Antiviral Research 80 (2008) 77–80

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short communication

In vitro susceptibility of sea lion poxvirus to cidofovir

Hendrik H. Nollens[a,*], Frances M.D. Gulland[b], Elliott R. Jacobson[a], Jorge A. Hernandez[c], Paul A. Klein[d], Michael T. Walsh[a], Richard C. Condit[e]

[a] Marine Mammal Health Program and Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, P.O. Box 100126, Gainesville, FL 32610, USA
[b] The Marine Mammal Center, 1065 Fort Cronkhite, Sausalito, CA 94965, USA
[c] Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, P.O. Box 100126, Gainesville, FL 32610, USA
[d] Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, P.O. Box 100275, Gainesville, FL 32610, USA
[e] Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, P.O. Box 100275, Gainesville, FL 32610, USA

ARTICLE INFO

Article history:
Received 26 October 2007
Accepted 19 March 2008

Keywords:
Isatin-beta-thiosemicarbazone
Rifampicin
Acyclovir
Cidofovir
Phosphonoacetic acid
Sea lion
Seal
Parapoxvirus
Poxvirus
Treatment

ABSTRACT

Parapoxviruses of seals and sea lions are commonly encountered pathogens with zoonotic potential. The antiviral activity of the antiviral compounds isatin-beta-thiosemicarbazone, rifampicin, acyclovir, cidofovir and phosphonoacetic acid against a parapoxvirus (SLPV-1) isolated from a Californian sea lions (*Zalophus californianus*) was evaluated. Cidofovir was able to reduce virus-induced cytopathic effect of SLPV-1 in confluent monolayers when used in concentrations greater than 2 μg/ml. A decreasing virus yield was observed in the presence of increasing concentrations of cidofovir, which confirmed the ability of cidofovir to inhibit SLPV-1 replication. The in vitro efficacy of cidofovir against SLPV-1 indicates the therapeutic potential of cidofovir for the treatment of infections of humans and pinnipeds with parapoxviruses of seals and sea lions. This study confirms the previously proposed therapeutic potential of cidofovir for the treatment of parapoxvirus infections.

The poxviruses of pinnipeds have been tentatively classified as parapoxviruses (Osterhaus et al., 1994; Nettleton et al., 1995; Becher et al., 2002; Nollens et al., 2006a). Poxvirus infections are a common complication in the treatment of stranded pinnipeds in marine mammal rehabilitation centers. The pinniped parapoxviruses are considered as zoonotic agents, as several seal handlers have acquired "sealpox" lesions (Hicks and Worthy, 1987; Clark et al., 2005). Other parapoxviruses (orf virus, bovine papular stomatitis virus and pseudocowpoxvirus) are known to infect man as well (Mercer et al., 1997). Infections of humans with the different parapoxviruses, including those of pinnipeds, are clinically indistinguishable. Lesions typically develop on the hands and fingers and usually resolve spontaneously within 6–8 weeks (Shelley and Shelley, 1983; Hicks and Worthy, 1987; Clark et al., 2005). Complications can occur and these include prolonged resolution time, ulceration, secondary bacterial infection, lymphangitis, lymphadenitis and bullous pemphigoid (Yirrell et al., 1991; Murphy and Ralfs, 1996; Reid, 1998).

Orf virus, bovine papular stomatitis virus and pseudocowpoxvirus infections of humans have been successfully treated via cryotherapy (Degraeve et al., 1999), excision of the lesions (Shelley and Shelley, 1983), amputation of the affected finger (Reid, 1998) and cidofovir (Geerinck et al., 1998). The in vitro antiviral activity of cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) against orf virus has been reported (Nettleton et al., 2000). Infections of humans with these parapoxviruses have been effectively treated using local, intranasal or intravenous administration of this drug (De Clercq, 2002a,b; McCabe et al., 2003).

Other compounds that are known to interfere with the effective replication of a number of DNA viruses include isatin-beta-thiosemicarbazone (IBT), rifampicin, phosphonoacetic acid (PAA) (foscarnet) and the nucleoside analogue acyclovir (9-(2-hydroxyethoxymethyl)guanine). IBT inhibits viral transcription termination and in vitro growth of vaccinia virus and cowpox virus

* Corresponding author at: Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, P.O. Box 100126, Gainesville, FL 32610, USA. Tel.: +1 352 392 2226x5775; fax: +1 352 392 5464.
E-mail addresses: NollensH@vetmed.ufl.edu (H.H. Nollens), gullandf@TMMC.org (F.M.D. Gulland), JacobsonE@vetmed.ufl.edu (E.R. Jacobson), HernandezJ@mail.vetmed.ufl.edu (J.A. Hernandez), paklein@ufl.edu (P.A. Klein), WalshM@vetmed.ufl.edu (M.T. Walsh), condit@ufl.edu (R.C. Condit).

0166-3542/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2008.03.007

(Condit et al., 1991). Rifampicin interferes with the function of a structural protein of vaccinia virus and consequently inhibits the maturation of virions (Szajner et al., 2005). PAA is a pyrophosphate analogue with activity against various DNA viruses via the inhibition of viral DNA polymerases (Crumpacker, 1992). Acyclovir and cidofovir are, respectively, guanine and cytosine nucleoside and nucleotide analogues that are FDA-approved for the treatment of human herpesviral infections. Cidofovir is converted exclusively via cellular enzymes to its active diphosphate form, whereas acyclovir requires both viral and cellular kinases to be phosphorylated into its active form (Elion, 1983; Morfin and Thouvenot, 2003). The active forms of acyclovir and cidofovir stop viral DNA replication via incorporation into and termination of the DNA chain, and via inactivation of the DNA polymerase (Elion, 1983; Magee et al., 2005). The antiviral activity of these compounds against the parapoxviruses of pinnipeds has not yet been determined. In this study we used a poxvirus (SLPV-1; Nollens et al., 2006b) isolated from a Californian sea lion (*Zalophus californianus*) as a model virus for testing the in vitro efficacy of IBT, rifampicin, acyclovir, cidofovir and PAA against the parapoxviruses of pinnipeds.

SLPV-1 was propagated in early passage California sea lion kidney (CZC-K) cells and a purified SLPV-1 stock was prepared on a Na-diatrizoate density gradient as previously described (Nollens et al., 2006a). This virus stock was used to evaluate sensitivity to the antiviral compounds. The titer of the purified SLPV-1 stock was determined via the cell culture infectious dose 50 ($CCID_{50}$) assay (Reed and Muench, 1938). CZC-K cells were grown to confluency in 24-well tissue culture plates. The culture media consisted of 85% (v/v) DMEM-F12 (Cellgro, Herndon, VA), 15% (v/v) cosmic fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine (Cellgro, Herndon, VA), 0.1 mM Na-pyruvate (Cellgro, Herndon, VA), 50 IU penicillin/ml and 50 μg streptomycin (Cellgro, Herndon, VA). All cultures were kept at 37.0 °C, 7.5% CO_2 and approximately 90% relative humidity. The SLPV-1 stock was serially diluted six times 1:10 in PBS. Ten wells were infected with 50 μl of each virus dilution and adsorbed for 90 min at 37.0 °C. Non-infected control wells were mock infected with PBS alone. The inoculum was removed and replaced with 1 ml of culture medium. After 5 days of incubation at 37.0 °C all cultures were inspected for cytopathic effect (CPE). Since SLPV-1 does not induce plaque formation, CPE was defined as rounding, clumping and detaching of the cells. The virus titer of the SLPV-1 stock was calculated at $10^{5.5}$ $CCID_{50}$/ml.

Five compounds were evaluated for their efficacy and cytotoxic effects: isatin-beta-thiosemicarbazone (IBT; Pfaltz and Bauer, Inc., Waterbury, CT), rifampicin (Sigma, St. Louis, MO), phosphonoacetic acid (PPA, Alfaproducts, Danvers, MA) and 9-(2-hydroxyethoxymethyl)guanine (Acyclovir, Calbiochem, La Jolla, CA). Cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) was kindly provided by Chimerix (La Jolla, CA). IBT was dissolved in acetone at a concentration of 5 mg/ml and then diluted with four volumes of 0.25 M NaOH immediately before use. Rifampicin, acyclovir and cidofovir were dissolved in ddH_2O. NaOH or HCl were added to adjust to a pH of 7.0. All compound solutions were diluted in ddH_2O to the desired concentrations and filter sterilized through a 200-μm pore filter prior to use. The range of concentrations were chosen based on minimum inhibitory concentrations of the compounds against other DNA viruses: IBT 22.5–90 μM, rifampicin 1–100 μg/ml, acyclovir 0.01–20 μg/ml, cidofovir 0.2–20 μg/ml and PAA 25–500 μg/ml (Baldick and Moss, 1987; Terry et al., 1988; Nettleton et al., 2000; American Society of Hospital Pharmacists, 2003; Tsankov and Angelova, 2003).

The antiviral activity of each compound was assayed via a CPE reduction assay. CZC-K cells were grown to confluency and all wells were infected with 32 $CCID_{50}$ of SLPV-1 and adsorbed for 90 min at 37.0 °C. The inoculum was removed and replaced with 1 ml of culture medium. Triplicate wells of CZC-K were incubated for 5 days in the presence of culture media containing the antiviral compounds at the various concentrations after which the presence or absence of CPE was recorded. IBT, rifampicin or acyclovir did not inhibit CPE formation. One and two infected wells incubated in the presence of 5 and 20 μg/ml cidofovir, respectively, were CPE-negative. Additionally, one infected well incubated in the presence of 200 μg/ml PAA was CPE-negative. The antiviral inhibitory activity of cidofovir and PAA was confirmed via a duplicate experiment using early passage Californian sea lion skin (CZC-S) cells following the exact same protocol. CPE reduction was confirmed using cidofovir at concentrations ≥2 μg/ml and when using PAA at concentrations ≥200 μg/ml. Antiviral activity was expressed as the minimum antiviral concentration (IC_{50}), defined as the compound concentration needed to prevent CPE in two or more wells. At any given concentration, PAA was unable to prevent CPE in more than one well. The IC_{50} for CPE of cidofovir was estimated between 5 and 20 μg/ml and between 2 and 5 μg/ml when using Californian sea lion kidney and skin cell cultures, respectively.

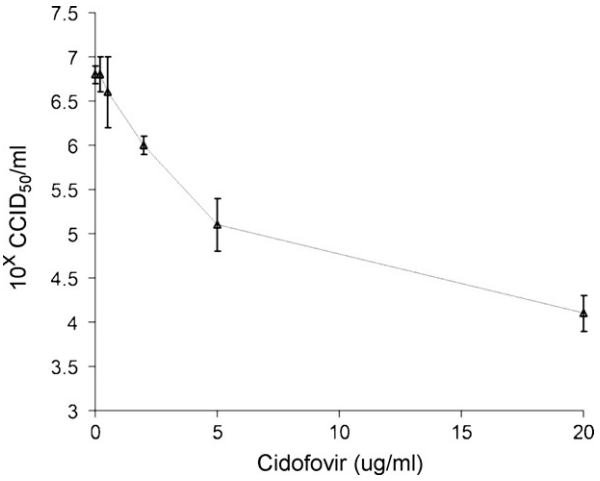

Fig. 1. Sea lion parapoxvirus (SLPV-1) yield (±S.E.) of infected CZC-S cells incubated in the presence of 0, 0.2, 2, 5 or 20 μg/ml of cidofovir for 5 days. A decreasing virus yield was observed in the presence of increasing concentrations of cidofovir.

To evaluate the effect of cidofovir on SLPV-1 yield, CZC-S monolayers of one drug-free control (0 μg/ml; Fig. 1) and of one well incubated at each drug concentration were harvested and the $CCID_{50}$ virus titer was determined. After 5 days of incubation, the calculated virus yield of cells infected in the absence of cidofovir (0 μg/ml) was $10^{6.8}$ $CCID_{50}$/ml. Virus yield decreased with increasing concentrations of cidofovir. The SLPV-1 yield of infected cells incubated in the presence of 0.2, 0.5 μg/ml of cidofovir was $10^{6.8}$, $10^{6.6}$ $CCID_{50}$/ml, respectively. The SLPV-1 yield of infected cells incubated in the presence of 2, 5 and 20 μg/ml of cidofovir was reduced to 10^{6}, $10^{5.1}$ and $10^{4.1}$ $CCID_{50}$/ml, respectively; or 13.4, 2.0 and 0.1% of the virus yield of the drug-free control.

A cytotoxic effect of the antiviral agent to CZC-S monolayers could be falsely interpreted as virus-induced CPE. The cytotoxic effect of cidofovir was therefore evaluated on both confluent and exponentially growing CZC-S cells. Triplicate confluent CZC-S monolayers were incubated for 5 days in the presence of culture media containing cidofovir at 0, 0.2, 0.5, 2, 5 and 20 μg/ml. After 5 days of incubation at 37.0 °C, the presence or absence of cytotoxicity, defined as apparent CPE, was recorded. No cytotoxicity was observed in the drug-free CZC-S control wells, but one of three monolayers incubated in the presence of cidofovir at a concentration of 20 μg/ml did show cytotoxicity.

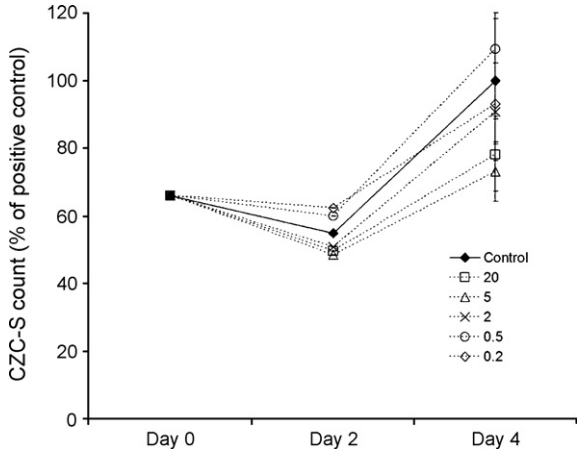

Fig. 2. Effect of cidofovir on the growth of early passage Californian sea lion skin cells incubated in the presence of 0, 0.2, 2, 5 and 20 μg/ml of cidofovir for 4 days. Depicted values are mean cell counts (±S.E.) for each drug concentration on day 0, 2 or 4, respectively. No significant cytotoxic effect of cidofovir on cell growth was detected ($p > 0.05$, Student's t-test).

The inhibitory effect of the cidofovir on the growth of CZC-S cells was also determined. CZC-S cells were manually counted and seeded into wells at a concentration of 28.6 ± 6.3 cells/mm^3. The cells were allowed to proliferate in fourfold replicates at 37.0 °C in the presence of culture media containing 0, 0.2, 0.5, 2, 5 and 20 μg/ml of cidofovir. After 2 days, the cells of one of each four wells were harvested and counted. The remaining wells were allowed to proliferate for an additional 2 days. All cell counts were expressed as the proportion (%) of cell count of the mean cell count in the drug-free controls on day 4 (Fig. 2). Mean proportions were compared using a Student's t-test for comparison of means. The calculated proportions were used to calculate CC_{50} (concentration of test compound required to inhibit the growth of host cells by 50%). Although after 2 days the cell counts in the presence of the higher cidofovir concentrations did appear to be lower, no significant cytotoxic effect of cidofovir on cell growth could be detected at the end of the experiment ($p \leq 0.05$). The CC_{50} of cidofovir for CZC-S cells was greater than 20 μg/ml. As a result, the calculated selectivity index of cidofovir, defined as the ratio of the measured IC_{50} to the CC_{50}, in CZC-S cells could not be accurately calculated. However, using the most conservative estimates ($IC_{50} = 5$ and $CC_{50} = 20$) the selectivity index was not greater than 0.25.

Our findings showed an unequivocal antiviral effect of cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) against the sea lion parapoxvirus SLPV-1. This confirms the findings of a previous study reporting the antiviral activity of cidofovir against other parapoxviruses (Nettleton et al., 2000). The sensitivity of SLPV-1 to cidofovir does appear to be lower than the sensitivities reported for these other parapoxviruses. This apparently atypical sensitivity of the SLPV-1 parapoxvirus could in part be due to limitations of the $CCID_{50}$ titering method used in this study. Alternatively, it should also be considered that the potency of cidofovir may, in part, depend on the efficiency of the intracellular metabolism and the activity of cidofovir may therefore differ depending on the cell type used.

Cidofovir does appear to have good therapeutic potential for the treatment of human cases of sealpox. The observed effective cidofovir concentrations for SLPV-1 are within the therapeutic dosage for humans. When administered IV at 10 mg/kg dose, peak serum levels are achieved of 23.6 ± 4.88 mg/l (Cundy, 1999), whereas cidofovir exhibited anti-SLPV-1 activity at concentrations of 2 μg/ml and higher. Zoonotic orf virus infection has been successfully treated via topical application of 1% cidofovir cream (McCabe et al., 2003). We therefore recommend cidofovir as a candidate therapeutic for the treatment of zoonotic sealpox infections as well. Cidofovir is presently of limited use for the treatment of parapoxvirus infections of wild seals and sea lions due to the need for handling and other practical constraints. Clinical trials are currently underway to test the safety and efficacy of CMX001, an ether-lipid analogue of cidofovir with excellent oral bioavailability (Buller et al., 2004; Quenelle et al., 2004; Parker et al., 2008). CMX001 may be an excellent candidate for the treatment of these parapoxvirus-infected pinnipeds.

Acknowledgements

The authors wish to thank Dr. John Wise and Dr. Carie Goertz (University of Southern Maine) for supplying the sea lion cell cultures. Cidofovir was kindly provided by Chimerix, La Jolla, CA. This work was conducted under NMFS permit number 1054-1731-00 to the University of Florida. Funding was provided in part by the Batchelor Foundation, College of Veterinary Medicine, by a grant from the Florida Fish and Wildlife Conservation Commission, and by John H. Prescott Marine Mammal Rescue Assistance grant number NA03NMF4390410 to E.R.J.

References

American Society of Hospital Pharmacists, 2003. American hospital formulary service drug information.
Baldick Jr., C.J., Moss, B., 1987. Resistance of vaccinia virus to rifampicin conferred by a single nucleotide substitution near the predicted NH2 terminus of a gene encoding an Mr 62,000 polypeptide. Virology 156, 138–145.
Becher, P., König, M., Müller, G., Siebert, U., Thiel, H.J., 2002. Characterization of sealpox virus, a separate member of the parapoxviruses. Arch. Virol. 147, 1133–1140.
Buller, R.M., Owens, G., Schriewer, J., Melman, L., Beadle, J.R., Hostetler, K.Y., 2004. Efficacy of oral active ether lipid analogs of cidofovir in a lethal mousepox model. Virology 318, 474–481.
Clark, C., McIntyre, P.G., Evans, A., McInnes, C.J., Lewis-Jones, S., 2005. Human sealpox resulting from a seal bite: confirmation that sealpox virus is zoonotic. Br. J. Dermatol. 152, 791–793.
Condit, R.C., Easterly, R., Pacha, R.F., Fathi, Z., Meis, R.J., 1991. A vaccinia virus isatin-beta-thiosemicarbazone resistance mutation maps in the viral gene encoding the 132-kDa subunit of RNA polymerase. Virology 185, 857–861.
Crumpacker, C.S., 1992. Mechanism of action of foscarnet against viral polymerases. Am. J. Med. 92, 3S–7S.
Cundy, K.C., 1999. Clinical pharmacokinetics of the antiviral nucleotide analogues cidofovir and adefovir. Clin. Pharmacokinet. 36, 127–143.
De Clercq, E., 2002a. Cidofovir in the therapy and short-term prophylaxis of poxvirus infections. Trends Pharmacol. Sci. 23, 456–458.
De Clercq, E., 2002b. Cidofovir in the treatment of poxvirus infections. Antiviral Res. 55, 1–13.
Degraeve, C., De Coninck, A., Senneseael, J., Roseeuw, D., 1999. Recurrent contagious ecthyma (Orf) in an immunocompromised host successfully treated with cryotherapy. Dermatology 198, 162–163.
Elion, G.B., 1983. The biochemistry and mechanism of action of acyclovir. J. Antimicrob. Chemother. 12 (Suppl. B), 9–17.
Geerinck, K., Lukito, G., Snoeck, R., De Vos, R., De Clercq, E., Vanrenterghem, Y., Degreef, H., Maes, B., 1998. A case of human orf in an immunocompromised patient treated successfully with cidofovir cream. J. Med. Virol. 64, 543–549.
Hicks, B.D., Worthy, G.A., 1987. Sealpox in captive grey seals (*Halichoerus grypus*) and their handlers. J. Wildl. Dis. 23, 1–6.
Magee, W.C., Hostetler, K.Y., Evans, D.H., 2005. Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate. Antimicrob. Agents Chemother. 49, 3153–3162.
McCabe, D., Weston, B., Storch, G., 2003. Treatment of orf poxvirus lesion with cidofovir cream. Pediatr. Infect. Dis. J. 22, 1027–1028.
Mercer, A., Fleming, S., Robinson, A., Nettleton, P., Reid, H., 1997. Molecular genetic analyses of parapoxviruses pathogenic for humans. Arch. Virol. Suppl. 13, 25–34.
Morfin, F., Thouvenot, D., 2003. Herpes simplex virus resistance to antiviral drugs. J. Clin. Virol. 26, 29–37.
Murphy, J.K., Ralfs, I.G., 1996. Bullous pemphigoid complicating human orf. Br. J. Dermatol. 134, 929–930.
Nettleton, P.F., Munro, R., Pow, I., Gilray, J., Gray, E.W., Reid, H.W., 1995. Isolation of a parapoxvirus from a grey seal (*Halichoerus grypus*). Vet. Rec. 137, 562–564.
Nettleton, P.F., Gilray, J.A., Reid, H.W., Mercer, A.A., 2000. Parapoxviruses are strongly inhibited in vitro by cidofovir. Antiviral Res. 48, 205–208.

Nollens, H.H., Gulland, F.M.D., Jacobson, E.R., Hernandez, J.A., Klein, P.A., Walsh, M.T., Condit, R.C., 2006a. Parapoxviruses of seals and sea lions make up a distinct subclade within the genus Parapoxvirus. Virology 349, 316–324.

Nollens, H.H., Jacobson, E.R., Gulland, F.M.D., Beusse, D.O., Bossart, G.D., Hernandez, J.A., Klein, P.A., Condit, R.C., 2006b. Pathology and preliminary characterization of a parapoxvirus isolated from a California sea lion (*Zalophus californianus*). J. Wildl. Dis. 42, 23–32.

Osterhaus, A.D., Broeders, H.W., Visser, I.K., Teppema, J.S., Kuiken, T., 1994. Isolation of a parapoxvirus from pox-like lesions in grey seals. Vet. Rec. 135, 601–602.

Parker, S., Touchette, E., Oberle, C., Almond, M., Robertson, A., Trost, L.C., Lampert, B., Painter, G., Buller, R.M., 2008. Efficacy of therapeutic intervention with an oral ether-lipid analogue of cidofovir (CMX001) in a lethal mousepox model. Antiviral Res. 77, 39–49.

Quenelle, D.C., Collins, D.J., Wan, W.B., Beadle, J.R., Hostetler, K.Y., Kern, E.R., 2004. Oral treatment of cowpox and vaccinia virus infections in mice with ether lipid esters of cidofovir. Antimicrob. Agents Chemother. 48, 404–412.

Reed, L.J., Muench, H., 1938. A simple method for estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

Reid, H.W., 1998. Poxviruses. In: Palmer, S.R., Soulsby, L.S.D.I.H. (Eds.), Zoonoses. Biology, Clinical Practice and Public Health Control. Oxford University Press, Oxford, pp. 415–421.

Shelley, W.B., Shelley, E.D., 1983. Surgical treatment of farmyard pox. Orf, milker's nodules, bovine papular stomatitis pox. Cutis 31, 191–192.

Szajner, P., Weisberg, A.S., Lebowitz, J., Heuser, J., Moss, B., 2005. External scaffold of spherical immature poxvirus partciles is made of protein trimers, forming a honeycomb lattice. J. Cell Biol. 170, 971–981.

Terry, B.J., Mazina, K.E., Tuomari, A.V., Haffey, M.L., Hagen, M., Feldman, A., Slusarchyk, W.A., Young, M.G., Zahler, R., Field, A.K., 1988. Broad-spectrum antiviral activity of the acyclic guanosine phosphonate (*R*,*S*)-HPMPG. Antiviral Res. 10, 235–251.

Tsankov, N., Angelova, I., 2003. Rifampin in dermatology. Clin. Dermatol. 21, 50–55.

Yirrell, D.L., Reid, H.W., Norval, M., Entrican, G., Miller, H.R., 1991. Response of efferent lymph and popliteal lymph node to epidermal infection of sheep with orf virus. Vet. Immunol. Immunopathol. 28, 219–235.