Intraocular Pharmacokinetics and Safety of a Humanized Monoclonal Antibody in Rabbits after Intravitreal Administration of a Solution or a PLGA Microsphere Formulation

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Poly(lactic-co-glycolic) acid (PLGA) bioresorbable microspheres are used for controlled-release drug delivery and are particularly promising for ocular indications. The objective of the current study was to evaluate the pharmacokinetics and safety of a recombinant human monoclonal antibody (rhuMAb HER2) in rabbits after bolus intravitreal administration of a solution or a PLGA-microsphere formulation. On Day 0, forty-eight male New Zealand white rabbits (2.3–2.6 kg) were immobilized with intramuscular ketamine/xylazine, and the test materials were injected directly into the vitreous compartment. Group 1 animals received rhuMAb HER2 in solution (n = 24/group). The dose for each eye was 25 µg (50 µl). After dosing, animals were sacrificed at 2 min, and on 1, 2, 4, 7, 14, 23, 29, 37, 44, 50, and 56 days (n = 2/timepoint/group). Safety assessment included direct ophthalmoscopy, clinical observations, body weight, and hematology and clinical chemistry panels. At necropsy, vitreous and plasma were collected for pharmacokinetics and analysis for antibodies to rhuMAb HER2, and the vitreal pellet (Group 1) was prepared for histologic evaluation. All animals completed the study per protocol—both treatments were well tolerated, and no suppurrative or mixed inflammatory cell reaction was observed in the vitreal samples (Group 1) at any of the time points examined. Antibodies to rhuMAb HER2 were detected in plasma samples by Day 7 in both treatment groups, but infrequently in vitreous samples. There were no safety implications associated with this immune response. The in vitro characterization of the PLGA microspheres provided reasonable projections of the in vivo rhuMAb HER2 release kinetics (Group 1). The total amount of antibody that was released was similar in vitro (25.9%) and in vivo (32.4%). RhuMAb HER2 (Group 2) was cleared slowly from the vitreous compartment, with initial and terminal half-lives of 0.9 and 5.6 days, respectively. The volume of distribution approximated the vitreous volume in a rabbit eye.

Key Words: formulation; kinetics; immunogenicity; intraocular; intravitreal; monoclonal antibody; pharmacokinetics; poly(lactic-co-glycolic) acid (PLGA) bioresorbable microspheres; recombinant protein; safety.

The limited permeability of the blood-ocular barrier often requires the use of high systemic levels of small-molecule drugs to achieve efficacy, while large molecules such as proteins do not cross this tight barrier. It may therefore be necessary to locally deliver these drugs in the eye to yield the desired therapeutic effect. Several recent reports have demonstrated that local delivery of small-molecule drugs may be accomplished by intravitreal injection of the drug entrapped in biodegradable microspheres (Giordano et al., 1993; Gould et al., 1994; Harper III et al., 1993; Kenley et al., 1987; Khoobehi et al., 1990, 1991; Morirea et al., 1991; Therin et al., 1992). Intravitreal delivery of drugs in depot devices is possible with a variety of different methods (for review see Metrinkin and Anand, 1994). Non-degradable devices require surgical removal and replacement with a new device, whereas biodegradable microspheres may be injected through a small-gauge needle. Several animal studies have been conducted using the biodegradable polymer, poly(lactic-co-glycolic) acid (PLGA) (Giordano et al., 1993; Khoobehi et al., 1990, 1991; Morirera et al., 1991). In these studies, intravitreal administration of PLGA microspheres in rabbit eyes resulted in a mild inflammatory response that was resolved over time, indicating the biocompatibility of these polymers.

In many ocular diseases, a potential therapeutic drug such as a protein or oligonucleotide may require direct intravitreal injection to maintain effective local concentrations. We sought to provide local delivery of a model protein, i.e., a recombinant human monoclonal antibody (rhuMAb HER2), into the vitreous by direct injection of the protein in either a soluble form or entrapped within PLGA microspheres. The pharmacokinetics and safety of both dosage forms were evaluated, providing a framework for future studies of intravitreal delivery of potential therapeutics to treat ocular diseases.
MATERIALS AND METHODS

Test materials. Recombinant human (rhu) MAb HER2 (Herceptin® trastuzumab, Genentech, Inc.) is a monoclonal antibody (MAb) directed against the extracellular domain of p185HER2 (Shepard et al., 1991). This antibody was selected to serve as a model compound for evaluation of in vitro and in vivo release kinetics of a large molecular weight protein (~148 kDa) from PLGA microspheres. rhuMAb HER2 was encapsulated in PLGA using a patented 2-step, spray freeze-drying microencapsulation process (Johnson et al., 1996). Briefly, the protein solution was atomized into liquid nitrogen and the resultant frozen droplets were lyophilized to form a solid powder. In the second step, the solid protein was homogenized with dissolved polymer and sprayed onto a frozen bed of ethanol contained in liquid nitrogen. The microspheres were hardened by extraction of the organic solvent into the ethanol at ~70°C for several days, then subjected to a fluidized bed of nitrogen at 5°C until dry. The protein load and in vitro release characteristics of the microspheres were then determined.

In vitro release kinetics. The protein load was calculated by dissolving the microspheres in sodium hydroxide and reading the absorbance of the solution by UV spectroscopy. The protein load was defined as the percent of the total amount of protein encapsulated in a specified amount of microspheres (%w/w). The release of the rhuMAb HER2 from the PLGA microspheres was determined by utilizing a modified microcentrifugation filter unit. A known amount of microspheres was placed in the filter unit, and the unit was capped and sealed. The release buffer (10 mM sodium succinate, 149 mM sodium chloride, 0.02% sodium azide, and 0.02% Tween 20, pH 5.0) was added to the microspheres. The filter unit was placed on a slow-rotating rocker at 37°C. Samples were collected by microcentrifuging the filter unit into pre-weighed Eppendorf tubes. The filter unit was then replenished with the release buffer and returned to the rocker at 37°C. Subsequent release samples were collected in a similar manner over 70 days. The rhuMAb HER2 released from the microspheres was then quantified using the bicinchoninic acid assay (BCA, Pierce Chemical, Rockford, IL) with the rhuMAb HER2 as the standard.

Animals. Forty-eight male New Zealand White rabbits (2.3–2.6 kg) (Western Oregon Rabbity, Philomor, OR) were housed in stainless steel cages in compliance with established standards of the Federal Animal Welfare Act and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Rooms were maintained at 65±2°F with 12-h light/dark cycles. Food and water were available ad libitum. Clinical signs were observed daily throughout the study (excluding weekends), and body weight was recorded before dosing, weekly, and at sacrifice.

Dosing. Animals were randomized to 2 groups by body weight (n = 24 rabbits/group). Each animal received intravitreal injections of test material into each eye (50 µl/eye) on Day 0 (t = 0) with a 25- or 28-gauge needle under light anesthesia [ketamine (40–50 mg/kg) and xylazine (5–10 mg/kg) intramuscularly]. Group 1 animals received rhuMAb HER2 in 50:50 lactide:glycolide PLGA microspheres (4.9% w/w rhuMAb HER2) in 0.9% NaCl (rhuMAb anesthesia [ketamine (40 –50 mg/kg) and xylazine (5–10 mg/kg) intramuscularly]), the supernatant was collected for pharmacokinetic and antibody analyses (see below), and the remaining pellet was fixed with 1 ml of 10% neutral buffered formalin and submitted for histologic evaluation. The pharmacokinetic and antibody samples were stored frozen at ~70°C prior to analysis.

Pharmacokinetic samples. Vitreous fluid from each eye and plasma were assayed for rhuMAb HER2 in the p185 HER2 extracellular domain (ECD) ELISA, which uses HER2 ECD for capture and goat anti-human IgG Fc HRP for detection (Organon Teknika Corp., Durham, NC). The lower limit of quantitation (LOQ) was 15.6 ng/ml, following a minimum 1:10 dilution in assay buffer.

Antibody samples. Vitreous fluid from each eye and plasma were evaluated for antibodies to rhuMAb HER2 in an ELISA which uses rhuMAb HER2 for capture and donkey anti-rabbit IgG HRP for detection (Amersham). The minimum titer in this assay was 2.0, following a minimum 1:100 dilution in assay buffer.

Clinical chemistry and hematology samples. Serum (0.5 ml) and blood (1 ml) were submitted for a complete chemistry panel and hemogram, respectively. The hematology assessment included red and white blood cell counts, hemoglobin, hematocrit, RBC indices, estimate of platelet numbers, and a leukocyte differential. Clinical chemistry profiles included chloride, potassium, sodium, calcium, inorganic phosphorus, blood urea nitrogen, creatinine, creatinine kinase, total bilirubin, glucose, total protein, albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, cholesterol, and triglycerides.

Ophthalmoscopy. Each eye was checked with an ophthalmoscope (Welch Allen, Model 11710) immediately prior to dosing, daily for the first week (excluding weekends), and weekly thereafter.

Histology. Formalin-fixed vitreal pellets from the Group 1 rabbits were pooled at each timepoint (n = 4 samples/day) into a single sample (due to the small amount of material available), embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for light microscopic evaluation.

In vitro kinetics. The in vitro release characteristics of the PLGA formulation were estimated by fitting the sum of exponentials, i.e., R = Ae^{-at} + Be^{-bt} to release rate (R) versus time (t) data. The data were weighted by $1/t^2$, where y is the observed concentration.

Plasma pharmacokinetics. rhuMAb HER2 concentrations in plasma were below 15.6 ng/ml (i.e., the lower limit of detection) for all samples except those from the Group 2 animals on Day 2 (26.4 and 26.9 ng/ml), Day 4 (27.9 and 42.5 ng/ml), and Day 7 (<15.6 and 45.0 ng/ml); no analyses were performed on these data. The absence of measurable circulating concentrations of rhuMAb HER2 most likely reflects rapid systemic clearance following the development of a significant immune response (see Results and Discussion).

Plasma pharmacokinetics. In vivo release characteristics of the microspheres, the supernatant was collected for pharmacokinetic and antibody analyses (see below), and the remaining pellet was fixed with 1 ml of 10% neutral buffered formalin and submitted for histologic evaluation. The pharmacokinetic and antibody samples were stored frozen at ~70°C prior to analysis.

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Intraocular pharmacokinetics. The average vitreal rhuMAb HER2 data from each group of animals were analyzed by 3 methods. Method I: the data from each group were evaluated by fitting a single exponential (i.e., C(t) = Ce^{-at}) or the sum of 2 exponentials (i.e., C(t) = De^{-at} + Ee^{-bt}) to the vitreal concentration-time data with weighting $1/t^2$, where y is the observed concentration. Method II: the average vitreal rhuMAb HER2 data from Group 2 were analyzed by fitting a 2-compartment model to the data with weighting $1/(SD)^2$. This model is illustrated as follows:

$$\frac{r_0}{X_1} \frac{r_1}{X_2} \frac{C}{X_1}$$

The dose is injected into $X_1$, and the variables $X_1$ and $X_2$ represent the amount of the drug in the vitreous compartment and the other exchanging compartment, respectively. The measured concentration in vitreous is $X_1/V$, where V is the volume of distribution in the vitreous compartment. This volume is a parameter that has to be identified in addition to the rate constants, $r_i$. Using the model parameters of this fitting (data from Group 2), the vitreal rhuMAb HER2 data from Group 1 were analyzed for the exponentially declining infusion rate and initial burst of rhuMAb HER2, in order to estimate the in vivo
release characteristics of the PLGA formulation. The burst represents the fraction of the dose that is instantaneously available (relative to the direct injection) and is reflected by the concentration at 2 min in the rhuMAb HER2/PLGA group. Method II: the vitreal rhuMAb HER2 concentrations for Group 1 were predicted using the Group 2 parameter estimates from the Method II fitting and the in vitro release kinetics as the input function.

Estimation procedures. Exponential components, as well as the model parameters, were estimated by a nonlinear, least-squares method using the Gauss-Newton-Marquardt-Levenberg procedure (Press et al., 1988). The model differential equations were solved numerically by the fourth-order Runge-Kutta method. The search routine was implemented on a Dell 450DE PC running HTBasic (HTBasic 386 for PC by TransEra, Provo, Utah 84604).

TABLE 1
The in vitro Release Characteristics of the PLGA Formulation Estimated by Fitting the Sum of Exponentials to the Release Rate Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (% dose/day)</td>
<td>1.53 ± 1.02</td>
</tr>
<tr>
<td>α (1/day)</td>
<td>0.498 ± 0.365</td>
</tr>
<tr>
<td>B (% dose/day)</td>
<td>0.287 ± 0.171</td>
</tr>
<tr>
<td>β (1/day)</td>
<td>0.0763 ± 0.0191</td>
</tr>
<tr>
<td>Burst (% dose)b</td>
<td>18.9</td>
</tr>
<tr>
<td>α release half-life (day)c</td>
<td>1.39 ± 1.02</td>
</tr>
<tr>
<td>% AUC for α-phase</td>
<td>44.3</td>
</tr>
<tr>
<td>β release half-life (day)c</td>
<td>9.08 ± 2.27</td>
</tr>
<tr>
<td>% AUC for β-phase</td>
<td>55.7</td>
</tr>
<tr>
<td>Cumulative % released</td>
<td>25.9</td>
</tr>
</tbody>
</table>

Note. See also Fig. 1.

aEstimate ± the standard error of the estimate.
bEstimated amount released in vivo (in percent dose) in one h.

cRelease half-life (days) = (ln 2)/α or (ln 2)/β
vitreous fluid (t = 2 min) were approximately 3.7 and 26.3 μg/ml for the PLGA and soluble form of rhuMAb HER2, respectively (Fig. 2). These concentrations were at or below 15.6 ng/ml at Day 56 (the last sample collection time) in both groups, indicating that the study was of sufficient duration to characterize the release characteristics of the PLGA formulation and the pharmacokinetics of rhuMAb HER2. The animals that had measurable antibody titers in the vitreous fluid were not excluded from the pharmacokinetic analysis because antibodies, when detected, did not correlate with any apparent pharmacokinetic changes (supporting a hypothesis that some vitreous samples were contaminated by blood).

Results of the pharmacokinetic evaluation for Method I are reported in Table 2. A single exponential function was fitted to the data from Group 1, since the two-exponential function was not identifiable. The elimination half-life was 5.1 days. As in the in vitro evaluation (Table 1), the PLGA formulation did not deliver 100% of the dose; in this case, only 32.4% of the rhuMAb HER2 was released into the vitreous. Incomplete release of a protein from PLGA is not unusual when the dosage form has not been optimized. In a recent report (Yeh et al., 1996), in vitro recovery of ovalbumin (45 kDa) from a PLGA formulation was less than 35% at 30 days; recovery was improved slightly, to approximately 40%, by the incorporation of Pluronic F127.

The data from Group 2 were best described by the sum of 2 exponentials. The antibody was cleared slowly from the vitreous, with initial and terminal half-lives of 0.9 and 5.6 days, respectively. The terminal half-life (5.6 days) is identical to the intravitreal half-life for rhuMAb HER2 in Rhesus monkeys (Mordenti et al., 1999). The initial volume of distribution (V₁) was 0.83 ml, and the volume of distribution at steady-state (Vₐ) was 1.78 ml. This volume approximates the vitreous volume in a rabbit eye (i.e., ~ 1.5 ml). The rabbit does not express the epitope for rhuMAb HER2 in the eye, therefore, this model does not have a specific antigen/antibody clearance component. The epitope for rhuMAb HER2 in humans is p185HER2. Expression of the rodent homologue (erbB2/neu) is negligible in the adult rodent (Birmingham-McDonogh et al., 1996). In addition, a murine antibody directed against p185HER2 (i.e., muMAb 4D5) did not cross react with rabbit tissues (N. Dybdal, personal communication). The pharmacokinetics and half-life of murine anti-VEGF antibody A4.6.1 and an anti-gp120 isotype control antibody were similar when compared in rabbits (dose = 20 μg/eye) and dogs (dose = 300 μg/eye) (L. Berleau, personal communication). These findings suggest that a specific ligand, if present, does not influence the intraocular pharmacokinetics at these doses.

Results of the pharmacokinetic evaluation for Method II are shown in Figure 2 and reported in Tables 3 and 4 for Groups 1 and 2, respectively. Different weighting schemes (1/y² and unweighted) were tried in addition to deleting the Day-37 concentration for Group 1 (rhuMAb HER2/PLGA), but the fitting did not improve or change appreciably. It is interesting to note that the in vivo burst estimate (12.1%) compares favorably with that of the in vitro measurement (18.9%); however, the dynamic characteristics of the release rate and those of the biological system are similar, which makes it difficult to recover the 2-exponential character of the in vitro release rate profile. The estimate of the in vivo exponential decay constant (0.144 day⁻¹) is between the two exponential coefficients of the

**TABLE 2**  
Pharmacokinetic Parameters of rhuMAb HER2 in the Vitreous of Male Rabbits after a Single Intravitreal Dose in PLGA Microspheres (Group 1) or in Saline (Group 2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1: rhuMAb HER2/PLGA</th>
<th>Group 2: rhuMAb HER2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (ng/ml)</td>
<td>25,300 ± 9890</td>
<td>796 ± 376</td>
</tr>
<tr>
<td>δ (1/day)</td>
<td>0.135 ± 0.013</td>
<td>0.123 ± 0.014</td>
</tr>
<tr>
<td>E (ng/ml)</td>
<td>0.9 ± 0.4</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>AUC (ng/ml*days)</td>
<td>71,400</td>
<td>74,060</td>
</tr>
</tbody>
</table>

Note. Treatment dose approximately 25 μg/eye (50 μl). Average data (n = 2 rabbits/group/time point) analyzed by Method I.

*Group 1 was analyzed by a single exponential function (see text).

*Estimate ± the standard error of the estimate.

*Estimated as 100*AUC (Group 1) / AUC (Group 2).
in vitro release rate and is close to the value of their geometric mean (i.e., 0.195 day⁻¹).

Results for Method III are shown in Figure 3, where the predicted Group 1 rhuMab HER2 vitreal concentrations are superimposed on the actual data. The similarity between the Method II Group 1 fitting (Fig. 2) and the Method III Group 1 simulation (Fig. 3) suggests that the release of rhuMab HER2 from PLGA is quite similar in vitro and in vivo. The most notable difference between Figures 2 and 3 is at the early time points, where the in vitro burst of rhuMab HER2 from the microspheres is blunted in the eye.

CONCLUSIONS

The in vitro characterization of the microspheres provided reasonable projections of the in vivo release kinetics of the PLGA formulation. The total amount of antibody that was released was similar in vitro and in vivo, 25.9% and 32.4%, respectively, and the difference is within the uncertainty of the estimates of the area under the curve (AUC). Intravitreal injection of rhuMab HER2 and the rhuMab HER2-loaded microspheres were well tolerated through Day 56 in rabbits, and no suppurative or mixed inflammatory cell reaction was observed in the vitreous samples at any of the time points examined. Antibodies to rhuMab HER2 were detected in the plasma samples within 7 days in both treatment groups, but infrequently in vitreous samples. There were no safety implications associated with this immune response. A human (homologous) protein in a human would not be expected to be immunogenic; however, safety testing in primates before the initiation of clinical studies would mitigate concerns regarding the intravitreal route of administration. Finally, rhuMab HER2 was cleared slowly from the vitreous compartment; the PLGA formulation did not offer any pharmacokinetic advantage over the solution formulation. The long terminal half-life (\(5.6\) days) suggests that injections of a therapeutic antibody in solution every 1 or 2 months may provide adequate concentrations without further formulation work. For shorter duration of exposure or greater tissue penetration, an Fab antibody fragment is recommended (Mordenti et al., 1999).

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