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Evaluation of Retinal Toxicity and Efficacy of the Anticytomegalovirus Compound 2-Amino-7-[(1,3-Dihydroxy-2-Propoxy)Methyl]Purine

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Compound 2242, also known as 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine, is the first known antivirally active nucleoside analog with the side chain substituted at the N-7 position of the purine ring system. Our purpose was to evaluate its retinal toxicity and assess the efficacy of its highest nontoxic concentration in a rabbit model of herpes simplex retinitis. Concentrations of the drug from 0.5 to 2,000 μM were injected intravitreally in twelve New Zealand White rabbits. Fundoscopic, histologic, and electrophysiologic data revealed no evidence of toxicity even at the highest dose of the compound. Dutch pigmented rabbits ($n = 34$) had their left eyes injected with herpes simplex virus type 1 3 days after, concurrently, or 3 days before intravitreal injection of either 2,000 μM compound 2242 or 480 μM ganciclovir (final concentration in the eye). Both compound 2242 and ganciclovir were equally effective compared with saline when administered simultaneously with the virus ($P < 0.0001$). In the 3-day pretreatment paradigm, compound 2242 was superior to ganciclovir ($P < 0.04$), but there was no clear difference between the two with regard to their effects on an established infection. The pharmacokinetics of compound 2242 in 10 rabbits injected intravitreally with 30 μM showed an intravitreal half-life of 8 h. This compound, which may be orally active in its pro form, has a very high therapeutic index in the eye and is more efficient than ganciclovir in this animal model of herpes retinitis.

Administration of ganciclovir or foscarnet is a useful treatment for patients with cytomegalovirus (CMV) retinitis (8, 16, 18, 28). However, both drugs can cause serious systemic toxicity, and during therapy with either drug, clinically resistant retinitis may develop in a significant proportion of these patients (9). For some patients, a change from one drug to the other does not ameliorate the retinitis, and combination therapy must also be used (22). Local intraocular antiviral therapy has been widely used for patients unable to tolerate systemic therapy (2, 4, 5, 14, 17, 32). Unfortunately, both ganciclovir and foscarnet have short intravitreal half-lives and durations of action, making weekly injections necessary, with all the risks associated with frequent procedures (11, 30). A recently developed intravitreal ganciclovir implant is capable of releasing ganciclovir for up to 8 months, but surgery with potential complications is required, and experience with the use of the device is quite limited (1, 24, 29). Although the systemic role of CMV is not yet clear, another concern with local therapy is that it does not treat possible clinically inapparent infection with CMV. In addition, strains of the virus resistant to both drugs have been isolated (7, 21). A continuing need for an orally administered drug for the management of CMV retinitis exists, and a safe intravitreal compound that would allow for the control of retinitis with infrequent injections is required.

Since the discovery of the selective antiherpes action of acyclovir, a multitude of purine and pyrimidine nucleoside analogs with antiherpes activity has been described (3). In the synthesis of these purine nucleosides with acyclic substituents, the N-9 region selectivity of the coupling of the heterocycle with the acyclic residue was considered essential, as natural purine nucleosides are substituted at N-9. Compound 2242 (2-amino-7-[(1,3dihydroxy-2-propoxy)methyl]purine) is the first known nucleoside analog with the side chain substituted at the N-7 position of the purine ring system that is active against herpesviruses (19, 20, 23, 26, 27). Activity against ganciclovir-resistant herpes simplex virus type 1 (HSV-1) and HSV-2 strains as well as against ganciclovir-resistant human CMV strains was also found, indicating a different mode of action (13, 25, 33). We report here the results of our investigations, which were designed to assess the retinal toxicity of compound 2242 and the duration of the antiviral effect of the compound after intravitreal administration in a focal, nonlethal, herpetic retinitis rabbit animal model (12), and we describe the ocular pharmacokinetics of intravitreally administered compound 2242 and the orally administered prodrug, HOE 961.

MATERIALS AND METHODS

Animals. A total of 56 New Zealand White and Dutch pigmented rabbits weighing between 2.5 and 3.5 kg were used. All the experiments were conducted in accordance with the guidelines of the University of California at San Diego Office of Veterinary Affairs and the Association for Research in Vision and Ophthalmology resolution on the use of animals for research.

For the surgical procedures, the rabbits were anesthetized with intramuscular injections of ketamine (26 mg/kg of body weight) and xylazine (7 mg/kg). The pupils were dilated with 2.5% phenylephrine hydrochloride and 1% cyclopentolate hydrochloride. The eye was then proptosed, and the injection site was prepared with povidone iodine applicators and then irrigated with sterile saline solution. An anterior chamber paracentesis was performed before drug delivery. The injections were performed with the aid of a surgical microscope. A 30-gauge needle was inserted 2 mm from the limbus into the vitreous cavity.

Toxicity study. Twelve New Zealand White rabbits were used for this study. Toxicity was assessed by electroretinography, weekly indirect ophthalmoscopy and slit-lamp examinations, and light and electron microscopy as previously described (6). Twelve eyes of six New Zealand White rabbits were used for each time point. Five different concentrations of compound 2242 (0.5, 5, 50, 500, and 2,000 μM per eye) were injected in 0.1-ml volumes of normal saline. Two eyes

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were used as controls for each time point; in these eyes, 0.1 ml of normal saline was injected intravitreally. The animals were sacrificed after 2 and 8 weeks. After being anesthetized as described above, the animals were euthanized with an intracardiac injection of 1.0 ml of pentobarbital sodium (390 mg/ml). Both eyes underwent triple fixation as previously described (6, 10, 31). The selected calottes were vertically sectioned through the optic nerve-inferior retina, which was prepared for thin-section light microscopy and electron microscopy.

Treatment study. Thirty-four Dutch pigmented rabbits were used for this study. Only the left eye of each animal was used. The procedures for the injection of the drug were the same as those described above. The animals were injected intravitreally either with compound 2242 (13 eyes) at its highest nontoxic dose (final intravitreal concentration, 2,000 μM) or with 200 μg of ganciclovir (10 eyes; final concentration in the eye, 480 μM) and were challenged with HSV-1 3 days after, concurrently, or 3 days before the treatment. Three animals used as controls for each group were injected intravitreally with 0.1 cm^3 of saline. We created a rabbit model of focal nonlethal retinitis by surgical inoculation of HSV-1 directly onto the retina. The challenge virus strain used was HSV-1 PH, which was grown and titered on Vero cells. The titer was $10^{7.5}$ 50% tissue culture infective doses per ml. We used this virus diluted 10^6-fold in sterile saline (12). For this virus, the 50% inhibitory concentration of compound 2242 was 5.23 μg/ml, that for acyclovir was 2.4 μg/ml, and that for ganciclovir was 1.1 μg/ml. The virus was inoculated onto the retinal surface at a point 2 mm inferior to the optic disc with a 30-gauge needle with the aid of a surgical microscope. Both eyes were examined by indirect ophthalmoscopy on days 1, 3, 5, 7, 10, 14, 21, and 28. Fundus diagrams based on indirect ophthalmoscopy as well as 50°-angle fundus photographs in selected cases were made.

The animals were sacrificed as described in the toxicity study paragraph above, and the eyes were immersion fixed in 4% paraformaldehyde and processed in paraffin. Immunostaining for HSV-1 was not routinely performed; the diagnosis of infection was based upon the clinical appearance in the paraffin sections. For purposes of statistical analysis, a grading scale based on the extent and date of the onset of optic nerve congestion, retinitis, and vitritis was devised. The scale ran from 0 to 4, with increasing severity being given a higher score. The classification of the animals was based on the worst clinical presentation of the eye during the first 14 days after viral inoculation. The grading of the eyes of the treated animals was done on the basis of comparisons with the eyes of control animals subject to the natural course of herpes retinitis in our model (12). Treated animals with a similar but delayed clinical presentation by comparison with that for animals subject to the natural course were given a score that was reduced by 0.5 for statistical purposes. Statistical analysis (see Table 1) of the retinitis grades by the Wilcoxon rank-sum test was performed with JMP software (SAS Institute, Carey, N.C.).

Pharmacokinetic studies. We did a limited intravitreal pharmacokinetic study to determine the half-life of this compound. We injected 30 μM compound 2242 in 0.1 ml intravitreally into 10 Dutch pigmented rabbits. Both eyes were used, and one animal was used as a control.

The animals were sacrificed as described above with the exception that the eyes were not fixed, at 1, 4, 8, 16, 24, 48, 72, 96, and 144 h after the injection. The eyes were enucleated, and vitreous samples for an analysis of the concentrations of compound 2242 and its main metabolite, compound 1225, by high-performance liquid chromatography (HPLC) were obtained. The elimination half-life was determined from a least-squares fit on the terminal log-linear portion of the concentration-time curve.

A study of the in vitro metabolism of the prodrug HOE 961 was done with 100 μg of HOE 961 per ml. The 9,000 × *g* fraction of the liver homogenate was incubated at 37°C for 3 h. The samples were lyophilized and extracted with methanol. After centrifugation (3,000 × *g* for 15 min), the supernatant was evaporated to dryness under a flow of N_2. The residue was dissolved in water and analyzed by HPLC and thin-layer chromatography.

Additional experiments to assess the concentrations of compound 2242 in the plasma and ocular fluid after continual oral administration of its oral prodrug HOE 961 were done. Four concentrations of HOE 961 (0.02, 0.1, 0.2, and 0.4%) were diluted in tap water and administered to Chinchilla rabbits for 4 to 7 days for the 0.02% concentration and for 2 days for the 0.1, 0.2, and 0.4% concentrations. Blood samples from the rabbit's marginal ear vein were collected once daily for 1 to 9 days after the start of the administration of the drug. Samples were centrifuged for 15 min at 1,800 × *g* at 4°C, and the separated plasma was centrifuged again and then frozen at −20°C until it was assayed for the concentrations of HOE 961 and the main metabolites. At the end of each time point, animals were sacrificed and their eyes were enucleated. Vitreous samples were obtained for the analysis of the concentrations of HOE 961 and its main metabolites.

The in vitro antiviral drug sensitivity of the HSV-1 strain used in our retinitis experiment was determined by plaque reduction assays of tissue cultures of HSV-1-infected Vero cells by methods described previously (19).

RESULTS

Toxicity study. The vitreous remained clear and the retina appeared normal in all animals throughout the period after the injection of compound 2242. The rabbit eyes had normal electroretinography findings for all concentrations tested (Fig. 1). Light microscopic examination showed normal retinal morphology at all times following the injection (Fig. 2). Transmission electron microscopy of the retinal tissue of an eye injected with 2,000 μM revealed no abnormalities, confirming the light microscopy findings (data not shown).

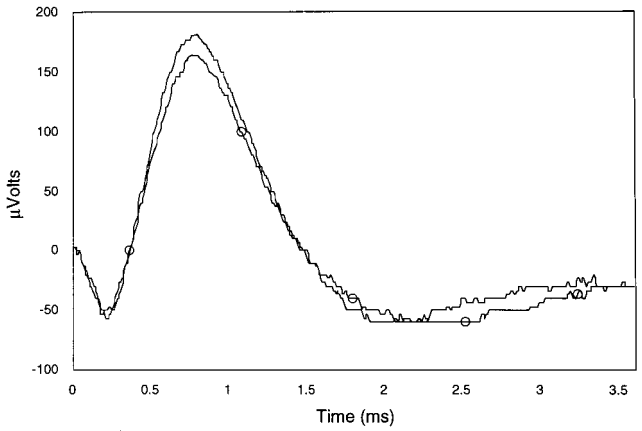

FIG. 1. Electroretinogram for an animal receiving 2,000 μM compound 2242, taken at baseline and at 2 weeks (line with circles). The b-wave tracings, form, and amplitudes are within normal limits. ms, milliseconds.

Treatment study. Compound 2242 at the dose of 2,000 μM and ganciclovir at the dose of 480 μM were equally effective compared with saline when administered simultaneously with the virus ($P = 0.025$) (Table 1). In the 3-day pretreatment paradigm, compound 2242 was superior to ganciclovir ($P = 0.034$); there was no clear difference between the two in the treatment of an established infection. All control eyes presented grade 4+ retinitis (12).

Pharmacokinetic studies. Noncompartmental analysis found a terminal elimination half-life of 8 h for compound 2242. The vitreous pharmacokinetic study showed that the data fit a two-compartment model with an initial half-life of 3.9 h and a longer terminal elimination half-life of 14.3 h. The main metabolite of compound 2242, compound 1225 (also known as 2-amino-8-hydroxy-7-[(1,3-dihydroxy-2-propoxy)methyl]purine), was not present at detectable levels. Testing of the in vitro metabolism of HOE 961 with rabbit liver showed that it is almost completely metabolized into three main metabolites. They are compound 2242 (40%), compound 0130 (15%), and compound 1225 (41%). Only 4% of the HOE 961 remained unmetabolized.

The concentrations of HOE 961 and compound 2242 in the plasma and vitreous after oral administration are summarized in Table 2. The concentrations of HOE 961 and its main metabolites in plasma correlate with the concentrations found in the rabbit liver. However, the level of the active parent compound 2242 in the eyes was considerably lower than that in plasma, and accumulation did not occur.

DISCUSSION

Compound 2242 (2-amino-7-[(1,3-dihydroxy-2-propoxy)-methyl]purine is the first known nucleoside analog antivirally active against herpesviruses. Its side chain is substituted at the N-7 atom position of the purine ring system. To date, all similar compounds had the sugar moiety attached to the N-9 atom of the purine ring system. This compound may act after phosphorylation on the corresponding triphosphate ester, exerting its anti-CMV activity via specific inhibition of viral DNA

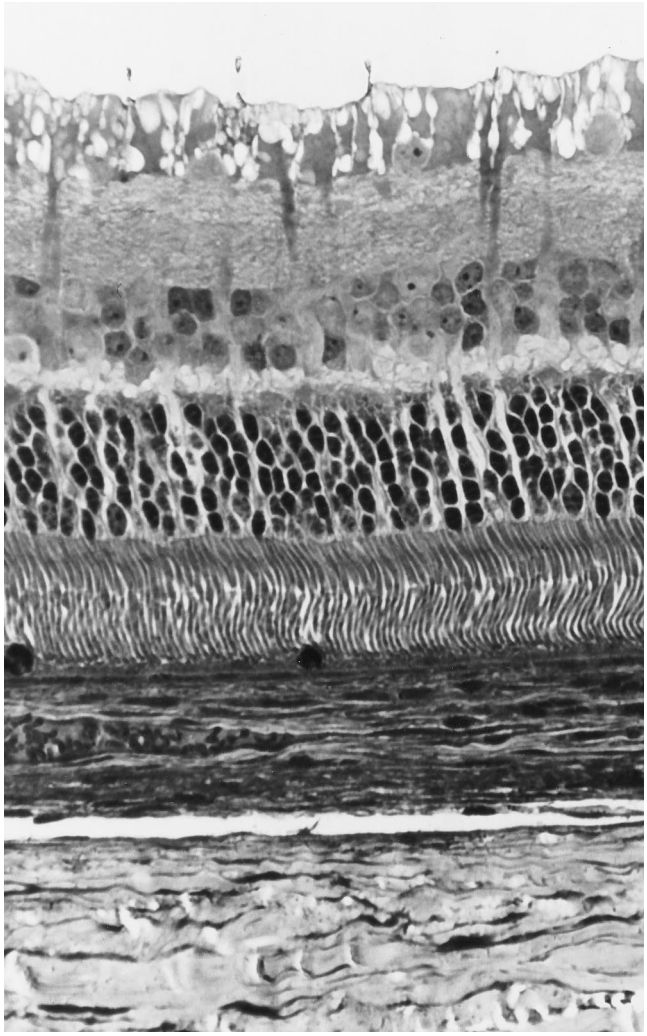

FIG. 2. Glycolmethacrylate section (thickness, 2 μm) of an eye after treatment with 2,000 μM compound 2242 for 2 weeks. The retinal cytoarchitecture is well preserved (toluidine blue staining; magnification, ×132).

synthesis (25). The efficacy of the intravenous treatment of murine CMV-infected mice with this compound is in the same range as that of treatment with ganciclovir, while intraperitoneal administration yields greater efficacy than that with ganciclovir (15). Pharmacokinetic studies in rhesus monkeys indicate that compound 2242 has an oral absorption rate at least twice that of oral ganciclovir. Its main metabolite is the 8-hydroxy derivative, which shows neither antiviral nor toxic effects (15). The cytotoxicity of the compound itself seems to be low. The only side effect observed in mice was a reversible atrophy of the testicles similar to that caused by ganciclovir.

Thus, compound 2242 has several interesting properties. It is structurally different from ganciclovir and related nucleoside analogs and may be active against ganciclovir-resistant strains of CMV or HSV. The pharmacokinetics and in vitro and in vivo metabolism of its diester derivatives in combination with the in vivo antiviral efficacy of compound 2242 led to the identification of an oral compound (diester prodrug HOE 961, also known as 2-amino-7-[(1,3-bis-acetoxy-2-propoxy)methyl]purine). Oral prodrugs are available, and the compound appears to have low cytotoxicity. We wished to quantitate the retinal toxicity of the parent compound and assess the efficacy of its highest nontoxic concentration in a rabbit model of focal herpes retinitis as well as compare its efficacy to that of ganciclovir. In this model, a reproducible focal retinitis develops and enlarges in a predictable fashion to involve the entire retina over a 2-week period. The retinitis is characterized by well-defined borders which are histologically well demarcated and by full-thickness retinal necrosis. These characteristics are in many ways similar to those of human CMV retinitis in immunosuppressed patients. Our results showed that the highest nontoxic concentration tested (2000 μM), which is near the solubility limit of the compound, has a very high anti-CMV intravitreal therapeutic index (40,000:1) in the eye, and treatment with the compound compares favorably with treatment with ganciclovir for the prevention of the development of retinitis. Intravitreally, the duration of action appears to be longer than that of ganciclovir, possibly because of its higher therapeutic index. It is 30 times more active against human CMV than HSV-1 (the virus used in our retinitis model), making it likely that compound 2242 would have a substantially longer duration of action against human CMV. In addition, the availability of an orally bioavailable prodrug for compound 2242 (its diacetate) makes it a particularly promising therapy, and if this

TABLE 1. Scores for treated and control animals[a]

Treatment	Pretreatment scores[b]	Simultaneous treatment scores[c]	Posttreatment scores[d]
Compound 2242	0, 0, 0, 0, 0, 0	0, 0, 0	0.5, 1.5, 2.5, 3.5
Ganciclovir	0, 0, 3.5, 3.5, 3.5	0, 0, 0	2.5, 2.5, 2.5, 3.5
Saline	4, 4, 4	4, 4, 4	4, 4, 4

[a] Scoring was based on the worst clinical presentation during the course of the infection. Treated animals with a similar but delayed clinical presentation by comparison with that for animals subject to the natural course of the disease were given a score that was reduced by 0.5 for statistical purposes.

[b] Pretreatment involved the intravitreal injection of drug or saline 3 days prior to inoculation with the virus. *P* values were determined by the Wilcoxon rank-sum test. Compound 2242 versus ganciclovir, $P = 0.034$; compound 2242 versus saline, $P = 0.0047$; ganciclovir versus saline, $P = 0.018$.

[c] *P* values were determined by the Wilcoxon rank-sum test. Compound 2242 and ganciclovir versus saline, $P = 0.025$.

[d] Posttreatment involved the intravitreal injection of drug or saline 3 days after inoculation with the virus (at the earliest sign of inflammation). *P* values were determined by the Wilcoxon rank-sum test. Compound 2242 versus ganciclovir, $P = 0.35$; compound 2242 versus saline, $P = 0.028$; ganciclovir versus saline, $P = 0.022$.

TABLE 2. Concentrations of compound 2242 and compound 1225 (main metabolite) in the plasma and the vitreous of the eyes after oral administration of the prodrug HOE 961

Time after cessation of drug therapy (h)	Days of dosing (no.)	Dose of HOE 961 (mg/kg/day)	Concn of compound 2242 (ng/ml)		Concn of compound 1225 (ng/ml)	
			Plasma	Vitreous	Plasma	Vitreous
48	7	247	<100	<100	360	<100
24	7	243	110	100	760	<100
0	4	22.6	<100	<100	170	<100
0	7	26.6	<100	<100	140	<100
0	2	181	310	110	800	180
0	2	151	200	<100	520	<100
0	2	334	990	250	1,970	230
0	2	281	440	110	790	120
0	2	649	1,050	460	1,200	260
0	2	613	5,780	840	7,530	770

prodrug proves to be nontoxic and effective, it might obviate the need for intravitreal therapy.

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