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Inhibitory effect of (*S*)-HPMPC, (*S*)-HPMPA, and 2′-nor-cyclic GMP on clinical ocular adenoviral isolates is serotype-dependent in vitro

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Summary

Currently, there is no effective treatment for ocular adenoviral infections that occur in epidemics worldwide, produce significant patient morbidity, and cause substantial economic losses. We tested several new antivirals in vitro, and found that (*S*)-HPMPC, (*S*)-HPMPA, and 2′-nor-cyclic GMP demonstrated significant serotype-dependent inhibitory activity by plaque reduction assay (ID_{50}= 0.017–17.0 μg/ml) against common clinical ocular isolates and standard adenoviral serotypes (Ad 1, Ad 5, Ad 8, and Ad 19). (*S*)-HPMPC was the least toxic (CD_{50} in A549 cells = 306 μg/ml), and (*S*)-HPMPC and (*S*)-HPMPA had high selectivity indices.

(*S*)-HPMPC; (*S*)-HPMPA, 2′-nor-cyclic GMP; Adenovirus

Ocular adenoviral infections occur world-wide, and are associated with community and medical facility epidemics (Ford et al., 1987). Currently, there is no effective topical or systemic antiviral therapy to control ocular adenoviral infections. Earlier studies with antiherpetic antivirals (Dudgeon et al., 1969; Pavan-Langston and Dohlman, 1972; Darougar et al., 1977), and interferon (Sundmacher et al., 1982; Isacsohn et al., 1983; Adams et al., 1984; Reilly et al., 1986; Mistchenko et al., 1987; Wilhelmus et al., 1987) failed to demonstrate

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efficacy. While vaccines provided effective prophylaxis against serotypes that cause respiratory disease in the military (Chaloner-Larsson et al., 1986), they have not been applied to the prevention or treatment of ocular disease in the general population.

Recent in vitro studies with newer broad-spectrum antivirals demonstrated inhibitory activity in vitro against several adenoviral serotypes. The most promising ones include: (*S*)-HPMPC (De Clercq et al., 1987; Snoeck et al., 1988; Bronson et al., 1989), (*S*)-HPMPA (De Clercq et al., 1986; Baba et al., 1987), 2′-nor cyclic GMP (Tolman et al., 1985; Baba et al., 1987), an antiherpetic agent, and novobiocin (D'Halliun et al., 1980), a substituted coumarin antibiotic. The present study compared the inhibitory effect of these promising antivirals in vitro against different adenoviral serotypes (clinical isolates and standard laboratory strains) known to cause significant ocular disease.

The standard human adenoviral serotypes – adenovirus type 5 (VR-5), adenovirus type 8 (Trim VR-1085), and adenovirus type 19 (VR-1096) – and the continuous cell line, A549 (CCL-185), an epithelial-like cell derived from human lung carcinoma, were obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD. Clinical adenoviral isolates were cultured from patients with typical adenoviral keratoconjunctivitis seen at the Eye & Ear Institute of Pittsburgh. All clinical isolates were typed by immunofiltration and serum neutralization. All adenoviral serotypes were successfully grown in A549 monolayers, at 37°C, 5% carbon-dioxide water-vapor atmosphere, and titered using a 96-well viral quantification assay. These stock suspensions of the different serotypes were then frozen, and stored (1 ml aliquots) at −70°C prior to use.

The antiviral agents (*S*)-HPMPA [(S)-9-(3-hydroxy-2-phosphonylmethoxy-propyl)adenine] and (*S*)-HPMPC [(*S*)-9-(3-hydroxy-2-phosphonylmethoxypro-pyl)cytosine were provided by A. Holý, Prague, Czechoslovakia. 2′-nor-cGMP (9-[(2-hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]guanineP-oxide), and DHPG {9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine}, were ob-tained from Merck Sharp & Dohme Research Laboratories, West Point, PA. Novobiocin was ordered from Sigma Chemical Co., St.Louis, MO. (catalogue number, N-1628).

The antiviral activity was evaluated in vitro by plaque reduction assay. Approximately 100 PFU of a known adenoviral serotype was added in triplicate to a 24-well plate with confluent A549 cell monolayers. Following adsorption of the virus suspension for 3 h, serial dilutions of a given antiviral in methylcellulose overlay media (1 ml/well) was added to each well. Controls included the addition of media alone to six virus-infected wells. The wells were examined daily using an inverted microscope for evidence of plaque formation (approximately 7–14 days depending upon Ad serotype). The medium was then aspirated from each well, and replaced with 1 ml of 0.5% gentian violet. Following staining for 30 min, the plates were washed with copious amounts of cold tap water, air-dried, and the plaques were counted under 25 × power

using a dissecting microscope. The data was plotted as antiviral concentration versus number of plaques, and the 50% inhibitory dose of the antiviral was determined from the graph. Each antiviral was tested in two independent trials.

Antiviral toxicity to uninfected A549 cells was determined by the inhibition of cell DNA synthesis (i.e., [*methyl*-^{3}H]thymidine uptake) in antiviral-treated and untreated A549 cells (Shigeta et al., 1983). In brief, 1–2 $\times$ 10^5 cells/ml were grown in sterile glass scintillation vials overnight. Media containing serial dilutions of antiviral and 0.5 μCi/ml [*methyl*-^{3}H]thymidine (sp. activity 121 Ci/mmol, Amersham Labs. Amersham, U.K.) were added to the cells in the vial. Each dilution was repeated in triplicate. The mixture was incubated for 24 h at 37°C in 5% carbon-dioxide water-vapor atmosphere. The media was then aspirated, and the cells treated with cold TCA for 30 min at 4°C. Each vial was washed with cold TCA four times, washed with cold 95% ethanol twice, and air-dried. Toluene scintillant was then added to each vial, and the preparation counted for radioactivity. The results are expressed as the concentration of antiviral which inhibits 50% host-cell DNA synthesis (CD_{50} for A549 = 50% A549 cell DNA inhibition) relative to media controls.

Selectivity indices were calculated for a given antiviral against a specific Ad serotype in A549 cells as the ratio of CD_{50} for A549 cell growth to ID_{50} for adenoviral plaque formation (Snoeck et al., 1988).

Table 1 summarizes the in vitro inhibitory effects of various antivirals in A549 cells against the standard ocular adenoviral serotypes obtained from the American Type Culture Collection: ATCC Ad 5, ATCC Ad 8, and ATCC Ad 19. In general, (*S*)-HPMPA and 2′-nor-cGMP demonstrated significant inhibitory activity against all three standard adenoviral serotypes. The selectivity indices for (*S*)-HPMPA were superior to 2′-nor-cGMP for all three serotypes: ATCC Ad 5 (720 vs. 370), ATCC Ad 8 (4500 vs. 1250), and ATCC Ad 19 (1200 vs. 82). Serotype-dependent inhibition was demonstrated by (*S*)-HPMPA and 2′-nor-cGMP, i.e., ATCC Ad 8 was the most sensitive by plaque assay (ID_{50} = 0.04 and 0.08 μg/ml respectively), and Ad 19 the most resistant (ID_{50} = 0.15 and 1.22 μg/ml respectively).

TABLE 1

Inhibitory activity of antivirals against standard ocular adenoviral serotypes (plaque assay – ID_{50} μg/ml)

	CD_{50} for A549 cell growth (μg/μl)	ATCC Ad 5	ATCC Ad 8	ATCC Ad 19
(*S*)-HPMPA	180	0.25 (720)[a]	0.04 (4500)	0.15 (1200)
2′-nor-cGMP	100	0.27 (370)	0.08 (1250)	1.22 (82)
DHPG	>2000	70.00 (28)	0.71 (72817)	26 (77)
Novobiocin	155	>100.0 (2)	>31.6 (5)	50.0 (3)

[a]Selectivity Index = CD_{50} for A549 cells/ID_{50} for Ad Serotype.

TABLE 2

Inhibitory activity of antivirals against clinical ocular adenoviral isolates (plaque assay – ID_{50} mg/ml)

	CD_{50} for A549 cell growth ($\mu g/\mu l$)	Ad 1 Kmetz	Ad 5 McEwen	Ad 8 Edmunds	Ad 19 Kowalski
(*S*)-HPMPC	306	0.38 (805)[a]	0.15 (2040)	0.017 (18000)	17.0 (18)
(*S*)-HPMPA	180	0.31 (581)	0.051 (3529)	0.023 (7826)	2.9 (62)
2′-nor-cGMP	100	3.7 (27)	1.40 (71)	0.15 (666)	3.8 (26)

[a]Selectivity Index = CD_{50} for A549 cells/ID_{50} for Ad Serotype.

Table 2 summarizes the in vitro inhibitory effects of (*S*)-HPMPC, (*S*)-HPMPA and 2′-nor-cGMP in A549 cells against clinical isolates recovered from patients with active keratoconjunctivitis. In general, all three antivirals demonstrated potent inhibitory activity (ID_{50} = 0.017–5.3 μg/ml) that again varied according to serotype (sensitivity: Ad 8 Edmunds > Ad 5 McEwen > Ad 1 Kmetz > Ad 19 Kowalski).

The selectivity indices for (*S*)-HPMPC and (*S*)-HPMPA were generally superior to 2′-nor-cGMP for most clinical isolates tested: Ad 8 Edmunds (18 000, 7826, and 666, respectively); Ad 5 McEwen (2040, 3529, and 71); Ad 1 Kmetz (805, 581, and 27); and Ad 19 Kowalski (18,62, and 26). (*S*)-HPMPC was less toxic to uninfected A549 cells than (*S*)-HPMPA and 2′-nor-cGMP (CD_{50} = 306, 180, and 100 μg/ml, respectively).

Our results are the first to directly compare (*S*)-HPMPC, (*S*)-HPMPA, and 2′-nor-cGMP against clinical ocular isolates, and to demonstrate serotype-dependency (Ad 8> Ad 5> Ad 1> Ad 19) among these clinical isolates. The relative resistance of Ad 19, an important cause of epidemic keratoconjunctivitis has not been previously described. Our results confirm and extend previous antiviral studies with (*S*)-HPMPC (De Clercq et al., 1987; Snoeck et al., 1988; Bronson et al., 1989), (*S*)-HPMPA (Baba et al., 1987; De Clercq et al., 1986), and 2′-nor-cGMP (Tolman et al., 1985), that demonstrated inhibitory activity against many adenoviral serotypes, as well as other herpesviruses. In contrast, our study failed to demonstrate any in vitro inhibitory activity for novobiocin against Ad 5, Ad 8, or Ad 19, although novobiocin reportedly inhibited (ED_{50} = 90 μg/ml) Ad 2 replication in KB cells (D'Halliun et al., 1980). DHPG demonstrated some inhibitory activity, but lacked the potency of the other compounds to warrant further study.

Adenoviral ocular infections are a significant public health problem worldwide. Epidemics associated with doctors' offices and health centers transmit a highly contagious disease that causes patient morbidity, and significant loss of time from work and school (Ford et al., 1987). The absence of effective treatment, and the limited ability to contain epidemics, continue to

stimulate the search for an effective clinical antiviral agent.

(*S*)-HPMPC, (*S*)-HPMPA, and 2′-nor-cyclic GMP all demonstrated significant inhibitory activity by plaque reduction against many common ocular adenoviral serotypes (Ad 1, Ad 5, Ad 8, and Ad 19). Further studies are indicated to evaluate their potential to meet the urgent clinical need for an effective topical antiviral agent to treat, and prevent the spread of, highly contagious ocular adenoviral infections. While ocular toxicity studies can be carried out at the present time in an animal model, the absence of a credible animal model of ocular adenoviral infection significantly impedes drug development. Immediate efforts should be directed toward the development of such an animal model of ocular adenoviral infection in order to facilitate future trials in humans.

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