Cyclosporine-Loaded Microspheres for Treatment of Uveitis: In Vitro Characterization and In Vivo Pharmacokinetic Study

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PURPOSE. A sustained intraocular level of immunosuppressive drug is desirable for the treatment of uveitis and other intraocular immune disorders. The objective of the present investigation was to assess the suitability of cyclosporine-loaded poly(lactic-co-glycolic acid) microspheres (CyS-PLGA-MS) to achieve this goal.

METHODS. A solvent-evaporation method was used in the preparation of CyS-PLGA-MS. These microspheres were characterized for drug loading, entrapment efficiency, and in vitro release by high-performance liquid chromatography, particle size by phase-contrast light microscopy and surface morphology by scanning electron microscopy. The 3H-CyS-PLGA-MS suspension was injected into the vitreous body of healthy rabbits, and the concentration of cyclosporine in various ocular tissues and blood at predetermined intervals was measured by a scintillation counting technique and the pharmacokinetic parameters were calculated. Intravitreous administration of 3H-CyS solution was conducted as the control.

RESULTS. The CyS-PLGA-MS was produced, with drug-loading ranging from 11% to 16% and a high entrapment efficiency from 86% to 98%. Microspheres were discrete, spherical particles with a diameter of approximately 50 μm. The CyS was constantly and slowly released from microspheres in the in vitro release experiment. Compared with CyS solution, microspheres prolonged the release of CyS and maintained therapeutic CyS concentrations for at least 65 days in disease-related tissues such as the choroid-retina and iris-ciliary body. The percentage of CyS released in vitro correlated well with the CyS distribution rate in vivo.

CONCLUSIONS. CyS-PLGA-MS, displaying sustained intraocular release of CyS and showing advantages over CyS solution, may meet clinical needs more efficiently. (Invest Ophthalmol Vis Sci. 2006;47:3983–3988) DOI:10.1167/iovs.05-1373

Uveitis is a common disease in ophthalmology that needs long-term therapy. Cyclosporine (CyS), a lipophilic cyclic polypeptide with immunosuppressive activity, has been shown to abate acute intraocular inflammation effectively and preserve vision, even when the uveitis is refractive to conventional therapy.1

Injection of drug into the vitreous cavity provides a therapeutic level at the target site and minimizes systemic side effects. This is especially important with CyS, in light of its considerable systemic adverse effects. Nevertheless, multiple intraocular injections not only bring patients discomfort, but also increase the risk of cataract formation, retinal detachment, and endophthalmitis. As an alternative to multiple intravitreous injections, sustained drug delivery systems have been proposed, such as biodegradable microspheres with the size of several to several hundred micrometers, ensuring that they readily pass through the syringe.2–5

Attention has also been paid to the intraocular use of CyS-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres (CyS-PLGA-MS) in ocular therapy, such as keratoplasty rejection.6,7 However, the potential for such microspheres in the treatment of uveitis remains unknown. In the present study, we prepared intraocular CyS-PLGA-MS for the treatment of uveitis. Obviously, one of the key points for such a drug delivery system is to maintain the therapeutic CyS concentrations for a long time in disease-related tissues, and so the in vitro release of CyS from the microspheres and in vivo pharmacokinetic behavior of CyS after intravitreous administration was emphasized in this study. 3H-CyS-PLGA-MS suspension was injected into the vitreous body of healthy rabbits. Then, the concentrations of CyS in various ocular tissues and blood were measured to characterize the pharmacokinetics of this microsphere preparation in comparison with solution.

MATERIALS AND METHODS

CyS was obtained from Huabei Pharmaceutical Group (Shijiazhuang, China) and 3H-CyS (radioactivity 1.0 mCi, radioactivity-specific 9 Ci/mmol, purified by HPLC, purification >99%) was supplied by Chinese Atomic Energy Research Institute (Beijing, China). PLGA was supplied by Birmingham Polymers Co. (Birmingham, AL). PEG1000, pluronic F68, para-phenylene-phenyloxazole (POPOP), and 2,5-diphenyloxazole (PPO) (fluid sparkle grade) were purchased from Sigma-Aldrich (St. Louis, MO). Polyvinyl alcohol (PVA), dimethylbenzene, and hydrogen peroxide were the product of Beijing Organic Chemical Plant (Beijing, China). Perchloric acid was from the Dongfang Chemical Plant (Tianjin, China).

Preparation of CyS-PLGA-MS

CyS-PLGA-MS for in vitro characterization were prepared from PLGA (molecular weight, 15,000; 75:25) by a solvent-evaporation process described in detail previously,8 with or without additives such as pluronic F68 (3%) or PEG 1000 (30%). The obtained microspheres were subsequently freeze dried and stored at −20°C before use. Three batches for each formulation were prepared.

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Particle Size Distribution and Morphology

The particle size distribution of microspheres was measured by phase-contrast light microscopy (model BX51; Olympus, Tokyo, Japan). The morphology of microspheres before and after the release experiment was examined by scanning electron microscope (SEM; model JSM-5600LV; JEOL, Tokyo, Japan).

Determination of CyS Content and Encapsulation Efficiency in Microspheres

The freeze-dried CyS-PLGA-MS were exactly weighed and dissolved in a mixture of methylene chloride and methanol, and the concentration of CyS was determined by high-performance liquid chromatography (HPLC) using the chromatographic conditions reported previously. The CyS content in microspheres was calculated according to the following equation:

\[ \text{CyS content} = \frac{W_{\text{CyS}}}{W_{\text{MS}}} \times 100\% \]

where \( W_{\text{CyS}} \) represents the weight of CyS found in microspheres and \( W_{\text{MS}} \) was the corresponding weight of microspheres.

To determine the encapsulation efficiency, the suspension of microspheres before freeze-drying was centrifuged and rinsed, the supernatant and rinsing solutions were mixed, and then the concentration of CyS in the mixture was analyzed by HPLC. Encapsulation efficiency was calculated according to the following equation:

\[ \text{Loading efficiency} = \frac{W_{t} - C_{s}V_{s}}{W_{t}} \times 100\% \]

where \( W_{t} \) represents the total CyS added, \( C_{s} \) is the concentration of CyS in the supernatant and rinsing solution, and \( V_{s} \) means the volume of the supernatant and rinsing solution.

In Vitro Release Study

Freeze-dried CyS-PLGA-MS were suspended in phosphate buffer (pH 7.4) containing 0.02% Tween-80 and 0.05% sodium azide. The system was maintained at 37°C and kept stirring at 100 rpm. At every predetermined time point, an aliquot (2 mL) of microsphere suspension was sampled and centrifuged for 5 minutes at 4440 \( g \), and the concentration of CyS in the supernatant was measured by HPLC. The fresh release medium (2 mL) was added to the remainder of the suspension.

Pharmacokinetic Study

Animals. Japanese White rabbits (Vital Laboratory Animal Center, Beijing, China) weighing between 2 and 3 kg were acclimated for at least 1 week under standardized temperature (25–28°C), humidity (50–60%), and light (12 hours light–dark) conditions before experiment. Animals were allowed to free access to standard food and tap water before and throughout the experiment. All care and handling of rabbits adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, with the approval of Institutional Authority for Laboratory Animal Care.

Preparation of \( ^{3} \text{H}-\text{CyS} \) Solution and \( ^{3} \text{H}-\text{CyS}-\text{PLGA-MS} \)

For the preparation of \( ^{3} \text{H}-\text{CyS} \) solution, CyS and \( ^{3} \text{H}-\text{CyS} \) were dissolved in anhydrous alcohol according to definite proportions, injection water was added, and the solution was mixed well, until clarity returned. The composition and preparation procedures of \( ^{3} \text{H}-\text{CyS}-\text{PLGA-MS} \) was the same as for in vitro characterization, except that we used \( ^{3} \text{H} \)-labeled CyS instead of CyS and the product was sterilized by radiation (2.5 M Rad, \( ^{60} \text{Co} \)).

Intravitreous Administration, Collection, and Analysis of Samples. Rabbits were randomly assigned into two groups. The solution group: An intravitreous injection of 0.1 mL \( ^{3} \text{H}-\text{CyS} \) solution (96 \( \mu \)g/mL) was given through the orbiculus ciliaris approximately 3 mm posterior to the limbus by means of a 30-gauge needle. Microsphere group: An intravitreous injection of 0.1 mL aseptic saline containing 10 mg \( ^{3} \text{H}-\text{CyS}-\text{PLGA-MS} \) (with 3% pluronic F68) was then administered by the same route as used in the solution group.

Sampling was performed on three rabbits of each group at 1, 3, 6, 12, 24, 48, 72, and 120 hours in the solution group and at 1, 3, 7, 14, 25, 35, 50, and 65 days for microsphere formulation. A blood sample was taken from the heart, and the animal was killed. Immediately, the aqueous humor was aspirated, and the conjunctiva, cornea, iris/ciliary body, sclera, lens, vitreous, and retina-choroid were dissected in situ.

Samples except the lens were digested by incubation in the mixture of perchloric acid and hydrogen peroxide (1:2, vol/vol) at 80°C until complete dissolution. Drug in the lens was extracted with methanol three times. The obtained digest and extract were analyzed by a scintillation counter (TRI-CARB2100; PerkinElmer, Wellesley, MA). The concentration of CyS was calculated according to the standard curve established with a series of standard solutions. The pharmacokinetic parameters were calculated with the computer program 3P97 (Chinese Association of Mathematical Pharmacology, Beijing, China).

In Vitro and In Vivo Correlation

The ratio of AUC\(_{\text{vis}} \) (the area under the drug concentration versus time curve for each sampling time point, \( (\mu g/mL) \times \text{time} \)) to AUC\(_{\text{oral}} \) for the total test period of 65 days, designated as AUC\(_{\text{vis}} \)/AUC\(_{\text{oral}} \) (percent), was calculated for each ocular tissue and blood to represent the distribution rate of CyS. The correlation between AUC\(_{\text{vis}} \)/AUC\(_{\text{oral}} \) (percent) and the in vitro cumulative release percent (%) for corresponding time points was statistically analyzed (SPSS ver. 10.0; SPSS Inc., Chicago, IL).

RESULTS

Both HPLC and the scintillation counting technique have been confirmed for in vitro and in vivo determination of CyS, with satisfactory accuracy and precision.

Characterization of CyS-PLGA-MS

Table 1 gives the data of drug content, encapsulation efficiency, and mean diameter of three microsphere formulations. As is shown in Figure 1, microspheres before the release experiment were discrete spheres with smooth surfaces.

Table 1. Formulation and Characteristics of CyS-PLGA-MS

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Additive</th>
<th>Drug Content (%/w/w)</th>
<th>Encapsulation Efficiency (%)</th>
<th>Mean Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>16.3</td>
<td>97.65 ± 3.21</td>
<td>49.87 ± 27.1</td>
</tr>
<tr>
<td>II</td>
<td>PEG1000 (30%)</td>
<td>13.0</td>
<td>97.24 ± 2.38</td>
<td>50.22 ± 29.8</td>
</tr>
<tr>
<td>III</td>
<td>Pluronic F68 (3%)</td>
<td>11.0</td>
<td>86.65 ± 3.84</td>
<td>49.81 ± 25.7</td>
</tr>
</tbody>
</table>

\( n = 3 \).
In Vitro Release of CyS-PLGA-MS

Figure 2 shows that the addition of PEG and pluronic F68 significantly accelerated the release of CyS. As is shown in Figure 1, it is clear that the morphology of PLGA-MS underwent great changes during the release experiment.

Pharmacokinetic Study

\textbf{3H-CyS Solution Group.} Figure 3 shows the \(^3\)H-CyS concentration versus time curves and Table 2 gives pharmacokinetic parameters for intravitreous injection of \(^3\)H-CyS solution. No compartment model was suitable for the concentration-time profile for the conjunctiva, so only the statistical moment parameter is presented for this tissue.

\textbf{3H-CyS-PLGA-MS Group.} As is shown in Figure 4, after intravitreous administration of \(^3\)H-CyS-PLGA-MS, the \(^3\)H-CyS concentration versus time profiles for the ocular tissues and blood were distinct from those for \(^3\)H-CyS solution. No comp-
mon compartment model was suitable for the calculation of their pharmacokinetic parameters, so we give statistical moment parameters in Table 3.

### In Vitro and In Vivo Correlation

From Figure 5 and Table 4, it is obvious that there was good correlation between the in vitro release rate of CyS and the in vivo distribution rate of CyS from the microspheres, although the data of zero point (0, 0) in Figure 5 has a remarkable effect on the linear regression.

### DISCUSSION

Intravitreous injection of CyS solution has been performed in the rat model with experimental autoimmune uveitis by Nussenblatt et al.,10 who found that intravitreous CyS therapy protects eyes from EAU without producing significant circulating CyS levels, which is consistent with our results. According to the report of Nussenblatt et al. and our observations, after intravitreous doses of the CyS solution, the elimination of CyS in the eye was rapid, and so multiple injections would be needed to treat chronic ocular diseases such as uveitis. Also, the local concentration of CyS in the solution group was so high that side effects may not be avoided.

Many sustained-release systems have been proposed for CyS intravitreous delivery, including biodegradable implants and nonbiodegradable implants.11–17 All implants described previously had a common disadvantage, in that these implants had to be placed by surgery which made the administration risky and inconvenient. To circumvent this difficulty, the microsphere system which could pass through 20- to 23-gauge needles and be readily injected has been investigated for ophthalmologic application.18, 19 As shown in our studies, the maximum concentration of CyS in most ocular tissues in the microsphere group was approximately 35 days, and the concentration of CyS in most ocular tissues was relatively steady over the whole period of 65 days compared with solution group. It was demonstrated that there is significant difference in pharmacokinetic behavior between the microsphere and solution formulation.

From the in vitro release profile, it is clear that CyS was released from microspheres at a relatively constant rate, which could be accelerated by addition of PEG1000 or pluronic F68. Because pluronic F68 accelerated the release of CyS more significantly and the pluronic F68-containing microspheres maintained their structural integrity after 2 months of release experiment, as was shown by SEM, pluronic F68 was incorporated in microspheres for in vivo investigation.

To establish the in vitro and in vivo correlation, there must be a proper parameter to represent the transportation of drug in vivo. In case of oral administration, the absorption rate could be obtained as the in vivo parameter for this purpose according to traditional pharmacokinetic models. As for the intraocular injection of microspheres, the distribution rate of drug may be applied since there is a course of distribution of drug molecules into different ocular tissues. No common compartment model was suitable, however, except the statistical moment. The AUC
is the most common parameter in statistical moment, and represents the amount of drug distributed into certain ocular tissues in this study. The $AUC_t(n)$ refers to the amount of drug distributed into tissue up to the sampling time point, $t(n)$, whereas the $AUC_{65d}$ means the total drug was distributed into issue during the test period of 65 days. Therefore, the ratio of $AUC_t(n)$ to $AUC_{65d}$ for each ocular tissue represented the distribution rate of CyS.

It was indicated by our studies that the linear in vivo/in vitro correlation for most tested tissues of the eye was good and only that for the choroid-retina seemed not to be as good, with a regression correlation coefficient lower than 0.9. Because in vitro release of CyS from the microspheres was quite steady, theoretically, the linear in vivo/in vitro correlation would be good if the change in distribution rate, which is predominated by the in vivo concentration fluctuation, is also steady. The great in vivo concentration fluctuation is shown for the choroid-retina in Figure 4, meaning that the changes in distribution rate of CyS represented by $AUC_t/AUC_{65d}$ (percent), began at a low rate and turned to a high rate in a certain period. For this reason, it is easy to understand that the linear correlation between the distribution rate in the choroid-retina and the in vitro release rate is relatively not so satisfactory. This rule seems applicable for other tissues of the eye. For instance, relatively fluctuating in vivo concentration and bad linearity were also found in the vitreous body and iris-ciliary body. On the contrary, relatively steady in vivo concentration and the good linearity were observed in the lens.

According to the report of Lallemand et al.,$^{13}$ the therapeutic concentration of CyS in vitreous for uveitis is approximately 100 ng/mL. In the present study, PLGA microspheres showed the potential for intraocular sustained-release of CyS, because the mean residence time was increased about 10 times compared with the CyS solution, and a therapeutic drug level was maintained for 65 days, especially in disorder-related tissues such as the choroid-retina, vitreous body, and iris-ciliary body, which are the primary target of uveitis. By contrast, the blood level of CyS was very low, indicating that CyS entering the blood circulation may not lead to severe side effects.

In summary, the present CyS-loaded PLGA microsphere system has been shown to be a useful drug delivery system to prolong the residence of the drug in the eye after intravitreous administration. Taking into account that intravitreous drug injection is the usual route for posterior segment disorder treatment and repeated injections of drug solution involve a high risk of complications and toxicity, our findings may lead to an improvement in the efficacy of the drug and the compliance for the patients.

### Table 3. Pharmacokinetic Parameters by Statistical Moment Analysis for the Eight Ocular Tissues and Blood after Intravitreous Administration of $^3$H-CyS-PLGA-MS

<table>
<thead>
<tr>
<th>Tissues</th>
<th>$T_{max}$ (day)</th>
<th>$C_{max}$ (ng/mL or ng/g)</th>
<th>$AUC_{65}$ (ng/mL·day or ng/g·day)</th>
<th>$AUC_{65d}/AUC_{65}$ Vitreous Body</th>
<th>$MRT_{65d}$ (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreous body</td>
<td>35</td>
<td>259.8</td>
<td>9978</td>
<td>1.000</td>
<td>33.5</td>
</tr>
<tr>
<td>Choroid-retina</td>
<td>35</td>
<td>406.48</td>
<td>13840</td>
<td>1.387</td>
<td>39.0</td>
</tr>
<tr>
<td>Iris-ciliary body</td>
<td>35</td>
<td>174.25</td>
<td>7717</td>
<td>0.773</td>
<td>34.5</td>
</tr>
<tr>
<td>Lens</td>
<td>7</td>
<td>4.84</td>
<td>140</td>
<td>0.014</td>
<td>29.6</td>
</tr>
<tr>
<td>Aqueous humor</td>
<td>35</td>
<td>21.52</td>
<td>845</td>
<td>0.085</td>
<td>34.2</td>
</tr>
<tr>
<td>Cornea</td>
<td>35</td>
<td>25.47</td>
<td>1151</td>
<td>0.115</td>
<td>31.8</td>
</tr>
<tr>
<td>Sclera</td>
<td>35</td>
<td>29.77</td>
<td>1246</td>
<td>0.125</td>
<td>33.3</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>35</td>
<td>35.28</td>
<td>1646</td>
<td>0.165</td>
<td>32.5</td>
</tr>
<tr>
<td>Blood</td>
<td>14</td>
<td>59.11</td>
<td>2872</td>
<td>0.288</td>
<td>30.4</td>
</tr>
</tbody>
</table>

$n = 6$.
Table 4. Linear Regression and Statistical Analysis

<table>
<thead>
<tr>
<th>Type of Tissues</th>
<th>Linear Regression Function*</th>
<th>$R^2$†</th>
<th>Significant‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreous body</td>
<td>$Y = 3.39X - 18.065$</td>
<td>0.911</td>
<td>0.004§</td>
</tr>
<tr>
<td>Choroid-retina</td>
<td>$Y = 2.85X - 16.844$</td>
<td>0.858</td>
<td>0.015§</td>
</tr>
<tr>
<td>Iris-ciliary body</td>
<td>$Y = 3.29X - 16.844$</td>
<td>0.915</td>
<td>0.004§</td>
</tr>
<tr>
<td>Lens</td>
<td>$Y = 3.59X - 18.638$</td>
<td>0.910</td>
<td>0.004§</td>
</tr>
<tr>
<td>Aqueous humor</td>
<td>$Y = 3.39X - 18.762$</td>
<td>0.908</td>
<td>0.005§</td>
</tr>
<tr>
<td>Cornea</td>
<td>$Y = 3.592X - 18.638$</td>
<td>0.928</td>
<td>0.002§</td>
</tr>
<tr>
<td>Sclera</td>
<td>$Y = 3.466X - 19.013$</td>
<td>0.910</td>
<td>0.005§</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>$Y = 3.473X - 17.563$</td>
<td>0.931</td>
<td>0.002§</td>
</tr>
<tr>
<td>Blood</td>
<td>$Y = 3.799X - 19.617$</td>
<td>0.936</td>
<td>0.002§</td>
</tr>
</tbody>
</table>

Data show the correlation between the in vitro cumulative CyS released (%) and the AUCt/AUC65 values (%) in various ocular tissues and blood after injection of $^3$H-CyS-PLGA-MS into the vitreous body of rabbits ($n = 6$).

* X, the in vitro cumulative CyS released (%); Y, AUCt/AUC65 values (%).
† Correlation Coefficient of the regression
‡ Statistical significance of the regression.
§ $P < 0.01$.
|| $P < 0.05$.

References