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ABSTRACT: We tested the *in vitro* sensitivity of Macropodid Herpesvirus 2 to eight commonly used anti-herpetic compounds using plaque reduction tests, March and April, 1995. The virus was most susceptible to inhibition by (E)-5-(2'-bromovinyl)-2'-deoxyuridine and adenine 9-β-D-arabino-furanoside. Both compounds have been used for anti-herpetic therapy in humans and may prove useful in the treatment of macropodoids in captivity.

Key words: Macropodid herpesvirus 2, herpesvirus, antiviral drugs, treatment, macropodoids, kangaroos.

Macropodid herpesvirus 2 (MHV2) has been associated with several epizootics of fatal disease among captive macropodoid populations. Based on serological surveys, the herpesviruses are widespread among captive macropodoids and may account for considerable morbidity in such environments (Webber and Whalley, 1978).

In 1975 a herpesvirus was implicated in the death of seven parma wallabies (*Macropus parma*) from a colony of captive animals held at Macquarie University in Sydney, Australia (Finnie et al., 1976). Upon necropsy, animals had congestion of the lung, liver, and spleen. A further nine animals developed overt clinical disease characterized by a high fever, respiratory rales, conjunctivitis, and the appearance of small vesicles in the ano-genital region (Finnie et al., 1976). Following careful genetic and biological characterization, the virus was tentatively classified as a group D Alphaherpesvirus and designated Macropodid Herpesvirus 1 (MHV1) (Whalley and Webber, 1979; Johnson and Whalley, 1990).

Based on plaque reduction neutralization studies, the virus, or a closely related herpesvirus, was widespread among captive and free-ranging macropodoids (Webber and Whalley, 1978). Serological evidence of macropodid herpesvirus infection was found in macropodoids from every Australian state (Webber and Whalley, 1979). Fatal herpesvirus infections have been observed among tammar wallabies (*Macropus eugenii*), brush-tailed rat kangaroos (*Bettongia penicillata*), rufous rat kangaroos (*Aepyprymnus rufescens*) (Dickson et al., 1980), grey dorcopsis wallabies (*Dorcopsis muelleri luctuosa*), western grey kangaroos (*Macropus fuliginosus*), and quokkas (*Setonix brachyurus*) (Callinan and Kefford, 1981; Wilks et al., 1981). Two herpesviruses were isolated from separated populations of dorcopsis wallabies from the same zoo and a third herpesvirus was isolated from a quokka (Callinan and Kefford, 1981). Based on serological studies and restriction endonuclease analysis, all three viruses were similar to one another but distinct from MHV1 (Wilks et al., 1981; Johnson et al., 1985). This new herpesvirus was designated Macropodid Herpesvirus 2 (MHV2) and was tentatively classified as a group E Alphaherpesvirus (Johnson and Whalley, 1987).

Herpesviruses appear widespread among captive macropodoid populations, with as many as 55% of animals having serological evidence of herpesvirus infection (Webber and Whalley, 1978). In addition to being an important cause of mortality in captive macropodoids, herpesviruses are responsible for significant morbidity. In particular, herpesvirus-like vesicles and lesions are often observed around the mouth and ano-genital region of captive macropodoids (Callinan and Kefford, 1981). Such lesions are not only undesirable in animals on public display but may develop into more serious ulcerations as a result of secondary bacterial infections. Our objec-

TABLE 1.   Inhibitory effect of varying concentrations of anti-herpetic compounds on the ability of MHV2 to form plaques in potoroo kidney cells.

Anti-herpetic compound	Concentration (μg/ml):				
	1.0	2.5	5.0	10.0	50.0
(E)-5-(2'-bromovinyl)-2'-deoxyuridine (BVDU)	38[a]	89	99	>99	>99
Acyloguanosine (ACV)	0	0	7	34	99
5-iodo-2'-deoxyuridine (IdU)	0	0	0	0	63
5-iodo-2'-deoxycytidine (IdC)	0	0	0	0	23
Triflurothymine deoxyribose (TFR)	0	0	0	12	55
Adenine 9-β-D-arabino-furanoside (Ara-A)	99	100	100	100	100
Cytosine β-D-arabino-furanoside (Ara-C)	0	25	32	70	100
Thymine 1-β-D-arabino-furanoside (Ara-T)	6	55	97	>99	>99

[a] Percent inhibition; based on the number of plaques observed in comparison to an average control inoculum of 130 plaque forming units per 25 cm² flask. Each test was performed in duplicate.

tive was to evaluate the therapeutic value of eight anti-herpetic compounds for the treatment of MHV2 infections.

The anti-herpetic compounds; (E)-5-(2'-bromovinyl)-2'-deoxyuridine (BVDU), acyloguanosine (9-[92-hydroxyethoxy)methyl]guanine) (ACV), 5-iodo-2'-deoxyuridine (IdU), 5-iodo-2'-deoxycytidine (IdC), triflurothymine deoxyribose (TFR), adenine 9-β-D-arabino-furanoside (Ara-A), cytosine β-D-arabino-furanoside (Ara-C) and thymine 1-β-D-arabino-furanoside (Ara-T) were purchased from Sigma Chemical Company, Crows Nest, New South Wales, Australia. Stock solutions of either 0.5 (BVDU, ACV, IdU, IdC, TFR) or 1.0 mg/ml (Ara-A, Ara-C, Ara-T) of each were prepared in water and sterilized by filtration through 0.22 μm membranes. The study was conducted in March and April, 1995.

A potoroo kidney cell line (PtK2) (Commonwealth Serum Laboratory Pty., Ltd., Melbourne, Victoria, Australia) was used as substrate for virus plaque inhibition assays (Fenner et al., 1968). Monolayers of PtK2, which had been grown to confluency in 25 cm² culture flasks (Corning, Corning, New York, USA) were decanted and inoculated with approximately 150 plaque forming units of MHV2. The virus was allowed to absorb at 37 C for 2 hr. Virus-containing supernatant was aspirated and each monolayer was overlayed with 5.0 ml

of a 1% nutrient agar overlay containing Minimum Essential Medium, 50 units/ml penicillin, 50 μg/ml streptomycin, 8.0 mM L-glutamine, 5% heat inactivated fetal bovine serum (Gibco-BRL, Mulgrave Victoria, Australia), 1% Nobel agar (Difco Laboratories, Detroit, Michigan, USA) and varying concentrations of the anti-herpetic compounds listed above. Each dilution of the various anti-herpetic compounds was tested in duplicate under identical conditions. After 5 days incubation at 37 C, each flask received a second 5.0 ml nutrient agar overlay containing 170 μg/ml of neutral red (Gibco-BRL, Mulgrave Victoria, Australia). Flasks were returned to 37 C for a further 16 hr and plaques were counted. Inhibition was calculated by averaging the plaque count of duplicate flasks and expressing the figure as a percentage of the average plaque count of the control virus in the absence of antiviral compounds.

All anti-herpetic compounds tested had some inhibitory effect on the in vitro replication of MHV2 (Table 1). While all showed some inhibitory effect, there was considerable variation in efficacy of the individual compounds. The most effective anti-herpetic compound against MHV2 was Ara-A. This compound was able to inhibit MHV2 plaque formation at the low concentration of 1.0 μg/ml. Only Ara-A and Ara-C completely inhibited MHV2

plaque formation at all of the concentrations tested. (E)-5-(2'-bromovinyl)-2'-deoxyuridine and Ara-T, which had greater than 95% plaque inhibition at a concentration of 5.0 μg/ml, were unable to completely inhibit plaque formation at a 10-fold higher concentration (50.0 μg/ml).

Since 1975, numerous antiviral compounds have been evaluated in both in vitro and in vivo trials. The activity of most of these compounds depends upon their selective phosphorylation by viral thymidine kinases (Kit, 1985). These compounds then are able to exert their antiviral activity through either selective incorporation into the nascent DNA chain, thus preventing further chain elongation, or by irreversibly binding to viral encoded DNA polymerases or a combination of both (Sim, 1990). Herpesvirus thymidine kinases have considerable heterogeneity at the nucleotide and amino acid level (Kit, 1985). Thus an anti-herpetic compound which is effective against MHV2 may not be equally effective against MHV1.

Of the eight compounds tested, IdU, IdC and TFR were least effective against MHV2 in this study but had the greatest toxicity for mammalian cells (Van Voris, 1984). Cytosine β-D-arabino-furanoside (Ara-C) which performed substantially better is associated with relatively high toxicity in mammalian cells (Van Voris, 1984). Thus these four anti-herpetic compounds are not recommended for further investigation as possible therapeutic agents in MHV2 infections.

Acycloguanosine (ACV), which has proven to be particularly effective in the treatment of herpesvirus infections in humans (Van Voris, 1984), was only moderately effective in inhibiting plaque formation in MHV2-infected cells. Indeed, the 50% inhibitory dose for MHV2 of between 10 and 50 μg/ml was 10- to 1,000-fold greater than that reported for herpes simplex virus 1 (HSV1) (Van Voris, 1984). Similarly, Ara-T was moderately effective in inhibiting MHV2. While this compound has not been tested as extensively as ACV,

it has a low level of toxicity for mammalian cells (Van Voris, 1984). Given the low toxicity associated with these drugs and their moderate inhibitory activity against MHV2, they may be worthy of more rigorous evaluation for the potential treatment of MHV2 infections.

The nucleoside analogue BVDU is highly effective in treating a range of herpesvirus afflictions including encephalitis, keratitis and orofacial lesions (Van Voris, 1984), has low toxicity for animals, and is particularly effective in the treatment of varicella zoster virus infections in immunocompromised patients (Sim, 1990). At a concentration of 2.5 μg/ml BVDU inhibited 89% of MHV2 plaque formation. The best performing compound, Ara-A inhibited 99% of MHV2 plaque formation at a concentration of 1.0 μg/ml, the lowest concentration tested. This dose is much lower than the 10 to 20 μg/ml needed to inhibit either herpes simplex virus 1 or varicella zoster virus in vitro (Van Voris, 1984). However the in vitro inhibitory dose of Ara-A varies depending upon cell line. The compound is used widely to treat a range of herpesvirus infections in humans, including infants. The drug can be administered by topical, intravenous or intramuscular routes and has no deleterious effect on immune function (Van Voris, 1984). Although animal trials will need to be conducted to establish the in vivo toxicity of BVDU and Ara-A in macropodoids, it is expected that either drug could be safely used to treat MHV2-infections in these animals.

Macropodid herpesvirus 2 has been associated with fatal disease epizooties among captive marsupials, is associated with vesicle eruptions in the ano-gential and oro-facial mucosa of macropodoids (Wilks et al., 1981), and would appear to be a significant cause of morbidity in captive colonies. In this study we have identified several compounds which have potent in vitro inhibitory effects on MHV2 plaque formation. At least four of these compounds, ACV, Ara-T, BVDU and Ara-

A appear to be suitable candidates for more through in vivo trials. Of the eight compounds examined in this preliminary study, Ara-A appears to be the most likely candidate for therapeutic intervention in suspected MHV2 disease. The compound completely inhibited MHV2 plaque formation at a concentration of 2.5 μg/ml, it has a low toxicity in animals, and it can be safely administered by a variety routes to treat a range of clinical manifestations associated with herpesvirus infections.

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