Marcin Kolodziej, Biological Threats Identification and Countermeasure Centre, Military Institute of Hygiene and Epidemiology, Lubelska 2, 24-100 Puławy, Poland
E-mail: mkolodziej@wihe.pulawy.pl

Received: 29 March 2012; accepted: 24 September 2012

drugs with various mechanisms of action may be crucial for the rapid and effective response of a national health service in case of the emergence of drug-resistant or genetically-modified virus strains [5, 7].

In smallpox, symptomatic treatment was usually used, and in the case of secondary bacterial infections, antibiotics were administrated. The smallpox vaccine used in the past belongs to the most dangerous vaccines because of the high probability of severe complications [6, 9, 10]. Symptoms such as eczema on vaccinated skin, systemic vaccinia, gangrenous vaccinia or postvaccinal encephalitis have been reported.

Cidofovir and other nucleoside analogues. For years, great importance in combating viral infections has been attributed to nucleoside analogues that can impair viral replication [5, 11]. In 2001, the US Food and Drug Administration (FDA) approved the use of cidofovir (HPMPC – (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) in the case of a smallpox outbreak [7, 12, 13]. The anti-smallpox activity of cidofovir in humans has not been fully described because the drug appeared after eradication of the disease. However, its effectiveness has been demonstrated in poxvirus infection in mice after intravenous, subcutaneous, topical, intranasal (as spray) and oral (as lipid prodrug) administration [14]. It has been confirmed that it is effective against vaccinia, cowpox, monkeypox, moluscum contagiosum and ecthyma. Cidofovir can be administered to people by the intravenous injection of an aqueous solution, but changing the formulation also allowed the use of the drug topically in the form of gels or creams, as well as in the form of an aerosol for inhalation or a lipid pro-drug (HDP-HPMPC) for oral administration [14, 15]. Cidofovir is officially registered as a medicine against CMV retinitis in patients with AIDS. It also shows activity against other viruses: HSV (type 1 and 2, varicella zoster, Epstein-Barr, and type 6, 7 and 8), polyoma, human papillomavirus, adenovirus, and poxvirus.

Although vaccinations are considered to be the best way to prevent smallpox in humans, cidofovir may be useful in preventing smallpox infection shortly before exposition or in the post-exposure treatment [14]. Cidofovir enters cells in the process of endocytosis and is then converted to its monophosphate (HPMPCp) and diphosphate (HPMPCpp) forms (half-life time 17-65 h), which compete with dCTP (deoxycytidyne triphosphate), a substrate of viral DNA polymerase. Inside the cells, it also forms a complex with choline (HPMPCp-choline, half-life time 87 h), which serves as a reservoir of cidofovir and promotes the long-lasting activity of the drug [14].

The usefulness of cidofovir in the treatment of poxvirus infections in animals has been confirmed in a study by Smee *et al.* [15], who demonstrated that intranasal infection with virulent strain of VACV caused pneumonia, weight loss and death in BALB/c mice. The researchers reported that a single intraperitoneal injection of cidofovir at a dose 100 or 30 mg/kg 24 h after exposure resulted in 90-100 % protection of animals, compared to the placebo group. The drug at a dose of 10 mg/kg did not influence the course of infection. At a dose of 100 mg/kg, 20- and 8-fold reduction in viral titer in the lung and oral cavities was observed on the third day of infection, when average blood oxygen saturation was significantly higher, indicating recovery in lung function. A statistically significant reduction in consolidation of lung tissue and inhibition of weight loss were noted.

Therapeutic potential of cidofovir was also confirmed by Baker *et al.* [5]. From 24 known antiviral compounds they isolated eight: cidofovir (HPMPC), cyclic cidofovir (cHPMPC), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine (HPMPA), ribavirin, tiazofurin, carbocyclic 3-deazaadenosine, 3-deazaneplanocin A and DFBA (a derivative of adenosine N_1-oxide), which inhibited *in vitro* replication of poxvirus strains VARV-BSH, VARV-YAM, VARV-GAR, VACV, CPXV and MPXV. The three most active compounds, cidofovir, cHPMPC and ribavirin, were also examined for the occurrence of drug resistance in 35 strains of poxviruses from different geographical regions. It was shown that the tested strains have similar sensitivity to these drugs without showing resistance to any of them. Additionally, cidofovir reduced viral titer in tissues and body fluids of animals.

Due to the poor oral bioavailability of cidofovir, its toxicity to kidneys and the occurrence of resistant strains [13], the search for analogues lacking these defects is still continuing [12]. Several ester conjugates of cidofovir with lipids, such as hexadecylopropanediol (HDP-HPMPC) and octadecylethanediol (ODE-HPMPC) were tested. They were designed so that they resemble natural lipids absorbed in the small intestine. In contrast to cidofovir, these compounds penetrate effectively into the blood and cells, yielding a weaker concentration in the kidneys than HPMPC. Their availability after oral administration and lesser nephrotoxicity make them useful drugs for the treatment of smallpox [7, 14]. Smee *et al.* [16] found that HDP-HPMPC given once orally at doses 100, 50 and 25 mg/kg protected 80-100% of animals infected intranasally with VACV. Lower doses (10 and 5 mg/kg) administered for five days protected 30% of animals. HOE961 (diacetate ester prodrug of 2-amino-7-[(1,3-dihydroxy-2propoxy)methyl]purine) administered orally for five days (100, 50 and 25 mg/kg) protected all animals, and the rate of recovery of the body weight depended on the drug dose. The obtained results indicate that oral administration of HDP-HPMPC or HOE961 has important therapeutic effects in the treatment of serious respiratory infections caused by poxviruses.

Keith *et al.* [17] studied the activity of pro-drugs of cidofovir (HPMPC), adefovir (PMEA), tenofovir (TDF, PMPA) and their cyclic and ester derivatives with regard to their ability to inhibit replication of VACV and CPXV. The most active compounds were derivatives of HPMPC and PMEA (while PMEA did not show activity against the viruses). The derivatives of tenofovir did not show greater activity than PMPA.

Sauerbrei *et al.* [18] studied *in vitro* (plaque assay) the activity of cyclic pronucleotides and acyclic nucleoside analogues, especially aciclovir, penciclovir, brivudin analogues, and phenolic polymers in the fight against VACV and CPXV infection. Among the chosen substances at doses non-toxic for Vero cells, three derivatives of aciclovir, one penciclovir derivative and two derivatives of brivudin appeared active. From 13 phenolic polymers, only a product of the oxidation of caffeic acid showed weak activity. Their action, however, was weaker in comparison with the reference compound – cidofovir, while their cytotoxicity was greater.

In order to find compounds with activity against poxviruses [6] hybridization of pyrazolon and pyrimidine nucleoside scaffolds were conducted, which are the basis of many drugs and biologically-active molecules that have

anti-inflammatory, antibacterial and anti-HSV properties. Two compounds created this way, 5-substituted pyrimidine nucleosides, showed activity against VACV and CPXV in *in vitro* studies.

Kane and Shuman [19] used an *in vitro* model to study the impact of ANO, i.e. adenosine N_1-oxide on synthesis of macromolecules of vaccinia virus. They noted that both viral DNA replication and late viral protein synthesis were completely blocked after the use of the investigated drugs. Early viral proteins also were not synthesized in the presence of ANO, despite the viral mRNA synthesis.

Khandazhinskaya *et al.* [20] studied ANO derivatives, such as N_1-alkoxy and N_6-alkyl, as well as analogues having a trihydroxycyclopentane ring instead of ribose, for their activity against poxviruses. The study was conducted in Vero and LLC-MK2 cells, which were infected with viruses: VACV, CPXV, MPXV, and ECTV. The activity of the ANO and its derivatives depended on the type of virus and cell line. ECTV and MPXV appeared to be the most sensitive to these compounds. Modifications of ANO at position N_6 did not cause an increase of effectiveness, and the N_1-derivative also had activity comparable to the original compound. Both derivatives, however, were significantly less cytotoxic.

Smee *et al.* [21] studied the effectiveness of N-methanocarbathymidine [(N)-MCT] in the treatment of infections caused by two strains of vaccinia virus – WR and IHD. (N)-MCT is a carbocyclic analogue of thymidine, which has been shown to inhibit replication of poxviruses *in vitro* and *in vivo*. Its activity *in vivo* was studied in comparison with cidofovir after oral and intraperitoneal administration. It was found that (N)-MCT was less toxic than cidofovir. (N)-MCT was effective in the treatment of infections in mice, its antiviral activity after oral and intraperitoneal administration was equal, but it was less effective than cidofovir in reducing viral titer in the lungs, oral cavity and brain of infected animals. Both compounds were capable of blocking the spread of viruses in liver and spleen. These results are promising, but it is advisable to investigate the action of (N)-MCT in primates, as it turned out that it was less active in monkey and human cells than in murine cells.

There are also reports describing the activity of cyclopentynyl nucleosides: adenine, cytosine and 5-F-cytosine regarding their anti-viral properties. Chu *et al.* [22] showed in an *in vitro* study that these compounds could effectively inhibit viral replication.

Non-nucleoside compounds with anti-poxvirus activity. Another well-characterized compound with activity against the vaccinia virus is a low molecular (376 Da) and non-toxic (CC_{50} > 40 μM) compound ST-246 (Tecovirimat) [23]. Its ability to inhibit virus spread from cell-to-cell has been confirmed in numerous studies *in vitro* and *in vivo* [23, 24, 25].

Quenelle *et al.* [24] tested ST-246 in *in vitro* and *in vivo* assays for potential against CPXV, VACV and ECTV. *In vitro* studies revealed that IC_{50} of the compound were: 0.48 μM, 0.05 μM and 0.07 μM, respectively. It was shown that ST-246 was more effective than cidofovir. *In vivo* studies confirmed the efficacy of ST-246 administered once a day for 14 days to mice infected with CPXV, beginning from 4th hour and 72nd hour after infection. When the compound was administrated to VACV-infected mice at a dose of 100 mg/kg for five days, a significant reduction in mortality was observed, even when treatment started 24 hours after exposure. The reduction was also noticed in CPXV and VACV replication in the liver, spleen and kidneys. ST-246 administration to animals infected with ECTV once a day for 10 days prevented them from death, even when the drug was given 72 hours after infection, with proven absence of the virus in liver, lungs and spleen. The bioavailability after oral administration and lack of side effects give strong evidence of a great therapeutic potential of ST-246.

Studies have been undertaken to investigate the specific targets of ST-246 action. In the CPXV strain that was resistant to ST-246, 1-aa mutation in V061 gene was discovered. Correction of this mutation restored susceptibility to the drug, which indicates that the V061 gene is a target site of ST-246. V061 has its counterpart in VACV (F13L gene), which encodes a large envelope protein (p37) necessary for the release of progeny virions [23]. *In vitro* studies on ST-246 revealed that it could inhibit the cytopathic effect and reduce 10-fold titer of released virions. Administration after intranasal infection with IHD-J strain of VACV protected mice from death. After secondary oral administration to animals which survived the disease it was observed that they were immune to intranasal infection. ST-246 also protected the mice infected with 40,000 × LD_{50} of ECTV, and the virus titer in organs after eight days of treatment was below the detection limit (10 PFU/ml), in contrast to negative control where virus titer remained high. Local lesions after caudal administration of the virus were also limited.

Inhibitors of enzymes. Silverman *et al.* [26] specifically designed their experiments to be able to isolate compounds that inhibit viral polymerase (E9) or affect the factors responsible for its processivity (A20 and D4). DNA polymerase of VACV demands two additional factors for effective DNA synthesis: A20 (putative DNA polymerase processivity factor) and D4 (uracil DNA glycosylase). The researchers succeeded in selecting a compound that inhibits DNA polymerase (NSC 55 636), and a compound that affects its processivity (NSC 123 526). Their activity has been demonstrated in plaque assay, with low toxicity. The selected compounds may have great therapeutic potential in cases of smallpox, due to the high sequence similarity between genes E9, A20 and D4 of VACV, and their counterparts in VARV.

Other studies have identified a compound having no nucleoside scaffold, which did not inhibit virus replication, but affected synthesis of late proteins [27]. Sequencing of mutants resistant to its action revealed a mutation in the peripheral region of a highly-conserved viral RNA polymerase subunit, indicating that this is the target site of this compound.

Thiosemicarbazones. Some potential in fighting poxvirus infections is attributed to long-known compounds, thiosemicarbazones, which have been used in the treatment of people exposed to VARV, and to relieve the side-effects of vaccination [28]. Numerous *in vitro* and *in vivo* tests were carried out, but no conclusive results about the actual effectiveness of these substances were obtained [29, 30, 31]. Quenelle *et al.* [32] tried to investigate two compounds from this group: isatin-β-thiosemicarbazone (IBT) and N-methyl-isatin-β-thiosemicarbazone (marboran, methisazone) in cells and mice. Preparations were administered intraperitoneally to animals infected intranasally with VACV or CPXV, or

by scarification. The compounds turned out to be active in the treatment of VACV infection, but marboran was more effective. In the case of CPXV, only the highest concentration of marboran reduced mortality. This compound also caused a reduction in VACV titers in organs, but in the case of CPXV, such an effect was not observed. Virus titer in swabs from infected skin was also not decreased. Lack of explicit limitation of virus replication confirms speculations [33] that thiosemicarbazones do not affect viral DNA polymerase (like cidofovir does). They rather limit transcription by interacting with RNA polymerase [34, 35].

Nigericin. Myskiw *et al.* [9] examined nigericin, an antibiotic and ionophore (chemical compound having the ability to connect to biological membranes and facilitate the exchange of monovalent cations for protons), for antiviral activity. The compound proved to be a potent inhibitor of VACV replication in the human cell lines HeLa, A549 and Huh7. In spite of the fact that there is no data about the mechanism of nigericin action, the results should be regarded as extremely promising.

Peptides and peptide aptamers. It is reported that peptides may also have antiviral potential [11, 36]. To combat viral infections, Saccucci *et al.* [12] used aptamers specific to A20 protein, a component of VACV replication complex. Aptamers are short pieces of DNA, RNA or peptides binding specifically to target agents, which can be aminoacids, antibiotics, peptides, proteins, viruses or whole cells. They are increasingly used as a useful tool in diagnostics and treatment [37]. The binding and blocking of A20 protein inside the replication complex can affect the synthesis of viral DNA. One of selected aptamers showed the ability to interfere with synthesis of viral DNA in cells and formation of progeny virions. These results may be a breakthrough in research on finding new compounds that inhibit the replication of VACV [12].

Altmann *et al.* [38] studied the activity of EB peptide against VACV. The peptide consists of 16-aa sequence of human fibroblast growth factor and an additional 'tag' of four soluble aminoacids. *In vitro* studies revealed that the activity of the peptide against vaccinia virus is associated with blocking virus-cell fusion and inhibition of virus penetration into host cells.

Imiquimod. Tarbet *et al.* [39] evaluated *in vivo* antiviral activity of imiquimod, an immunomodulator which helps to stimulate macrophages and monocytes to produce interferon-α and cytokines. It is used to treat genital warts and some forms of skin cancer: basal-cell carcinoma or squamous-cell carcinoma. The study was conducted on hairless mice with immunosuppression, which were infected with vaccinia virus by scarification (giving 25 ml of virus (2.5×10^5 PFU) on damaged skin-sites). In the first group of animals, damaged skin was treated with a cream containing 1% imiquimod, in the second group a cream with 1% cidofovir was applied. Mortality, skin lesions and viral titer were assessed in tissue samples taken at various times after infection. It was found that mice treated with imiquimod for 3, 4 and 5 days, and with cidofovir for 7 days lived longer by 4.5, 5, 4, and 5 days, compared with the control group. In all groups of animals a significant reduction in skin lesions was observed. Treatment with cidofovir and imiquimod delayed the appearance of the virus in lungs.

Phospholipids. Perino *et al.* [40] examined the surface phospholipids from lungs, i.e. PC (phosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine), PG (phosphatidylglycerol), DPPG (dipalmitoylphosphatidylglycerol) and PE (phosphatidylethanolamine) in terms of their impact on infection with poxviruses, using two VACV strains, Lister (VACV-List) and Western Reserve (VACV-WR) in the experiment. In preliminary studies on interaction between the viruses and phospholipids, DPPG was selected because of its strongest ability to associate with the viruses. Studies on human A549 cells demonstrated that after pre-incubation of VACV with SUVs (SUVs are phospholipids organized in small unilamellar vesicles, allowing their use in the aquatic environment), inhibition of infection occurred only in the case of DPPG-SUV (titer reduction 40% for VACV-WR and 45% for VACV-List). It was shown that both the pre-incubation of virus with DPPG-SUV and pre-treatment of cells with DPPG-SUV inhibited the development of infection. The interaction between virus and DPPG-SUV relies on the roundup of viral particles by phospholipids, which was observed with the use of electron microscopy. The results of experiments on murine lung epithelial cells confirmed the ability of DPPG to inhibit infection, which encourages further research.

Since the mice infected intranasally with VACV-WR pre-incubated with SUV-DPPG survived after the challenge with lethal dose of virus, it was suggested that dipalmitoylphosphatidylcholine plays an important role in natural protection of animals against poxviruses. Further research may allow the use of this strategy in the fight against infections with this virus group.

New directions of research on ways of fight poxvirus infections. The deficiency of effective antiviral drugs and high frequency of genetic changes occurring in the genomes of pathogens cause an urgent need to increase efforts to develop new ways to combat the most dangerous viral infections. Many researchers are engaged in the search for new molecular factors which could be targets for new potential pharmaceuticals. Enzymes and structural proteins important for virulence are agents which currently attract scientists' attention..

EGFR inhibitors. EGFR is a protein receptor located on the surface of cells and is activated by binding of specific ligands (e.g. EGF – epidermal growth factor, VGF – vaccinia virus growth factor). The epidermal growth factor receptor is composed of three domains: extracellular, membrane and intracellular that has an activity of tyrosine kinase. Stimulation of EGFR leads to autophosphorylation of the tyrosine kinase, located in the intracellular part of the receptor, followed by activation of intracellular signaling pathways which, through the involvement of some transcription factors, induce the expression of genes affecting proliferation, adhesion, migration, differentiation and cell survival [41, 42]. The growth factor encoded by the vaccinia virus, which is similar to VARV growth factor, stimulates host cells via the EGFR signaling pathway (being a ligand for EGFR), facilitating viral replication and spread in host cells. Knowledge about the participation of EGFR in the pathogenesis of vaccinia virus provides new molecular targets for the design of new antiviral drugs. Langhammer *et al.* [43] studied the activity of small molecule compounds

PD153035, Vandetanib and Gefitinib inhibiting tyrosine kinase of EGFR. Gefitinib proved to be the most effective of these compounds. *In vitro* studies on epithelial cells infected with VACV or CPXV showed dose-dependent reduction in the size and number of plaques. Blocking of phosphorylation of EGFR resulted in abolition of signaling, and thus limited the spread of poxviruses.

Impact on E3L gene. The E3L gene, which is crucial for the pathogenesis of smallpox, has also become the object of many research investigations [44, 45, 46]. It is a key gene for resistance of VACV to interferon and it is necessary for replication of the virus in many hosts [45]. Expression of the E3L gene is observed at early stages of infection [45]. E3L encodes a 190-aa protein that consists of two domains: C-terminal dsRBD – a highly conserved domain that determines the resistance to IFN and is able to bind dsRNA, inhibiting antiviral dsRNA-dependent kinase, and the N-terminal Zα – a domain that binds Z-DNA.

Brandt *et al.* [44], in their studies on mice, attempted to explain whether both domains are essential for disease development. In their experiments they used recombinant VACV strains devoid of whole gene encoding protein E3 (VVΔE3L), or sequences encoding its N- or C-end. It was shown that intranasal infection with wild type of virus resulted in 50% falls in mice and weight loss, while infection with VVΔE3L did not lead to the disease. It was also found that both domains of E3 protein are required for full development of symptoms in animals, although *in vitro* assays showed that Zα domain was not necessary for virus multiplication.

In order to assess neurovirulence, VVΔE3L was administered directly to the brain by intracranial injection. VACV wild type (strain WR, a highly neurovirulent strain) spread systemically, whereas VVΔE3L was found only in the respiratory system, which proves that lack of the E3L gene reduces infection in mice [44].

Kwon and Rich [45] investigated the role of E3L as a regulator of the transcription of genes related to apoptosis and immune response. After experiments on HeLa cells, they came to the conclusion that E3L inhibits apoptosis induced by hygromycin B, which is a consequence of Z-DNA binding by the Zα domain. They also found that E3L activates transcription of human genes IL-6, NF-AT (nuclear factor activating T cells), p53, and that Z-DNA binding by the N-terminal domain of E3L is essential for its activity. These results suggest that the role of E3L in the pathogenesis of VACV relies on the modulation of gene expression in host cells.

Rice *et al.* [46] investigated the role of E3L and K3L genes of vaccinia virus, as well as the functions of proteins PKR and RNase L, which are key elements of the mouse response to intratracheal infection. To determine the function of the genes, they exploited recombinant VACV strains with deletion of K3L or E3L gene VVΔK3L, VVΔE3L, and mice in which genes PKR and Rnase L were suppressed individually or simultaneously. In poxvirus infection, interaction between E3L and K3L genes, which regulate the expression of RNase L and PKR host genes, respectively, is used to prevent dsRNA-dependent induction of interferon. Synthesized by VACV, dsRNA is a potent activator of two IFN-induced antiviral enzymes, i.e. PKR and RNase L. PKR activated by interaction with dsRNA is capable of inhibiting proteins in the infected cell. It was shown that VACV caused fatal disease in all tested strains of mice. Animals with a single suppressed gene, RNase L or PKR, were more susceptible to disease when compared to wild-type animals, whereas animals with a double gene knockout were the most susceptible to infection. Infected with a recombinant virus VVΔE3L, wild-type mice were insensitive to infection, suggesting that E3L plays a key role in controlling host immune response.

RNase L(-) mice did not show any symptoms of disease, while 20% of PKR(-) mice died. In contrast, all RNase L(-) PKR(-) mice died. After infection with a recombinant VVΔK3L strain there were no differences in the course of the disease in any of murine constructs, suggesting that PKR is not the sole target of K3L. Since the VVΔK3L strain did not spread from the lungs to other tissues, it is believed that the cause of death in this experimental model was disease of the respiratory system. These results suggest that K3L gene facilitates the spread of the viruses.

Many viruses have developed strategies to overcome host immune responses, e.g. poxviruses evolved mechanisms to minimize the effects of interferon, inactivate the complement system, or to prevent activation of NK cells [47]. By binding of dsRNA, which is synthesized in early stages of infection, the C-terminal domain of E3 protein prevents activation of cellular effector particles – PKR and Rnase L. It is likely that the mechanism to counteract RNA interference also belongs to repertoire of viral defence strategies.

Studies by Li *et al.* [48] on insect cells showed that expression of E3 protein caused disturbance in RNA interference. Lantermann *et al.* [49] attempted to clarify the influence of E3 on RNA interference in mammalian cells (HeLa, 293T) and revealed that the expression of a marker gene of VACV was effectively inhibited by siRNAs, independently of the presence of E3 protein. The authors suggest that these results, different from the results by Li *et al.* [48], may be associated with different cellular systems used in experiments. Attempts to determine the effectiveness of siRNA targeting E3L gene were also undertaken by Dave *et al.* [47]. *In vitro* studies showed that VACV replication was inhibited in 97% in HeLa cells and 98% in 293T cells, which suggests that a strategy based on silencing E3L gene expression can be an effective approach to fight poxvirus infections.

Impact on C6 protein. In order to identify new viral proteins that may be involved in inhibiting of signaling pathways of innate immunity, Unterholzner *et al.* [50] investigated the role of functional proteins of VACV-WR strain and early C6 protein, which is present in nucleus and cytoplasm. Analysis showed that C6 inhibited PRRS-induced secretion of IFN β. PRRS are receptors that recognize viral DNA or RNA. It was also found that mutants with a deletion of C6L gene, or viruses mutated in a way that did not allow the gene to be expressed can replicate normally in cell culture, whereas *in vivo* in comparison with wild strains, they showed weaker virulence. C6 protein, conservative in most of poxviruses, is therefore an attractive target to design or seek new antiviral substances.

Inhibition of complement system. The pathogenesis of smallpox has still not been sufficiently investigated. Lack of advanced research techniques in the days before eradication of disease meant that we do not have full knowledge about mechanisms that contribute to the high mortality of smallpox. Genes, which formerly were not considered important, may

affect the immune system of the host and sharpen severe symptoms. It is possible that among them there are genes related to inhibition of the activity of INF, NK cells and the complement system [47, 51].

The complement system may be affected by particular viral genes that encode specific inhibitors (PICEs – poxviral inhibitors of complement enzymes). It is an innate immune mechanism that can recognize, bind and eliminate pathogens, and infected cells by lysis [52]. The action of complement system is under the control of host genes, encoding inhibitors of the enzymatic cascade. These genes have probably been 'hijacked' by poxviruses during their evolution and have become a part of their genome. Liszewski *et al.* [53] compared the activity of inhibitors of the complement system originating from various poxviruses: SPICE – from VARV, VCP (VICE) – from VACV, and MOPICE – from MPXV. SPICE appeared to be the most effective PICE. Scientists who have been trying to characterize regulation of the complement system found the close analogy between sites of SPICE that bind membrane glycosaminoglycans (e.g. heparin) and binding sites present in host's inhibitors. Chimerical combination of SPICE and VCP turned out to be 200 times more potent than VCP. L131 residue seems to be critical for the activity of both factors. Antibodies that neutralize PICEs *in vivo* were also developed [53].

SUMMARY

The search for effective drugs against the smallpox virus is now an urgent need due to a possible use of the virus in bioterrorist attacks. In light of the fact that prophylactic vaccination ceased after eradication of smallpox, pharmaceuticals may be beneficial in the case of an emergency. The present review describes the groups of preparations which give the most promising antiviral effects in the research investigations, raising hope for their introduction as therapeutic agents in the near future.

REFERENCES

1. Niemiałtowski M, Szulc L, Boratyńska A, Martyniszyn L, Struzik J, Karandys J. Ospa prawdziwa: patrząc wstecz od Edwarda Jennera, a nawet trochę dalej *(Smallpox: looking back to Edward Jenner and even a little further).* Post Mikrob. 2009; 48(4): 289-298 (in Polis).
2. Duraffour S, Meyer H, Andrei G, Snoeck R. Camelpox virus. Antivir Res. 2011; 92(2): 167-186.
3. Henderson DA, Inglesby TV, Bartlett JG, Ascher MS, Eitzen E, Jahrling PB, et al. Smallpox as a biological weapon. JAMA. 1999; 281: 2127-2137.
4. Johnson RF, Yellayi S, Cann JA, Johnson A, Smith AL, Paragas J, et al. Cowpox virus infection of cynomolgus macaques as a model of hemorrhagic smallpox. Virology. 2011; 418: 102-112.
5. Baker RO, Bray M, Huggins JW. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antivir Res. 2003; 57: 13-23.
6. Fan X, Zhang X, Zhou L, Keith KA, Kern ER, Torrence PF. A pyrimidine-pyrazolone nucleoside chimer with potent *in vitro* anti-orthopoxvirus activity. Bioorg Med Chem Lett. 2006; 16: 3224-3228.
7. Painter GR, Hostetler KY. Design and development of oral drugs for the prophylaxis and treatment of smallpox infection. Trends Biotechnol. 2004; 22(8): 423-427.
8. Fenner F, Henderson DA, Arita I, Ježek, Ladnyi ID. Smallpox in non-endemic countries. In: Fenner F, Henderson DA, Arita I, Ježek, Ladnyi ID. Smallpox and its eradication. WHO, Geneva 1988.p.1069-1101.
9. Myskiw C, Piper J, Huzarewich R, Booth TF, Cao J, He R. Nigericin is potent inhibitor of the early stage of vaccinia replication. Antivir Res. 2010; 88: 304-310.
10. Aquino LL, Wu JJ. Cutaneous manifestations of category A bioweapons. J Am Acad Dermatol. 2011; 65(6): 1213.e1-1213.e15.
11. Joniec J, Kołodziej M, Bartoszcze M, Kocik J, Knap J. Research on prevention and treatment of hemorrhagic fevers. Ann Agr Env Med. 2012; 19(2): 165-171.
12. Saccucci L, Crance J-M, Colas P, Bickle M, Garin D, Iseni F. Inhibition of vaccinia virus replication by peptide aptamers. Antivir Res. 2009; 82: 134-140.
13. Becker MN, Obraztsova M, Kern ER, Quenelle DC, Keith KA, Prichard MN, et al. Isolation and characterization of cidofovir resistant vaccinia viruses. Virol J. 2008; 5(58).
14. De Clercq E. Cidofovir in the therapy and short-term prophylaxis of poxvirus infections. Trends Pharmacol Sci. 2002; 23(10): 456-458.
15. Smee DF, Bailey KW, Wong M-H, Sidwell RW. Effects of cidofovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice. Antivir Res. 2001; 52: 55-62.
16. Smee DF, Wong M-H, Bailey KW, Beadle JR, Hostetler KY, Sidwell RW. Effects of four antiviral substances on lethal vaccinia virus (IHD strain) respiratory infections in mice. Int J Antimicrob Ag. 2004; 23: 430-437.
17. Keith KA, Hitchcock MJM, Lee WA, Holý A, Kern ER. Evaluation of Nucleoside Phosphonates and Their Analogs and Prodrugs for Inhibition of Orthopoxvirus Replication. Antimicrob Agents Ch. 2003; 47(7): 2193-2198.
18. Sauberei A, Meier C, Meerbach A, Schiel M, Helbig B, Wutzler P. *In vitro* activity od *cyclo*Sal-nucleoside monophosphates and polyhydroxy-carboxylates against othopoxvirus. Antivir Res. 2005; 67: 147-154.
19. Kane EM, Shuman S. Adenosine N_1-Oxide Inhibits Vaccinia Virus Replication by blocking Translation of Viral Early mRNAs. J Virol. 1995; 69(10): 6352-6358.
20. Khandazhinskaya AL, Shirokova EA, Shipitsin AV, Karpenko IL, Belanov EF, Kukhanova MK *et al.* Adenosine $\boldsymbol{N}_1$-Oxide Analogues as Inhibitors of Orthopox Virus Replication Collect Czech Chem C. 2006; 71(7); 1107-1121.
21. Smee DF, Hurst BL, Wong M-H, Glazer RI, Rahman A, Sidewell RW. Efficacy of N-methanocarbathymidine in treating mice infected intranasally with the IHD and WR strains of vaccinia virus. Antivir Res. 2007; 76: 124-129.
22. Chu CK, Jin YH, Baker RO, Huggins J. Antiviral Activity od Cyclopentenyl Nucleosides Against Orthopox Viruses (Smallpox, Monkeypox and Cowpox). Bioorg Med Chem Lett. 2003; 13: 9-12.
23. Yang H, Pevear DC, Davies MH, Collett MS, Bailey T, Rippen S, et al. An Orally Bioavailable Antipoxvirus Compound (ST-246) Inhibits Extracellular Virus Formation and Protects from Lethal Orthopoxvirus Challenge. J Virol. 2005; 79(20): 13139-13149.
24. Quenelle DC, Buller RML, Parker S, Keith KA, Hruby DE, Jordan R, et al. Efficacy of Delayed Treatment with ST-246 Given Orally against Systemic Orthopoxvirus infections in Mice. Antimicrob Agents Ch. 2007; 51(2): 689-695.
25. Huggins J, Goff A, Eric M, Twenhafel N, Chapman J, Tate M, et al. Successful Treatment in the Monkeypox and Variola Primate Models of Smallpox by the Oral Drug ST-246. Antivir Res. 2007; 74: A1-A74(A35).
26. Silvermann JEY, Ciustea M, Shudofsky AMD, Bender F, Shoemaker RH, Ricciardi RP. Identification of polymerase and processivity inhibitors of vaccinia DNA synthesis using a stepwise screening approach. Antivir Res. 2008; 80: 114-123.
27. Dower K, Filone CM, Hodges EN, Bjornson ZB, Rubins KH, Brown LE, et al. Identification of pyridopyrimidinone inhibitor of orthopoxviruses from a diversity-oriented synthesis library. J Virol. 2012; 86(5): 2632-2640.
28. Bauer DJ, St Vincent L, Kempe CH, Downie AW. Prophylactic treatment of smallpox contacts with N-metylisatin β-thiosemicarbazone (compound 33t57, marboran). Lancet. 1963; 35: 494-496.
29. Borysiewicz J, Witaliński W. Effect of *N,N'*-bis(methylisatin-β-thio-semicarbazone)-2-methylpiperazine on vaccinia virus replication *in vitro* and *in vivo*. Brief report. Antimicrob Agents Ch. 1979; 62(1): 83-86.
30. Woodson B, Joklik WK. The inhibition of vaccinia virus multiplication by isatin-β-thiosemicarbazone. PNAS. 1965; 54(3): 946-953.
31. Neyts J, De Clercq E. Therapy and short-term prophylaxis of poxvirus infections: historical background and perspectives. Antivir Res. 2003; 57: 25-33.
32. Quenelle DC, Keith KA, Kern ER. *In vitro* and *in vivo* evaluation of isatin-β-thiosemicarbazone and marboran against vaccinia and cowpox virus infections. Antivir Res. 2006; 71: 24-30.
33. Prins C, Cresawn SG, Condit RC. An Isatin-β-thiosemicarbazone-resistant vaccinia virus containing a mutation in the second largest subunit of the viral RNA polymerase is defective in transcription elongaction. J Biol Chem. 2004; 279(43): 44858-44871.

34. Prichard MN, Kern ER. Orthopoxvirus Targets for the Development of Antiviral Therapies. Curr Drug Targets – Infections Disorders. 2005; 5: 17-28.
35. Black EP, Condit RC. Phenotypic Characteriszation of mutans in Vaccinia Virus Gene G2R, a Putative Transcription Elongation Factor. J Virol. 1996; 70(1): 47-54.
36. Kołodziej M, Joniec J, Bartoszcze M, Mirski T, Gryko R. Peptydy – nowe możliwości zwalczania zakażeń wirusowych. Przegl Epidemiol. 2011; 65: 477-482.
37. Meyer C, Hahn U, Rentmeister A. Cell-Specific Aptamers as Emerging Therapeutics. J Nucleic Acids. 2011; doi:10.4061/2011/904750.
38. Altmann SE, Jones JC, Schultz-Cherry S, Brandt CR. Inhibition of Vaccinia virus entry by a broad spectrum antiviral peptide. Virology. 2009; 388: 248-259.
39. Tarbet EB, Larson D, Anderson BJ, Bailey KW, Wong M-H, Smee DF. Evaluation of imiquimod for topical treatment of vaccinia virus cutaneous infections in immunosuppressed hairless mice. Antivir Res. 2011; 90: 126-133.
40. Perino J, Crouzier D, Spehner D, Debouzy J-C, Garin D, Crance J-M, et al. Lung surfactant DPPG phospholipid inhibits vaccinia virus infection. Antivir Res. 2011; 89: 89-97.
41. Deptała A, Soborczyk A, Krajewska K. Leczenie interferujące z funkcją receptora dla naskórkowego czynnika wzrostu (EGFR) u chorych na raka gruczołu krokowego *(The treatment interfering with EGFR in prostate cancer patients).* Onkol Prak Klin. 2011; 7(4): 208-214 (in Polish).
42. Wcisło G, Szczylik C. Szlak sygnałowy receptora naskórkowego czynnika wzrostu (EGFR) i potencjalne zastosowanie kliniczne jego blokowania w raku nerki *(Inhibition of molecular signaling of epidermal growth factor receptor (EGFR) and its clinical potential for treating renal cell cancer).* Onkol Prak Klin. 2011; 7(4): 197-207.
43. Langhammer S, Koban R, Yue C, Ellerbrok H. Inhibition of poxvirus spreading by the anti-tumor drug Gefitinib (Iressa). Antivir Res. 2011; 89: 64-70.
44. Brandt TA, Jacobs BL. Both Carboxy- and Amino-Terminal Domains of the Vaccinia Virus Interferon Resistance Gene, E3L, Are Required for Pathogenesis in a Mouse Model. J Virol. 2001; 75(2): 850-856.
45. Kwon J-A, Rich A. Biological function of the vaccinia virus Z-DNA-binding protein E3L: Gene transactivation and antiapoptotic activity in HeLa cells. PNAS. 2005; 102(36): 12759-12764.
46. Rice AD, Turner PC, Embury JE, Moldawer LL, Baker HV, Moyer RW. Roles of Vaccinia Virus Gene E3L and K3L and Host Genes PKR and RNase L during Intratracheal Infection of C57BL/6 Mice. J Virol. 2011; 85(1): 550-567.
47. Dave RS, McGettigan JP, Quereshi T, Schnell MJ, Nunnari G, Pomerantz RJ. siRNA targeting Vaccinia virus double-stranded RNA binding protein [E3L] exerts potent antiviral effects. Virology. 2006; 348: 489-497.
48. Li W-X, Li H, Lu R, Li F, Dus M, Atkinson P, et al. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. PNAS. 2004; 101(5): 1350-1355.
49. Lantermann M, Schwantes A, Sliva K, Sutter G, Schnierle BS. Vaccinia virus double-stranded RNA-binding protein E3 does not interfere with siRNA-mediated gene silencing in mammalian cells. Virus Res. 2007; 126: 1-8.
50. Unterholzner L, Sumner RP, Baran M, Ren H, Mansur DS, Bourke NM, et al. Vaccinia Virus Protein C6 Is a Virulence Factor that Binds TBK-1 Adaptor Proteins and Inhibits Activation of IRF3 and IRF7. PLoS Pathog. 2011; 7(9): e1002247. doi:10.1371/journal.ppat.1002247.
51. Rosengard AM, Liu Y, Nie Z, Jimenez R. Variola virus immune evasion design: Expression of a highly efficient inhibitor of human complement. PNAS. 2002; 99(13): 8808-8813.
52. Liszewski MK, Leung MK, Hauhart R, Mark R, Buller L, Bertram P, et al. Structure and Regulatory Profile of the Monkeypox Inhibitor of Complement: Comparison to Homologs in Vaccinia and Variola and Evidence for Dimer Formation. J Immunol. 2006; 176(6): 3725-3734.
53. Liszewski MK, Leung MK, Hauhart R, Fang CJ, Bertram P, Atkinson JP. Smallpox Inhibitor of Complement Enzymes (SPICE): Dissecting Functional Sites and Abrogating Activity. J Immunol. 2009; 183(5): 3150-3159.