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Development of A Specific Monoclonal Antibody for the Quantification of Artemisinin in *Artemisia annua* and Rat Serum

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Abstract

Artemisinin, extracted from *Artemisia annua*, and its derivatives are important frontline antimalarials. To produce specific antibodies for the detection and quantification of artemisinin, artemisinin was transformed to 9-hydroxyartemisinin by microbial fermentation, which was used to prepare a 9-succinate artemisinin hapten for conjugation with ovalbumin. A monoclonal antibody (mAb), designated as 3H7A10, was selected from hybridoma cell lines which showed high specificity to artemisinin. No competitive inhibition was observed with artesunate, dihydroartemisinin, and artemether for up to 20,000 ng mL⁻¹. An indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed, which showed a concentration causing 50% of inhibition (IC₅₀) for artemisinin as 2.6 ng mL⁻¹ and a working range of 0.6–11.5 ng mL⁻¹. The icELISA was applied for the quantification of artemisinin in crude extracts of wild *A. annua* and the study of pharmacokinetics of artemisinin in rat serum after intraperitoneal injection. The results were highly correlated with those determined by HPLC-UV analysis ($R^2=0.9919$). In comparison with reported anti-artemisinin mAbs which have broad cross reactivity with other artemisinin derivatives, the high specificity of 3H7A10 for artemisinin will enable development of methods for quantification of artemisinin in *Artemisia* plants and antimalarial drugs such as Arco®, and for pharmacokinetic studies.

Graphical Abstract

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

Keywords

Artemisinin; iELISA; monoclonal antibody; specificity; quantification

Introduction

Malaria is still a major public health problem in many countries. According to the recent estimates, 198 million cases of malaria occurred globally in 2013, resulting in 584 000 deaths.¹ Artemisinin is a sesquiterpenoid compound extracted from *Artemisia annua* L., which has outstanding antimalarial efficacy. Currently, artemisinin-based combination therapies (ACTs) have been adopted as first-line treatment of falciparum malaria in most *Plasmodium falciparum* endemic regions.¹ Among them, the artemisinin-naphthoquine combination has been developed as a new ACT (Arco®) for treating uncomplicated *P. falciparum* malaria in both adults and children.² In addition, artemisinin has many other antimicrobial activities (e.g., against *Campylobacter jejuni*³ and *Babesia gibsoni*⁴) as well as antitumor activity.⁵ Hence, there is a need for developing fast and reliable methods of detecting and quantifying artemisinin without the requirement of sophisticated equipment.

Currently, there are a number of analytical methods for detecting and quantifying artemisinin, including high-performance liquid chromatography (HPLC),^{6–8} HPLC-electrochemical detection (HPLC-ECD),^{9,10} HPLC-evaporative light scattering detection (HPLC-ELSD),¹¹ gas chromatography-flame ionization detection (GC-FID),¹¹ liquid chromatography-mass spectrometry (LC-MS),¹² thin layer chromatography-densitometry,¹³ and gas chromatography-mass spectrometry (GC-MS),¹⁴ and radioimmunoassay (RIA)¹⁵.

Immunoassays such as the enzyme-linked immunosorbent assay (ELISA) have been developed for quantifying artemisinin and its derivatives. Since all reported antibodies against artemisinin were produced with immunogens through conjugation of a carrier protein to position 12 of artemisinin,^{15–19} antibodies generated in this way usually cross react with artesunate and dihydroartemisinin. To develop specific monoclonal antibodies against artemisinin, it is necessary to conjugate the artemisinin at positions opposite to

position 12 so that the peroxide group and derived groups at position 12 are fully exposed. In the present work, we showed the development of a specific monoclonal antibody to artemisinin using an unreported hapten derived from position 9, which exhibited almost no cross reactivities to dihydroartemisinin, artemether, or artesunate. Using this artemisinin-specific antibody, we developed an indirect competitive ELISA (icELISA), which can be used for quantification of artemisinin in plants, animals, and possibly humans.

MATERIALS AND METHODS

Materials

Cunninghamella elegans (ATCC 9245) was from the American Type Culture Collection. Artemisinin, artesunate, dihydroartemisinin, and artemether were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Quinine and primaquine phosphate were purchased from J&K Chemical (Beijing, China). Chloroquine diphosphate salt, pyrimethamine, lumefantrine, hypoxanthine, aminopterin, and thymidine (HAT), hypoxanthine and thymidine (HT) medium supplements, penicillin, streptomycin, l-glutamine, horseradish-peroxidase-labeled goat anti-mouse IgG, complete and incomplete Freund's adjuvant were purchased from Sigma (St Louis, MO, USA). Cell culture medium (Dulbecco's modified Eagle's medium, DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Paisley, Scotland). All other chemicals and organic solvents used were of analytical grade and purchased from Sinopharm Chemical Reagent (Beijing, China).

Preparation of 9-hydroxyartemisinin

9-Hydroxyartemisinin was obtained by microbial transformation of artemisinin with *C. elegans* (Scheme 1) as described previously.²⁰ *C. elegans* was grown at 28 °C in twenty 500-mL culture flasks with each flask containing 200 mL of medium. A total of 1000 mg of artemisinin (in 40 mL of ethanol) was evenly distributed among the 24 h-old stage II cultures. After 14 days, the incubation mixtures were pooled and filtered to remove the cells and the filtrate (4 L) was extracted three times with ethyl acetate. The combined extracts were dried over anhydrous Na₂SO₄ and evaporated to dryness at 35 °C under reduced pressure to obtain a brown residue. The residue was purified with a silica gel column (30 g, 25 cm) using a petroleum ether (60–90 °C)-ethyl acetate (5/2, v/v) mixture as the eluting system to obtain 9-hydroxyartemisinin. HRMS (ES⁺) calcd for C₁₅H₂₂NaO₆ (M + Na)⁺ 321.1309, found, 321.1313; ¹H-NMR (CDCl₃, 300 MHz): δ 5.94 (1 H, s), 3.37 (1 H, m), 3.24 (1 H, m), 2.43 (1 H, m), 2.1 (1 H, m), 1.9–2.1 (1 H, m), 1.9–2.1 (2 H, m), 1.3–1.6 (1 H, m), 1.3–1.6 (2 H, m), 1.47 (3 H, s), 1.0–1.2 (2 H, m), 1.19 (3 H, d), 1.10 (3 H, d); ¹³C-NMR (CDCl₃, 75 MHz): δ 171.6, 105.4, 93.4, 78.6, 73.5, 47.9, 44.4, 42.1, 35.7, 32.5, 32.1, 25.7, 24.7, 15.4, 12.3.

Preparation of Artemisinin Hapten

Succinic anhydride (60 mg) was added to 80 mg of 9-hydroxyartemisinin in 4 mL anhydrous CH₂Cl₂ and stirred at 4 °C. DMAP (38.9 mg) was added subsequently and stirred at 0–5 °C for 30 min. The reaction was warmed to room temperature naturally and stirred for 3.5 h. Chemical synthesis was monitored by TLC developed with ethyl acetate/petroleum ether

(3/1, v/v). The reaction solution was poured into 4 mL water, and the mixture (~pH 7.0) adjusted to pH 3.0 using 10% hydrochloric acid. The solution was washed with water (3×4 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure to obtain the hapten 9-O-succinylartemisinin (Fig. 2). HRMS (ES⁺) calcd for C₁₉H₂₆NaO₉ (M + Na)⁺ 421.1469; found, 421.1467; ¹H-NMR (CDCl₃, 300 MHz): δ 5.94 (1 H, s), 3.37 (1 H, m), 2.70 (2 H, m), 2.63 (2 H, m), 2.43 (1 H, m), 2.13 (1 H, m), 1.9–2.1 (1 H, m), 1.9–2.1 (2 H, m), 1.3–1.6 (1 H, m), 1.3–1.6 (2 H, m), 1.28 (3 H, s), 1.0–1.2 (2 H, m), 1.18 (3 H, d), 1.10 (3 H, d); ¹³C-NMR (CDCl₃, 75 MHz): δ 174.5, 172.7, 172.4, 105.2, 93.9, 78.6, 75.34, 48.22, 41.3, 40.9, 35.3, 32.5, 28.9, 28.3, 27.7, 24.4, 24.0, 14.2, 11.4.

Preparation of Immunogen and Coating Antigen

The resulting hapten 9-O-succinylartemisinin was conjugated to OVA and BSA as immunogen and coating antigen, respectively (Scheme 2). Briefly, 2.1 mg EDC and 1.38 mg NHS were added to 2 mg of 9-O-succinylartemisinin in 0.5 mL of DMSO. The solution was stirred overnight at 4 °C. The reaction mixture was added dropwise to 23 mg of BSA or 14.76 mg of OVA dissolved in 4 mL of 0.01 M phosphate buffered saline (PBS) and stirred overnight at 4 °C. The mixture was dialyzed against 2 L of 0.01 M PBS (pH 7.5) containing 0.15 M NaCl for 3 days with two changes per day, then lyophilized and stored at –20 °C.

Preparation of Specific mAb against Artemisinin

The mAb against artemisinin was prepared according to the procedures described previously.^{16, 21} Balb/c mice were immunized with 100 μ g of the immunogen using equal volume of complete Freund's adjuvant. Mice were subsequently injected two more times with the immunogen emulsified with incomplete Freund's adjuvant at 2-week intervals. The best-performing mouse was boosted with 100 μ g of immunogen in 100 μ L PBS 3 days before fusion. Spleen cells collected from the mouse were fused with the Murine SP2/0 myeloma cells which were grown in complete medium (DMEM supplemented with 15% FBS, 0.2 M glutamine, 50,000 U/L penicillin, and 50 mg/L streptomycin). The hybridomas were selectively cultured in complete medium supplemented with 1% (v/v) HAT for approximately 2 weeks and the supernatants were screened by ELISAs as described below. The resulting mAbs were generated by inoculating the selected hybridoma cells into Balb/c mice treated with mineral oil. Anti-artemisinin mAbs were purified from ascite fluids by ammonium sulfate precipitation.²²

Indirect Competitive ELISA (icELISA) and Indirect ELISA (iELISA)

The protocol for icELISA and iELISA was the same as that described previously.²³ The specificity of the mAbs was evaluated by cross-reactivity with artemisinin derivatives, quinine, primaquine phosphate, chloroquine diphosphate salt, pyrimethamine and lumefantrine utilizing icELISA. The stock solutions at 1 mg mL^{–1} were prepared in absolute ethanol (quinine), water (chloroquine diphosphate salt, primaquine phosphate), acetonitrile (artemisinin and derivatives, pyrimethamine), and chloroform (lumefantrine).

Sample Extraction

The dried samples of wild *A. annua* were collected from several provinces in China. *Artemisia* samples were extracted according to the method of Zhao and Zeng with modifications described by Han et al.²⁴ Briefly, the dried leaf sample was powdered by a high-speed grinder and 5 mL of petroleum ether (boiling range 30–60 °C) was added to 50-mg portions of the powder in tubes (Corning, NY, USA), which were kept for 12 h at room temperature. The next day it was extracted by ultrasonication (SB5200, Branson, Shanghai, China) for 2 min, followed by filtration under vacuum. The filtrate obtained was evaporated to dryness at room temperature under a mild stream of nitrogen. The residue was then dissolved in 5 mL of methanol and centrifuged at $10,000 \times g$ for 5 min. The supernatant was collected as the final extract and kept at –20 °C until ELISA and HPLC analyses. The extract was diluted directly in PBSTG (0.1 M phosphate buffer containing 0.9% NaCl, 0.1% (v/v) Tween-20 and 0.5% (w/v) gelatin, pH 7.5) at a ratio of 1: 1,000–2,000 for ELISA. As for HPLC, 0.5 mL of extract was pipetted into a 5-mL volumetric flask followed by the addition of 2 mL of 0.2% (w/v) NaOH. After incubation at 50 °C in a water bath for 30 min, the mixture was cooled to room temperature with tap water, and then 0.05 M acetic acid was added up to a total volume of 5 mL and mixed thoroughly. The final solution was filtered through a 0.45- μ m membrane prior to HPLC analysis.

HPLC Analysis of Artemisinin

Standards and *A. Annua* samples were analyzed by HPLC according to the procedure of Zhao and Zeng.⁷ The HPLC system consisted of a Waters 600E multisolvent delivery system and a Waters 2487 dual λ absorbance detector (Milford, MA, USA). The mobile phase, standards, and sample extracts obtained above were filtered through a 0.45- μ m filter prior to HPLC. A C18 reverse-phase column (250 $\times$ 4.6 mm, 5 μ m particle size; Thermo, Vantaa, Finland) was used to separate artemether. The mobile phase was 60% methanol in 0.01 M PBS (pH 7.0) at a flow rate of 1 mL/min. The UV absorption was detected at 260 nm. The injection volume was 5 μ L. The retention time of artemisinin was approximately 8.1 min. All data were collected and analyzed by Waters Millennium 32 software.

Recovery Test for Artemisinin Spiked in *A. annua* Samples and Rat Serum

The 50-mg ground *A. annua* samples, of which the artemisinin contents were quantified by icELISA, were spiked with artemisinin at concentrations ranging from 78 to 2,500 μ g/g. Samples with no artemisinin added were used as the blank controls. After overnight incubation at 4 °C, the samples were extracted as described for ELISA. The extracts were then analyzed with icELISA. Three separate extracts were taken for each spiked sample, and each extract was analyzed in triplicates.

Rat serum samples were spiked with artemisinin at concentrations ranging from 3.13 to 25 ng mL^{–1}. The rat serum samples with no artemisinin added was used as the blank controls. The serum samples were then analyzed with icELISA. Three rat serum samples were taken for each spiked sample, and each extract was analyzed in triplicates.

Animal Studies

The assay method described above was applied to study the pharmacokinetics of artemisinin in rat serum after intraperitoneal injection. This study was performed in strict accordance to the standards described in the “Guide for the Care and Use of Laboratory Animals” (National Research Council Commission on Life Sciences, 1996 edition). Preanulated Sprague-Dawley rats (200–220 g) were obtained from Vital River Laboratories (Beijing, China). These rats were housed in standard cages and fasted for 12 h before dosing but allowed free movement and access to water during the whole experiment. Rats ($n = 6$) were treated with artemisinin (1 mg mL^{-1}) in 3% DMSO (in normal saline) at doses of 10 mg/kg by intraperitoneal injection. Each time $250 \mu\text{L}$ of blood were collected from each rat using an indwelling cannula before dosing and from the same rats at predetermined time intervals after dosing. Serum was separated from the blood samples by centrifugation at $3000 \times g$ for 10 min at 4°C and stored at -20°C . Seventy-five μL of the serum was diluted with equal volume of PBSTG and analyzed directly with icELISA. The pharmacokinetic parameters were calculated by using the ELISA results.

Analysis of artemisinin concentrations in plasma was performed with a computerized program (DAS, version 2.0; Mathematical Pharmacology Professional Committee of China, Shanghai, China). Peak plasma concentration (C_{max}) was obtained from observed data. The area under the plasma concentration versus time curve (AUC) was determined by trapezoidal rule and was extrapolated to 12 h. AUC values were determined for the period 0–12 h. C_{max} was the observed peak value. Plasma clearance (CL) and terminal half-life ($t_{1/2}$) were also calculated.

RESULTS AND DISCUSSION

Artemisinin hapten synthesis and development of a mAb

Due to difficulties in deriving an active group at position 9 in the artemisinin rings by chemical synthesis, we employed a microbial fermentation procedure using the fungus *C. elegans*. From 1000 ml of the fungal culture and 1 g of artemisinin, we were able to purify approximately 200 mg of 9-hydroxyartemisinin, which was used to react with succinate to produce 9-succinate artemisinin. A total of 2 mg of 9-succinate artemisinin were conjugated to OVA and BSA, which were used to immunize mice and as coating antigen for ELISA, respectively. Antisera collected from the mice after the fourth immunization were screened against the coating antigen. The titer of the antibody was defined as the fold dilution giving an absorbance of 1.0 in iELISA. The mouse with the highest titer and the best percentage inhibition in icELISA was used for further study. Three positive hybridomas were cloned by limiting dilution. A positive clone, designated as 3H7A10, was found to secrete mAbs against artemisinin. Subsequently, 3H7A10 was expanded and used to produce ascites.

Specificity of the mAb

The cross-reactivity of the mAb 3H7A10 was tested using artemisinin derivatives (artesunate, dihydroartemisinin, and artemether), and additional antimalarial drugs (quinine, primaquine phosphate, chloroquine diphosphate salt, pyrimethamine, and lumefantrine) in

icELISA (Table 1). No competitive inhibition was observed for all tested drugs up to 20,000 ng mL⁻¹.

Competitive inhibition

The optimal concentrations of coating antigen, mAb, and peroxidase-labeled goat anti-mouse IgG were screened by checkerboard titration. A standard inhibition curve for artemisinin was established by icELISA under the optimized conditions (Figure 1). The IC₅₀ value and the working range based on 20 to 80% of inhibition were 2.6 ng mL⁻¹ and 0.6–11.5 ng mL⁻¹, respectively.

Recovery of artemisinin from fortified *A. annua* samples and rat serum

The average recoveries for 78–2,500 µg/g of spiked artemisinin from *A. annua* samples ranged from 86% to 111%, and the overall average was 102% (Table 2). The average recoveries from artemisinin-spiked (3.13–25 ng/g) rat serum ranged from 85.3% to 115.2%, and the overall average was 95.5% (Table 3).

Analysis of *A. annua* samples with icELISA and HPLC

The artemisinin contents in nine *A. annua* samples were determined by using icELISA and HPLC (Table 4). The artemisinin content varied remarkably in different wild *A. annua* samples and ranged from 0.26 to 2.47 mg/g. The artemisinin content of the *A. annua* samples from Sichuan Province was higher than that from other provinces in China. The HPLC results were similar to those of icELISA (Figure 2). The correlation coefficient (R^2) between the two assays was larger than 0.99.

Pharmacokinetics in rats

As a pilot study to validate this method for pharmacokinetic study, we used rat serum samples after intraperitoneal injection of artemisinin. Figure 3 shows the plasma concentration time profile for artemisinin in rats. The artemisinin concentration increased rapidly to the peak level at 5 min after intraperitoneal administration, which was decreased quickly. The corresponding pharmacokinetic parameters are shown in Tables 5. The value of the peak concentration ($C_{\max}$) was 29.15 ng/mL and the $t_{1/2}$ was 0.38 h. The short half-lives indicate that the compound was removed rapidly from the blood, which is consistent with the short half-lives observed for all artemisinin derivatives in human.

CONCLUSIONS

The position in the target molecule used for conjugation to a carrier protein is very important for specific antibody development. The epitopes distant from the site of conjugation tend to be better recognized by antibodies, whereas epitopes neighboring the coupling site tend to be less well recognized.²³ Artemisinin and its derivatives have similar chemical structure and differ at position 12 (e.g., O-H, βO-CH₃, βO-CH₂CH₃, and O-COCH₂CH₂COOH). The reported antibodies to artemisinin have all been prepared with immunogens on which the haptens were conjugated to position 12. Thus antibodies derived from these conjugations generally have low specificities. The mAbs reported by He *et al.*¹⁶ recognized artesunate, dihydroartemisinin, artemether with cross-reactivity of 650%, 57%, 3%, respectively. The

mAbs generated by Tanaka et al.¹⁷ showed 630% cross-reactivity for artesunate and 30% for dihydroartemisinin, while the polyclonal antibodies prepared by Song et al.¹⁵ recognized artesunate, dihydroartemisinin, and artemether equally well. The mAbs raised by artelinic acid-BSA conjugate bound artemisinin and artemether at approximately the same level.¹⁸ In the present work, we employed bio-transformation by the fungus *C. elegans* to introduce a hydroxy group at position 9 of artemisinin. Using 9-hydroxyartemisinin to generate the immunogen, we successfully developed a highly specific mAb against artemisinin. Using this specific mAb, we developed an icELISA, which allows accurate quantification of artemisinin with a working range of 0.6–11.5 ng mL⁻¹. The limits of detection of HPLC-UV and HPLC-MS were approximately 5 µg/mL^{8, 25, 26} and 50 ng mL⁻¹^{27, 28}, respectively, which are almost 10,000 and 100-fold lower than the immunoassay described here. The high sensitivity of this immunoassay can afford up to 1,000- to 2,000-fold dilutions of the *A. annua* samples to completely eliminate matrix interference, thus increasing the assay accuracy. Compared with reported instrumental methods, the newly developed ELISA is rapid, cost-effective, selective, and sensitive and allows a much higher sample throughput. It should have a good potential for routine monitoring of artemisinin contents in *A. annua*. Furthermore, it may also enable the development of a point-of-care assay in the format of dipsticks for qualitative and semi-quantitative analysis of artemisinin contents in ACT drugs.

We have shown the applicability of the immunoassay for pharmacokinetic studies of artemisinin using rats. The C_{max} and AUC values were in a reasonable range with two earlier studies. Oral administration of artemisinin in rats at the dosage of 10 mg/kg resulted in a peak plasma concentration of <20 ng/mL and the AUC_{0-3h} value of 36.4 ng mL⁻¹ h,²⁹ while intraperitoneal injection of artemisinin at the same dosage resulted in a peak plasma concentration of 0.25 µM (70.5 ng/mL) and AUC_{0-24h} value of 0.132 µM/h.³⁰ In comparison, in humans the C_{max} and AUC values were much larger (623 ng mL⁻¹ and 2,611 ng mL⁻¹ h in one study, 167 ng mL⁻¹ and 754 ng mL⁻¹ h in another study).^{31, 32} In goat, however, the metabolite dihydroartemisinin peaked at 12 h and large amount of unabsorbed artemisinin was eliminated in feces, suggesting slow absorption and low bioavailability.³³ In dogs, oral artemisinin, both in low-dose continuous and high-dose intermittent treatments, was well tolerated but bioavailability appeared low, and blood concentrations of artemisinin and dihydroartemisinin were <0.1 µM.³⁴

The T_{max} was 5 min in our study, which reflects the rapid absorption through the intraperitoneal delivery route. While this is shorter than several other studies using injection as the delivery route, the C_{max} was found at the first sample time points in these studies, suggesting earlier T_{max} might have been missed.^{30, 35} In comparison, oral delivery route resulted in longer T_{max}.²⁹ In all these studies, the half-life of artemisinin is short (~30 min in rats, ~2 h in humans),^{31, 32} which might be due to rapid metabolism and elimination by the liver and to a lesser extent by other organs such as lungs and kidneys.³⁶ In human, artemisinin is metabolized in the liver by cytochrome P450s including CYP2B6 and CYP3A4,³⁷ yielding a number of metabolites that are excreted in urine,³⁸ while urinary excretion of unchanged artemisinin is negligible.³¹ The low C_{max} may also partially be due to irreversible decomposition by erythrocytic heme,³⁹ and binding to serum albumin.⁴⁰

Further, the variation may also depend on the delivery routes used, since the low aqueous solubility of artemisinin may cause a poor, erratic absorption upon oral administration.

Pharmacokinetic analysis of artemisinin relies on a highly sensitive assay. In these analyses, the limited volumes of rat serum make it more difficult for designing time-course analysis. In our pilot pharmacokinetic study of artemisinin, the icELISA required only 75 μ L of serum, which is especially advantageous for pharmacokinetic studies in children. We showed that the mAb 3H7A10-based ELISA allowed the quantification of artemisinin in rat serum without the need for extraction. This result is promising and further validation of the test for pharmacokinetic studies in human subjects is warranted.

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Figure 1.

A standard inhibition curve of artemisinin in icELISA format. B_0 and B are absorbance in the absence and presence of a competitor, respectively. The concentration causing 50% inhibition (IC_{50}) by artemisinin was 2.6 ng mL^{-1} . Each value represents the mean of three replicates

Figure 2.
Correlation between artemisinin content of *A. annua* samples determined by icELISA and by HPLC

Figure 3.
Mean serum concentration time profile of artemisinin after intraperitoneal injection of artemisinin into rats (n = 6) at 10 mg/kg

Scheme 1.

Microbial transformation of artemisinin. The scheme shows the desired addition of the -OH group to the position 9 of artemisinin through microbial transformation

Scheme 2.

Preparation of artemisinin hapten and protein-hapten conjugates

Table 1

Cross-reactivity of mAb 3H7A10 with antimalarial drugs

Analytes	IC ₅₀ (ng/mL)	Cross-reactivity ^a (%)
Artemisinin	2.60 ± 0.12 ^b	100 ± 4.6
Dihydroartemisinin	NI ^c	0
Artemether	NI ^c	0
Artesunate	NI ^c	0
Quinine	NI ^c	0
Primaquine phosphate	NI ^c	0
Chloroquine diphosphate salt	NI ^c	0
Pyrimethamine	NI ^c	0
Lumefantrine	NI ^c	0

^aCross-reactivity (%) = (IC₅₀ of artemisinin/IC₅₀ of other compound) × 100^bData represent means of triplicate ± SD^cNo inhibition was observed with up to 20,000 ng mL⁻¹ of the analytes

Table 2Recovery of artemisinin spiked in *A. annua* samples

Artemisinin (µg/g)		SD	Mean recovery (%; n=3)
Spiked	Detected ^a		
0	260	1	0
78	342	11	105
156	434	15	111
313	606	1	111
625	853	5	95
1250	1332	5	86
2500	2874	2	105

SD, standard deviation

^aData were the means of triplicate samples. Recoveries were determined after subtraction of the background concentration of artemisinin

Table 3

Recovery of artemisinin spiked in rat serum

Spiked concentration (ng/mL)	SD	Mean recovery (%; n=3)
3.13	1	85.3
6.25	3	115.2
12.50	7	91.7
25.00	3	89.5

SD, standard deviation.

Table 4ELISA and HPLC analysis of artemisinin in *A. annua* samples

Sample ^a	ELISA(mg/g)	HPLC(mg/g)
Guangxi	0.39 ± 0.02 ^b	0.41 ± 0.01 ^b
Hebei	0.39 ± 0.01	0.42 ± 0.02
Anhui sample 1	1.18 ± 0.04	1.19 ± 0.01
Anhui sample 2	0.66 ± 0.01	0.65 ± 0.01
Anhui sample 3	1.15 ± 0.04	1.15 ± 0.01
Hunan Zhangjiajie	1.21 ± 0.03	1.28 ± 0.01
Henan Nanzhao	0.26 ± 0.01	0.29 ± 0.01
Shandong Weifang	0.70 ± 0.04	0.60 ± 0.02
Sichuan Zigong	2.47 ± 0.06	2.30± 0.01

^a Each sample was analyzed in triplicate^b Data represent mean ± SD

Table 5

Pharmacokinetic parameters of artemisinin after intraperitoneal injection of 10 mg/kg into rats (n = 6) following a non-compartmental model using DAS software

Parameters	Unit	Mean
C _{max}	ng/mL ± S.D.	29.15 ± 3.00
t _{1/2}	H	0.38
CL	L/h/kg	78.55
AUC _{0-12h}	ng/h/mL	43.67

Values are expressed as mean ± S.D. (n = 6)