ABSTRACT:

The present study was carried out to delineate the ocular pharmacokinetics of ganciclovir (GCV) following intravitreal administration. Another objective was to achieve sustained therapeutic concentrations of GCV in the vitreous over a prolonged period of time using its acyl monoester prodrugs (acetate, propionate, butyrate, and valerate). New Zealand albino male rabbits (2–2.5 kg) were implanted in the anterior chamber across the cornea using a 25-gauge needle, and a linear microdialysis probe was implanted in the anterior chamber across the cornea using a 25-gauge needle. The probes were perfused with isotonic phosphate buffer saline (pH 7.4) at a flow rate of 2 μl/min. The drugs were administered (0.2 μmoles) intravitreally and the samples were collected every 20 min over a period of 10 h. The vitreal terminal elimination half-life (t1/2β) of GCV was found to be 426 ± 109 min. The hydrolysis rate and vitreal clearance of the prodrugs increased with the ascending ester chain length. The vitrebral elimination half-lives (t1/2β) of GCV, acetate, propionate, butyrate, and valerate esters of GCV were 170 ± 37, 117 ± 50, 122 ± 13, 55 ± 26, and 107 ± 14 min, respectively. A parabolic relationship was observed between the vitreal elimination rate constant and the ester chain length. Mean residence time (MRT) of the regenerated GCV following prodrug administration was found to be three to four times the value obtained after GCV injection. In conclusion, these studies have shown that the ester prodrugs generated therapeutic concentrations of GCV in vivo, and the MRT of GCV could be enhanced by 3- to 4-fold through prodrug modification.

HUMAN CYTOMEGALOVIRUS (HCMV) retinitis is the most common opportunistic infection occurring in about 15 to 42% of the patients with acquired immunodeficiency syndrome (AIDS), which often leads to blindness if untreated (Holland et al., 1983; Freeman et al., 1984; Gross et al., 1990). Ganciclovir (GCV), a 2′-deoxyguanosine analog, was the first Food and Drug Administration approved drug available in the United States with significant activity against HCMV. It was shown to be 26 times more potent than acyclovir against HCMV in vitro (Morse et al., 1993). Previous studies reported excellent in vitro activity of GCV against human herpes virus type 6, human herpes simplex viruses types 1 and 2, varicella-zoster virus, and Epstein-Barr virus (Smee et al., 1983; Andrei et al., 1991; Shigeta et al., 1991; Snoeck et al., 1991; Konno et al., 1993). It was approved for the induction and maintenance therapy of HCMV retinitis in AIDS patients in 1989 and has been widely used. GCV also gained significant importance in the treatment of HCMV infection in solid organ recipients (Dunn et al., 1991; Markham and Faulds, 1994; Tsinontides and Bechtel, 1996; Murray, 1997).

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1 Abbreviations used are: HCMV, human cytomegalovirus; AIDS, acquired immunodeficiency syndrome; GCV, ganciclovir; GCVMA, monoacetate; GCVM/P, monopropionate; GCVMB, monobutyrate; GCVMV, monovalerate; AUC, area under the curve; MRT, mean residence time.

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Intravenous administration of GCV alone or in combination with other anti-HCMV agents like foscarinet, cidofovir, zidovudine etc., is currently the treatment of choice for HCMV retinitis (Markham and Faulds, 1994; Manion et al., 1996). As it is only virostatic, indefinite maintenance therapy is required in addition to the induction therapy to prevent any relapse (Drew, 1992). The most common adverse effect in patients receiving intravenous GCV is the hematological toxicity, mainly neutropenia (up to 50%) (Faulds and Heel, 1990; Goodrich et al., 1993; Winston et al., 1993). Other toxic effects include granulocytopenia, thrombocytopenia, azoospermia, and rise in the serum creatinine (Faulds and Heel, 1990). Relatively high systemic toxicity caused by intravenous therapy has lead to the intravitreal administration of GCV (Henry et al., 1987; Daikos et al., 1988; Ussery et al., 1988; Cantrill et al., 1989; Cochereau-Massin et al., 1991), which enabled considerable improvement and/or stabilization of HCMV retinitis in most (80–100%) of the patients. Intravitreal administration has the advantage of maintaining high concentrations of the drug for effective control of ocular infections with minimal systemic adverse effects. Clinical trials reported good response rates to intravitreal induction therapy with GCV (Cochereau-Massin et al., 1991). However, very few studies have been carried out to date delineating the intravitreal pharmacokinetics of GCV (Henry et al., 1987; Ashton et al., 1992; Morlet et al., 1996). The major constraint to the development and assessment of posterior segment pharmacokinetics of drugs in animal models is the inaccessibility of the ocular fluids for continuous serial sampling. Microdialysis has gained wide recognition as a standard technique for sampling various tissues and fluids such as brain, liver, kidney, skin, tumor, bile, and blood (Diaz et al., 1992;
Robinson, 1995; Maggs et al., 1997), as well as anterior and vitreous chambers of the eye (Stemple et al., 1993; Hughes et al., 1996; Sato et al., 1996; Waga and Ehinger, 1997; Rittenhouse et al., 1999; Macha and Mitra, 2001a,b). We have developed an animal model in our laboratory for simultaneous and continuous sampling of the vitreous and aqueous humors in rabbits using microdialysis technique (Macha and Mitra, 2001a). The proposed animal model would delineate complete ocular pharmacokinetics of drugs after intravitreal administration.

The present study was carried out to determine the intravitreal pharmacokinetics of GCV. Another objective was to achieve sustained concentrations of GCV, using its acyl monoester prodrugs, for a prolonged period of time. In this study, we have also demonstrated that the requirement for repeated intravitreal injections of GCV could be avoided by the prodrug approach. The detailed investigation of the pharmacokinetics of GCV and its monoester prodrugs may provide valuable information for the future development of more effective GCV therapy.

Experimental Procedures

Materials. GCV was a generous gift from F. Hoffmann-La Roche (Nutley, NJ). The monoester prodrugs of GCV [monoacetate (GCVMA), monobutyrate (GCVMB), monovalerate (GCVMV)] were synthesized according to a method previously published from our laboratory (Gao and Mitra, 2000).

The concentric probes (CMA/20, 0.5 × 10 mm polycarbonate membrane and 14 mm shaft) used for sampling the vitreous chamber were obtained from CMA/Microdialysis (North Chelmsford, MA). The linear probes (MD-2000, 0.32 × 10 mm polycrylonitrile membrane and 0.22 mm diameter tubing) for aqueous humor sampling were acquired from BAS Bioanalytical Systems Inc. (West Lafayette, IN). A microinjection pump (CMA/100) was used for perfusing isotonic phosphate buffer saline.

Methods. Animal model. New Zealand albino rabbits weighing 2 to 2.5 kg were used for the experiments. The animals were kept under anesthesia throughout the experiment using ketamine HCl (35 mg/kg) and xylazine (3.5 mg/kg) given intramuscularly every hour. Prior to the implantation of the microdialysis probes, pupils were dilated with two drops of 1% tropicamide. For the implantation of the concentric probe in the vitreous chamber, a 22-gauge needle was inserted carefully into the eye about 3 mm below the corneal scleral limbus so that it traverses through the center of the anterior chamber to the opposite end of the cornea as evidenced by microscopic examination. The needle was inserted into the vitreous chamber using a 25-gauge needle. The needle was inserted across the cornea just above the corneal scleral limbus so that it traverses through the center of the anterior chamber to the opposite end of the cornea as evidenced by microscopic examination. The sample-collecting end of the linear probe was inserted carefully into the bevel edge end of the needle. The needle was slowly retracted leaving the probe in the vitreous chamber. The outlets of both the probes were fixed to prevent any disturbances during sample collection. A diagrammatic representation of the implanted microdialysis probes within the eye is presented in Fig. 1. The probes were perfused with isotonic phosphate buffer saline (pH 7.4) at a flow rate of 2 μl/min using a CMA/100 microinjection pump.

After probe implantation, the animals were allowed to stabilize for 2 h prior to the initiation of any study. One hundred microliters of the standard solution of GCV, or its monoester prodrug (0.2 μmoles), was carefully administered into the midvitreous using a specially prepared cannula containing a 30-gauge needle. The samples were collected every 20 min over a period of 10 h. At the end of an experiment, euthanasia was performed under deep anesthesia with an intravenous injection of sodium pentobarbital through the marginal ear vein.

In vitro probe calibration. Microdialysis probe recovery was determined in an aqueous solution containing a known concentration of the compound maintained at biological temperature. The probe was continuously perfused at a constant flow rate of 2 μl/min, and samples were collected every 20 min. The recovery of a compound of interest is calculated according to eq. 1.

\[ \text{Recovery}_{\text{in vitro}} = \frac{C_{\text{out}}}{C_{\text{i}}} \]  

\( C_{\text{out}} \) is the concentration in the outflow solution and \( C_{\text{i}} \) the concentration in the medium. The dialysate concentrations were transformed into the actual anterior and vitreous concentrations using eq. 2.

\[ \bar{C} = \frac{C_{\text{out}}}{\text{Recovery}_{\text{in vitro}}} \]  

\( \bar{C} \) is the substance concentration in the vitreous and aqueous humors, and \( C_{\text{out}} \) is the concentration of the compound in dialysate.

HPLC analysis. HPLC system (Waters 600 pump; Waters Corp, Milford, MA), equipped with a fluorescence detector (HP 1100) and a reversed phase C18 column (4 μ, 250 × 4.6 mm, Synergy-max; Phenomenex, Torrance, CA), was used for the quantification of the samples. The GCV samples were analyzed using an isocratic method with an eluent containing 15 mM potassium phosphate buffer (pH 2.5) and 0.2% acetonitrile at a flow rate of 1 ml/min. The separation of GCV and its monoesters was achieved using a gradient method with 15 mM potassium phosphate buffer (pH 2.5): acetonitrile in proportions of 99.8:0.2 as phase A and 1:1 as phase B at a flow rate of 1 ml/min. All the samples were analyzed at an excitation wavelength of 265 nm and emission wavelength of 380 nm. The limit of quantification was 20 ng/ml for GCV and 50 to100 ng/ml for the prodrugs.

Data analysis. The experiments were carried out at least in triplicates, and the results are given as mean ± S.D. The statistical significance between the parameters was determined using analysis of variance at \( p < 0.05 \) unless otherwise specified.

The rate constants for elimination from vitreous chamber were determined by nonlinear regression analysis (Kinetica 2000, version 3.1; InnaPhase Corporation, Philadelphia, PA) of the concentration-time data. The best fit model for the vitreous concentration-time data of GCV and its monoester prodrug was selected based on the coefficient of variation percentage, Akaike’s information criterion, F-test, Run-test, and residual plots. The vitreous concentration-time profiles are represented with the best fit line drawn using the parameters determined from the best fit model. Noncompartmental analysis was carried out for the anterior chamber profiles of GCV.

Results

The representative anterior and vitreous chamber concentration-time profiles of GCV following intravitreal administration is shown in Fig. 2. Vitreous concentration of GCV appeared to decline biexponentially. The vitreous concentration-time profiles of GCV were mod-
eled using a two-compartment open system (eq. 3) with the major component of elimination from the central compartment.

\[ C_V = \frac{X_0(k_{21} - \alpha)}{V_1(\beta - \alpha)} e^{-\alpha t} + \frac{X_0(k_{21} - \beta)}{V_1(\alpha - \beta)} e^{-\beta t} \]  

(3)

where, \( \alpha + \beta = k_{10} + k_{12} + k_{21} \), \( \alpha \beta = k_{10} k_{21} \), \( X_0 \) is the dose administered and \( V_1 \) is the volume of the central compartment. The constant \( k_{10} \) is the apparent first-order elimination rate constant from the central compartment, and \( k_{12} \) and \( k_{21} \) are the intercompartmental transfer rate constants.

The anterior chamber concentration-time profiles of GCV after intravitreal administration were analyzed using a noncompartmental model, in which the initial anterior chamber concentration was considered to be zero.

The vitreous concentration-time profiles of the monoesters are depicted in Figs. 3 to 6. The anterior chamber concentrations of the monoesters after intravitreal administration were below the quantitation limits. The vitreous concentration of acetate and butyrate esters of GCV was observed to decline biexponentially, and their disposition was modeled according to a two-compartment system. The propionate and valerate esters showed a monoeponential decline and were modeled as one-compartment system. The distribution phase of the metabolite (GCV) was obscured by the kinetics of its formation in vivo; therefore, a single compartment was used to model the vitreous concentration-time profiles of the metabolite.

The three-compartment system used to describe the disposition of acetate and butyrate esters of GCV is depicted in Fig. 7. Two of the compartments are for the monoester, which was assumed to be eliminated from the central compartment (compartment 1). In this model, the monoesters distribute into the peripheral compartment (compart-
The disposition of the propionate and valerate esters of GCV was described by a two-compartment system. The monotertiary concentration-time profiles of acetate and butyrate esters were described based on eq. 4 and the regenerated GCV using eq. 5.

\[
C_v = \frac{X_0(k_{21} - \alpha)}{V_1(\beta - \alpha)}e^{-\alpha t} + \frac{X_0(k_{21} - \beta)}{V_1(\alpha - \beta)}e^{-\beta t}
\] (4)

\[
C_v = \frac{k_{13}X_0(k_{21} - \gamma)}{V_3(\alpha - \gamma)(\beta - \gamma)}e^{-\gamma t} + \frac{k_{13}X_0(k_{21} - \alpha)}{V_3(\gamma - \alpha)(\beta - \alpha)}e^{-\alpha t} + \frac{k_{13}X_0(k_{21} - \beta)}{V_3(\alpha - \beta)(\gamma - \beta)}e^{-\beta t}
\] (5)

where, \(\alpha + \beta = k_{10} + k_{12} + k_{24} + k_{13}\), \(\alpha \beta = k_{10}k_{24} + k_{13}k_{21}\), \(\gamma = k_{30}\), and \(X_0\) is the intravitreal dose. The constant \(k_{10}\) is the apparent first-order elimination rate constant of the drug from the central compartment, \(k_{12}\) and \(k_{21}\) are the intercompartmental transfer rate constants, and \(k_{13}\) and \(k_{30}\) are the apparent first-order formation and elimination rate constants, respectively, of the metabolite.

The disposition of the propionate and valerate esters of GCV was described by a two-compartment system. The monoesters were assumed to distribute instantaneously (compartment 1) and simultaneously metabolize to GCV, which is represented by the compartment 3. The vitreous concentration-time profiles of propionate and valerate esters were described based on eq. 6 and the regenerated GCV using eq. 7.

\[
C_v = \frac{X_0}{V_1}e^{-\alpha t}
\] (6)

where, \(\alpha = k_{10} + k_{13}\).

\[
C_v = \frac{k_{13}X_0}{V_1(\beta - \alpha)}e^{-\alpha t} + \frac{k_{13}X_0}{V_1(\alpha - \beta)}e^{-\beta t}
\] (7)

where, \(\alpha + \beta = k_{10} + k_{30} + k_{13}\), and \(\alpha \beta = k_{10}k_{30} + k_{13}k_{30}\). The constants \(k_{10}\), \(k_{13}\), and \(k_{30}\) are as defined previously.

The concentration-time data of all the drugs was fitted into their respective models and the final pharmacokinetic parameters were determined using nonlinear, least-squares program Kinetica 2000 (version 3.1). The pharmacokinetic model parameters for GCV and its monoesters are shown in Table 1.
increased with the ester chain length, which is in accordance with the results of GCV would provide therapeutic concentrations over prolonged intervals for the treatment of cytomegalovirus retinitis. The monoester prodrugs of GCV, such as the acetate, propionate, and butyrate esters, although the elimination rate constant (k10) was higher, the prodrugs generated GCV primarily due to the esterase activity, acetycholine and butyrylcholine esterases, in all the ocular tissues (i.e., retina-choroid, iris-ciliary, lens, vitreous and aqueous humors) (Lee et al., 1985). Previously, pharmacokinetic studies have been carried out in human patients by collecting a sample of the vitreous and/or aqueous humors prior to intraocular surgery. In case of animal studies, the subjects were sacrificed for collecting the vitreous and/or aqueous humors at each time point. These techniques yield inadequate data and, moreover, introduce a considerable amount of intersubject variability. A dual probe microdialysis technique has been developed in our laboratory to study the ocular pharmacokinetics in rabbits following intravitreal or systemic administration. The animal model was validated by measuring intraocular pressure, protein concentrations in the aqueous and vitreous humors, and fluorescein kinetics after systemic and intravitreal administration. These studies have proved the integrity of the blood ocular barriers during the term of an experiment.

The terminal elimination (t1/2) half-life of GCV after intravitreal administration was found to be 426 ± 109 min, which is consistent with a previous report (Lopez-Cortes et al., 2001). Lopez-Cortes et al. (2001) have reported terminal half-lives of 7.14 and 8.66 h after the intravitreal administration of 196 and 800 µg of GCV, respectively. The biexponential decline of GCV indicates its distribution/penetration into the intraocular tissues such as retina, lens, iris-ciliary body etc. The proportion of GCV eliminated through the anterior chamber pathway was found to be only 1%, thus indicating that the retinal pathway is the major route of elimination for this drug. The vitreal elimination half-life of GCV obtained in our studies support intravitreal administration. The MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the propionate ester prodrug as such. MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the acetate ester prodrug. The MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the acetate ester prodrug. The MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the acetate ester prodrug. The MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the acetate ester prodrug. The MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the acetate ester prodrug. The MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the acetate ester prodrug.

GCV was detected in the vitreous within 10 to 30 min after the administration of the prodrugs. The Tmax for GCV regeneration following prodrug administration was observed to range from 150 to 210 min, and the therapeutic concentrations of GCV were achieved within 1 h. Cmax values for the regenerated GCV increased with the ester chain length possibly due to an ascending metabolism rate from acetate to valerate esters. The acetate and propionate ester prodrugs degraded at an optimum rate with matching GCV generation and elimination rates thus providing sustained concentrations of GCV over the experimental time period. In case of butyrate and valerate esters, the Cmax values were found to be twice the Clast values, probably due to the rapid metabolism in vivo and elimination of the prodrug as such. MRT of the regenerated GCV from acetate and propionate esters was found to be three to four times as compared with the direct administration of GCV.

The percentage of the acetate, propionate, butyrate, and valerate ester prodrugs metabolized to produce GCV in vivo were 26, 27, 35, and 31%, respectively. The percentage metabolized increased with the ester chain length and the hydrolysis rate of the prodrugs. These results indicate that the prodrugs are also eliminated as such from the vitreous, and their elimination rate accelerates with lipophilicity. GCV formed in vivo has lower rate of elimination compared with the prodrugs, thus improving the residence time of GCV in the vitreous chamber. In addition, it has been assumed that equilibrium is estab-
lished between the vitreous humor and retina, and the drug levels become similar in both the tissues (Henry et al., 1987; Morlet et al., 1996). The in vitro tissue hydrolysis studies carried out in our laboratory showed that the prodrugs are hydrolyzed in the retina at a much faster rate compared with that in the vitreous humor. A major portion of the prodrugs penetrating as such into the retina might hydrolyze to generate more GCV in the tissue, thus providing higher retinal concentrations of GCV than detected in the vitreous humor.

In conclusion, the microdialysis technique can be effectively used for determining the drug and metabolite concentrations simultaneously. Chemical modification of GCV was found to provide effective concentrations of GCV over a prolonged period of time. Based on the MRT values, the frequency of administration could be reduced by at least three to four times using acetate and propionate esters of GCV, with proper dosage adjustments. This approach appears to be useful in development of new formulations for direct intraocular administration, as the prodrugs studied have demonstrated optimal physicochemical properties. Ocular drug delivery through prodrugs appears to be a better approach compared with the sustained release implants, which require complicated surgery for the implantation and removal of the device from the vitreous chamber.

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References
1033.