Pharmacokinetic and Pharmacodynamic Evaluation of Intramuscular Artesunate in Healthy Beagle Dogs

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Abstract. Pharmacokinetic and pharmacodynamic responses were evaluated after intramuscular (IM) injection of artesunate (AS). Twelve dogs were injected with IM AS at 2.5, 5, or 10 mg/kg into the left gluteal muscle. A second injection of only diluent was given in the right gluteal muscle. At 24 hours post-injection, plasma creatine kinase (CK) concentrations were elevated above normal. Muscle biopsies showed myocyte necrosis and acute inflammation, which was worse on the treated side. At 7 days after injection, CK concentrations were normal. Muscle biopsies showed mineralization, fibrosis, and chronic inflammation with less difference between sides. Compared with intravenous administration, IM AS resulted in a prolonged half-life for both AS and DHA. Intramuscular AS also had a lower mean dose-adjusted \( C_{\text{max}} \) and a higher mean dose-adjusted area under the curve; but produced similar concentrations of dihydroartemisinin. These findings suggest that adverse reactions to IM artesunate are minor and temporary which justify further study of this route in treating severe malaria.

INTRODUCTION

Severe and complicated malaria kills more than two million people each year.1,2 Most of these deaths occur in pregnant women and children in sub-Saharan Africa in areas with limited medical care. Although many artemisinin-derived compounds—including a Chinese formulation of intravenous (IV) artesunate (AS)—are already being used around the world for the treatment of severe and complicated malaria, there are not any IV artemisinin products that are approved by the US Food and Drug Administration (FDA) for this indication.1–5 Currently, quinidine is the only approved IV medication available for treatment of patients with severe malaria in the United States.6 Unfortunately, quinidine has several potential adverse effects, which make it desirable to have an alternative treatment available. The Walter Reed Army Institute of Research (WRAIR) is developing a formulation of IV AS as a potential alternative to IV quinine. The WRAIR formulation of AS (WR 256283) is reconstituted using a 0.3 M phosphate buffer solution (PBS) rather than 5% sodium bicarbonate (NaHCO\(_3\)) like the Chinese formulation.7 Studies of the bioequivalence of these two formulations are ongoing and will be reported soon.

Although it is our intention to seek only an IV indication for our product, it is likely that intramuscular (IM) use of the WRAIR formulation of AS will occur in countries with limited health care services and in poorly staffed hospitals. Intramuscular administration can be anticipated because of the relative ease of this route compared with IV therapy. Additionally, IM administration could be an important route of administration in patients with difficult venous access. Finally, it is likely that treatment with IM AS will be initiated in the severely ill during transport to hospitals from outlying clinics. Therefore, we attempted to evaluate the pharmacokinetics and local tolerability of the IM administration of WR 256283.

MATERIALS AND METHODS

We obtained 12 adult male beagle dogs from Harland Sprague-Dawley (Alder Ridge Farms, Lakewood, PA). The dogs were between 0.9 and 1.1 years old and weighed between 7.3 and 14.4 kg at purchase. The dogs were housed singly in 4 × 10-ft aluminum runs. They were fed a commercial laboratory canine ration (Canine Diet; PMI Feeds, St. Louis, MO) and \textit{ad libitum} water. The dogs were acclimated to the interior environment for > 2 weeks before any study initiation. On arrival, the dogs were given complete physical examinations, including evaluation of their chemistry profile, hematology, fecal, and urinary analysis by a veterinarian. Approximately 4 months before beginning this study, the dogs were used in a two-period, two-sequence, two-formulation bioequivalence study of the WRAIR and Chinese formulations of artesunate (Q Li and others, unpublished data). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. The study was approved by the Institutional Animal Care and Use Committee (IACUC) and carried out in a facility that was fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Treatment groups were assigned using the random number generator function in Microsoft’s Excel 2003 package.

For this study, we used AS [4-(10’ dihydro-artemisinin-oxymethyl] succinate] manufactured by Stanford Research Institute (SRI, Menlo Park CA). The drug was dry-filled in 20 mL vials with 110 mg of AS per vial. The buffer was manufactured as the phosphate salt with 0.3 M PBS at a pH of 8.1 by Afton Scientific Corporation (Charlottesville, VA). The AS was reconstituted with 2.2, 4.4, or 8.8 mL of PBS to achieve drug concentrations of 50, 25, or 12.5 mg/mL. A fixed volume of 0.2 mL/kg was used for each injection. Injections were administered within 1 hour of reconstitution of the AS.

Twelve adult beagle dogs were randomly assigned to be injected with a single dose of IM artesunate at 2.5, 5, or 10 mg/kg after reconstitution with PBS into the left gluteal muscle region. The lowest dose level used in this study was approximately equivalent to the human IV loading dose of
artesunate used in the South East Asian Quinine Artesunate Malaria Trial (SEAQUAMAT). The other two dose levels represent a 2- and 4-fold increase over the IV loading dose used in the SEAQUAMAT study. Four dogs were injected at each dose level. An identical volume of only PBS was injected into each dog’s contralateral gluteal muscle. This was done so that inflammation from the two injection sites could be compared for each dog. To facilitate comparison, each injection site was shaved and marked externally with a black marker at injection. During injection, each animal was observed and given a subjective grading of its pain response by an investigator blinded to the concentration of AS injected.

Blood samples were taken by an IV catheter in the anterior cephalic vein at 0, 5, 15, 30, 45, 60, 90, 120, 240, and 360 minutes after drug administration for pharmacokinetic (PK) parameter determination. The dogs were divided into two equal cohorts, with each cohort having two dogs dosed at each of the three dose levels. The first cohort of dogs was killed 24 hours after AS administration (Cohort 1). The second cohort of dogs was killed at 168 hours after AS dosing (Cohort 2). Complete blood counts (CBCs) and plasma creatine kinase (CK) laboratory analyses—although planned for both cohorts—were obtained at baseline from only Cohort 1 secondary to an unintentional protocol deviation. Both cohorts had blood samples drawn for CBC and CK analyses before death.

After death, all dogs had extensive muscle biopsies of each of the injection sites, which were harvested and labeled with an identification number and side of biopsy. Next, after serial sectioning and fixation, a veterinary pathologist blinded to the side and dose of drug treatment evaluated the muscle biopsies using routine histopathologic evaluation for type and severity of inflammation present. The pathologist assigned a semi-quantitative score to the inflammatory changes of each slide by evaluating for the presence of necrosis, myositis, and perimysial inflammation. Each of these changes was assigned a score (necrosis, 3; myositis, 2; perimysial inflammation, 1) and a severity score (severe, 3; moderate, 2; mild, 1). The inflammation score for each slide was obtained by multiplying the inflammatory change score by the severity score and summing for all changes present on the slide. For example, if a slide contained moderate necrosis, severe myositis, and mild perimysial inflammation, the inflammation score for that slide would be 
\[
(2 \times 3) + (3 \times 2) + (1 \times 1) = 13.
\]
An inflammation score for the slide could range from 0 to 18. The total inflammation score for each side of a dog was obtained by summing the inflammation scores from all the slides of each treatment side. The average inflammation score for each side of each dog was calculated by dividing the total inflammation score for that side by the number of slides examined from that side for each dog.

Determination of the plasma concentrations of both AS and dihydroartemisinin (DHA) were performed by Midwest Research Institute (MRI; Kansas City, MO) using validated liquid chromatography tandem mass spectrometry methods. The limits of quantification were 9.48 ng/mL for AS and 5.15 ng/mL for DHA. Any out-of-trend concentration values were reanalyzed, with the average of three repeated values used in PK parameter determination. Complete conversion from AS to DHA was assumed. The PK data were analyzed using WinNonlin 5.0 (Pharsight, Mountain View, CA). Conventional pharmacokinetic parameters were determined from plasma concentration versus time data using non-compartmental analysis, in which the model of the elimination phase with the optimal correlation was chosen.

Statistical analysis was performed using STATA 7.0 (Stata Corp., College Station, TX). Comparison of the average inflammation scores between the treated and untreated sides for all the dogs was done using the paired t test. A two-tailed level of significance of 0.05 was assumed. Pharmacokinetic parameters are reported as the mean value and SE.

RESULT

All dogs seemed to tolerate the injections well. No difference in observed pain could be discerned by side of injection or dose. Two dogs rubbed their injection sites on the ground immediately after injection. Five dogs vocalized at the time of injection. Although all the dogs ate poorly the day of injection, all were eating normally by the next day. Most dogs lost a small amount (<5%) of weight over the study period. Two dogs developed redness at the injection site. One of the two dogs (ID 32024) developed redness over the right gluteal muscle 3 days after injection. The other dog (ID 32532) developed redness over the left gluteal muscle 5 days after injection.

The dose-adjusted maximum concentration (Cmax_D) for AS ranged from 402.9 to 1,798.4 kg · ng/mL/mg. The Cmax_D for DHA ranged from 111.4 to 472.6 kg · ng/mL/mg. The mean Cmax_D for AS was 1,148.9 ± 411.2 and 209.9 ± 102.5 kg · ng/mL/mg for DHA. The mean elimination half-life (t1/2) was 0.53 ± 0.38 hours for AS and 1.1 ± 0.98 hours for DHA. The dose-adjusted area under the curve from zero to infinity (AUCINF_D) for AS ranged from 232.2 to 1,320.7 h · kg · ng/mL/mg. The AUCINF_D for DHA ranged from 85.1 to 315.9 h · kg · ng/mL/mg. The mean AUCINF_D was 528.4 ± 268.3 h · kg · ng/mL/mg for AS and 214.6 ± 70.8 h · kg · ng/mL/mg for DHA (Table 1).

All the dogs in Cohort 1 had normal white blood cell (WBC) and red blood cell (RBC) counts at baseline and normal WBC counts at death. One dog from Cohort 1 had a RBC count that was slightly lower than the normal range (2.4% less than lower limit of normal) at death. Although Cohort 1 dogs had normal CK levels at baseline, all had CK levels that were elevated outside of the normal range at 24 hours after injection. The CK levels for Cohort 1 dogs ranged from 288 to 2,000 U/L (Table 2). Except for one, all Cohort 2 dogs had normal WBC and RBC counts at 7 days after injection. Dog 32071 had a slightly elevated WBC count (1.2% over the upper limit of normal) at death but no clear infection. All of Cohort 2’s CK levels were in the normal range at 7 days after injection and ranged from 115 to 221 U/L (Table 2).

Muscle biopsies obtained 24 hours after IM injection showed myocyte necrosis, hemorrhage, and acute inflammation with mainly neutrophils (Figures 1 and 2). Biopsies from most of the dogs killed 24 hours after injection had...
more inflammation on the artesunate-treated side than the side treated with PBS only (Table 3). Muscle biopsies from the dogs killed 7 days after injection showed necrosis, mineralization, fibrosis, and chronic inflammation with macrophages, lymphocytes, and plasma cells (Figures 3 and 4). However, on average, the differences in inflammation between the biopsies from the artesunate- and the PBS-treated sides of the dogs from Cohort 2 were not as evident as the differences in inflammation seen on the biopsies of the dogs from Cohort 1. However, when the average inflammation scores for the artesunate-treated sides were compared with the PBS only–treated sides for all dogs, regardless of day of death, the average inflammation scores from the AS-treated side were higher than the inflammation scores from the control-treated side (P = 0.03). There was no statistically significant dose-dependent increase in inflammation observed.

**DISCUSSION**

Both the AS and PBS injections seemed well tolerated by the dogs. All the dogs were able to bear weight and walk within a minute of receiving the IM injections of AS and diluent in the left gluteal muscle and diluent alone in the right gluteal muscle. In addition to whining at injection, two dogs transiently dragged their hind quarters on the ground for a short time after the injections. Because the drug-diluent admixture was injected first in each case, it is possible that some of the reactions observed after the second injection of diluent alone were caused by the dog anticipating the shot. This would have the effect of causing an overestimation of pain caused by the diluent. However, because no significant differences were observed between the reactions to the two injections, it is doubtful that the AS-PBS injection is significantly more painful than the PBS injection.

All but three dogs lost weight over the course of the study. The dogs in Cohort 1 had a relatively small amount of weight change (+0.7% to −4.0% of baseline weights), which may be explained by their not eating or drinking much during the drug dosing day and because they were killed the following day. Most of the dogs in Cohort 2 also lost a small amount (−0.74% to –4.5%) of their baseline weights. However, Dog 32153 from Cohort 2 gained 0.9 kg (8.6% of baseline weight) over the 7-day period from study initiation to death. It is unclear if the weight loss most dogs experienced was a result of the AS or some other confounding factor.

Both dogs that developed redness at the injection site were in Cohort 2. The redness seen on Dog 32532 was linear and resembled a scratch. Dog 32024 had redness over the entire shaved injection area overlying the right gluteal muscles. Although this rash could have resulted from the diluent, it also could have been a reaction to being
shaved or could have resulted from the dog licking the site. Neither animal was tender to palpation of the reddened areas. In addition, neither animal had an elevated WBC at death.

Mean PK parameter estimates from the first period of a bioequivalence study that used the WRAIR formulation of IV AS in dogs were compared with the PK parameter estimates from this study (Table 1). In the bioequivalence study, a single 20 mg/kg IV dose of WRAIR’s formulation of AS resulted in a mean estimate of the C_max-D of 3,559.7 ± 924.8 kg · ng/mL/mg for AS and 240.0 ± 56.7 kg · ng/mL/mg for DHA. The mean elimination t_{1/2} was 0.28 ± 0.10 hours for AS and 0.72 ± 0.33 hours for DHA in that study. The mean AUCINF_D estimate was 376.0 ± 88.1 h · kg · ng/mL/mg for AS and 176.3 ± 39.5 h · kg · ng/mL/mg for DHA in this study.

When compared with estimates from the IV bioequivalence study, administration of IM AS resulted in a lower mean dose-adjusted maximum concentration estimate and a higher mean dose-adjusted area under the curve estimate. These differences likely result from a slower, more prolonged absorption with the IM route. However, the mean C_max-D and mean AUCINF_D estimates for DHA were similar between the IM and IV routes of administration. The elimination half-life estimates for both AS and DHA were longer with IM administration.

It should be noted that the wide range of variation in individual parameter values used in the mean parameter estimations could result in an incorrectly skewed parameter value. This variation may have resulted from improper IM admin-

### Table 3

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* Cohort 1 dogs were killed 24 hours after injection.
† Cohort 2 dogs were killed 168 hours after injection.

FIGURE 2. Biopsy from Dog 31854 collected 1 day after intramuscular injection of only phosphate buffer solution showing area of myocyte necrosis with moderate amounts of disrupted architecture, edema, and acute inflammation (inflammation score = 12). Magnification, ×20.

FIGURE 3. Biopsy from Dog 30421 collected 7 days after intramuscular injection with 10 mg/kg of intramuscular artemesunate showing large area of myocyte loss with chronic-active inflammation, hemorrhage, and myocyte regeneration (inflammation score = 15). Magnification, ×20.

FIGURE 4. Biopsy from Dog 30421 collected 7 days after intramuscular injection with only phosphate buffer solution showing localized area of myocyte loss and regeneration (inflammation score = 3). Magnification, ×20.
istration resulting in poor drug absorption. Improper injection administration may have resulted from missing the muscle secondary to an unexpected movement by the dog at the time of injection. Indeed in at least one case (Dog 31833), it seems that the injection missed the muscle as evidenced by the pathologist comment that the injection appeared to be in the deep fascial plane. Although improper IM administration was more likely to have occurred with the PBS injections—as happened in the aforementioned case—because they were given second; it can not be guaranteed that improper administration did not also occur with the AS injections. If AS was injected in a fascial plane rather than muscle, it is speculated that absorption would be slow or limited because fascia is less vascular than muscle.

Median PK values of IM AS were also calculated to examine the influence of outliers on the parameter estimation. The median $C_{max,D}$ was 1,093.4 and 187.4 kg · mg/mL for AS and DHA, respectively. The median $t_{1/2}$ estimates were 0.44 hours for AS and 0.59 hours for DHA. The median AUCINF,D estimates were 476.2 h · kg · mg/mL for AS and 211.7 h · kg · mg/mL for DHA. Therefore, the use of mean values instead of median values in calculating PK parameters did not yield a different interpretation when comparing the pharmacokinetics of AS between IV and IM administration.

A single IM injection of AS did not seem to affect either the WBC or RBC counts in this study with healthy dogs. However, elevated CK concentrations were seen in all six of the dogs killed at 1 day after injection. These CK concentrations ranged from 1.02 to 7.09 times the upper limit of normal. Although some of the elevation in CK was likely caused by the trauma of receiving two IM injections, it is doubtful that all of the elevation was attributable to the injections. Rather, it is likely that at least some of the elevation in CK concentrations was caused by the acute myocyte necrosis. Because the CK concentrations from all the dogs in Cohort 2 were normal and the inflammation scores were lower by 7 days after AS injection, it is unlikely that there is any worsening necrosis or long-term, local muscle effects.

Pathologic examination of the muscle biopsies of all dogs from Cohort 1 except Dog 30822 showed acute changes that were worse on the drug-treated side than on the diluent only–treated side. Histologic examination of the muscle biopsies of the dogs from Cohort 2 showed more chronic changes that were not significantly different between the AS–treated and diluent only–treated sides. However, except for Dog 32071, the average and high inflammation scores were also higher on the drug-treated side than the control side in Cohort 2. Ignoring dose level and day of death, the drug-treated side had statistically higher inflammation than the PBS side. However, no dose- or concentration-related increase in inflammation was evident in this study. This failure to show a dose-related increase in inflammation could have resulted from having sample sizes that were too small at each of the different dosing levels. Alternatively, this might mean that the acute local inflammation and myocyte necrosis seen with IM administration of artesunate is not dose related.

In addition to the small sample sizes, some of the limitations of our study included our using dosing levels that differed from the dose level that was used in the IV bioequivalence study. Unfortunately, this required calculating PK estimates that were dose normalized to compare the PK parameters between studies. Therefore, we make the assumption that AS has linear pharmacokinetics over the range of doses tested. In addition, the dogs used in this study were not AS naïve. However, we feel that because several months had passed between the first AS exposure and this study, it was doubtful that the kinetics of AS was altered.

An additional limitation was that we chose not to use a tissue marker to mark the injection site secondary to concerns of confounding the evaluation of AS’s local toxicity. Unfortunately, this meant that we could not be sure that the biopsies were of the correct site. We attempted to prevent this by shaving the injection sites, externally marking the injection sites, and by taking wide surgical specimens for evaluation. Also, it is possible that interspecies or disease state differences in the metabolism or tissue blood flow between healthy dogs and patients with severe malaria may make PK parameter comparisons misleading. Finally, this study was not designed to address the question of the efficacy of IM AS. It may be that the delayed, smaller $C_{max}$ seen with IM AS may translate into less robust antiparasitic activity.

Despite these limitations, some conclusions can be made based on this study. First, although acute muscle necrosis was seen with IM administration of AS at 1 day after injection, there seemed to be no worsening of muscle necrosis at the injection sites 7 days after injection. Additionally, adverse effects following IM administration of WR 256283 seemed to be minor and transient. Finally, similar concentrations of DHA were observed when selected pharmacokinetic parameters were compared between IM and IV AS. These findings suggest that IM administration of the WRAIR formulation of AS is well tolerated. Furthermore, based on the current use of the Chinese AS formulation, it is likely that the WRAIR formulation of IV artesunate will be used off-label in areas with malaria and limited medical resources when it gets licensed. Therefore, further clinical studies are warranted to determine whether IM administration of the WRAIR formulation of artesunate is safe and effective in the treatment of severe and complicated malaria.

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