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Pharmacokinetics of artemisinin delivered by oral consumption of *Artemisia annua* dried leaves in healthy vs. *Plasmodium chabaudi*-infected mice

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Abstract

Ethnopharmacological Relevance—The Chinese have used *Artemisia annua* as a tea infusion to treat fever for > 2,000 yrs. The active component is artemisinin. Previously we showed that when compared to mice fed an equal amount of pure artemisinin, a single oral dose of dried leaves of *Artemisia annua* (pACT) delivered to *Plasmodium chabaudi*-infected mice reduced parasitemia at least fivefold. Dried leaves also delivered >40 times more artemisinin in the blood with no toxicity. The pharmacokinetics (PK) of artemisinin delivered from dried plant material has not been adequately studied.

Material and Methods—Healthy and *P. chabaudi*-infected mice were oral gavaged with pACT to deliver a 100 mg kg⁻¹ body weight dose of artemisinin. Concentrations of serum artemisinin and one of its liver metabolites, deoxyartemisinin, were measured over two hours by GCMS.

Results—The first order elimination rate constant for artemisinin in pACT-treated healthy mice was estimated to be 0.80 hr⁻¹ with an elimination half-life (T_{1/2}) of 51.6 min. The first order absorption rate constant was estimated at 1.39 hr⁻¹. C_{max} and T_{max} were 4.33 mg L⁻¹ and 60 min, respectively. The area under the curve (AUC) was 299.5 mg·min L⁻¹. In contrast, the AUC for pACT-treated infected mice was significantly greater at 435.6 mg·min L⁻¹. Metabolism of artemisinin to deoxyartemisinin was suppressed in infected mice over the period of observation.

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The authors declare no conflict of interest.

Serum levels of artemisinin in the infected mice continued to rise over the 120 min of the study period, and as a result, the elimination $T_{1/2}$ was not determined; the C_{max} and T_{max} were estimated at 6.64 mg L⁻¹ and 120 min, respectively. Groups of healthy mice were also fed either artemisinin or artemisinin mixed in mouse chow. When compared at 60 min, artemisinin was undetectable in the serum of mice fed 100 mg AN kg⁻¹ body weight. When plant material was present either as mouse chow or *A. annua* pACT, artemisinin levels in the serum rose to 2.44 and 4.32 mg L⁻¹, respectively, indicating that the presence of the plant matrix, even that of mouse chow, had a positive impact on the appearance of artemisinin in the blood.

Conclusions—These results showed that artemisinin and one of its drug metabolites were processed differently in healthy and infected mice. The results have implications for possible therapeutic use of pACT in treating malaria and other artemisinin-susceptible diseases.

Keywords

Plasmodium chabaudi; malaria; *Artemisia annua*; artemisinin; deoxyartemisinin; pACT

1.0 INTRODUCTION

Malaria is one of the worst vector-borne infectious diseases in the developing world and the World Health Organization (WHO) estimated that 215 million cases of malaria occurred, with >655,000 deaths; half the world's population is at risk of contracting the disease (WHO, 2012). Artemisinin, delivered in combination with an alternate drug as Artemisinin-based Combination Therapy (ACT), is currently the preferred treatment against the disease in order to prevent emergence of drug resistance.

Artemisinin (AN; Fig. 1) is produced, extracted, and purified from the “generally regarded as safe” (GRAS; Duke, 2001) plant *Artemisia annua* L. (Asteraceae). The drug also has shown promise against a wide variety of human (Efferth, 2009) and livestock diseases (Ferreira et al., 2011); many are common to the developing world. Although new sources of the drug are emerging from chemical synthesis (Zhu and Cook, 2012) and engineered microbes (Paddon et al., 2013), artemisinin is currently only commercially available from *A. annua* with insufficient supply to treat malaria, let alone other artemisinin-susceptible diseases. To obviate emergence of drug resistance, ACT co-drugs are also needed, which usually increases cost (O'Connell et al., 2011).

In an effort to improve the efficacy of artemisinin therapy and lower cost, we recently showed that when delivered orally to *Plasmodium chabaudi*-infected mice, a single dose of dried leaves of *A. annua* quelled parasitemia at least fivefold more than an equal amount of the pure drug (Elfawal et al., 2012). Beyond the abnormalities associated with infection, analysis of blood toxicology showed no toxicity, results consistent with some human trials using dried *A. annua* leaves (Onimus et al., 2013; ICIPE, 2005). We also previously showed that healthy mice fed dried *A. annua* leaves had > 40 times more artemisinin in their bloodstream than mice fed a corresponding amount of pure drug (Weathers et al., 2011). The measured serum levels exceeded by eight fold the minimum concentration of artemisinin (~10 µg L⁻¹) required for lethality against *P. falciparum* (Alin and Bjorkman, 1994). Together these results suggested that more artemisinin was delivered from whole plant

treatments than from the pure drug treatment. Indeed, in a recent simulated digestion study, > 50% of dry leaf-delivered AN was still available in the intestinal digestate (Weathers et al., 2014a). It is thought that besides artemisinin, the combination of other parasite-killing substances normally present in the plant (flavonoids, monoterpenes etc.; Liu et al., 1992; Elford et al., 1987; Lehane and Saliba, 2008; van Zyl et al., 2006) may be responsible for the observed responses either by improving bioavailability and/or improving therapeutic efficacy. In short, the plant may itself be providing endogenous combination drugs with its artemisinin. We thus termed this a plant-based artemisinin combination therapy, hereafter referred to as pACT.

To better assess the potential of pACT, we conducted a longer pharmacokinetic study to answer two main questions: were the pharmacokinetics of pACT different between healthy and infected mice, and was the plant matrix critical to the appearance of artemisinin in the blood? These results will further our understanding of how the drug moves into the blood when orally delivered as dried leaves of the whole plant.

2.0 MATERIALS AND METHODS

2.1 Plant material

Artemisia annua L. (SAM clonal cultivar; voucher MASS 00317314; vernacular names: annual wormwood; *q ngh o*; sweet annie, sweet wormwood) containing $1.48 \pm 0.06\%$ artemisinin (dry weight) as determined by GCMS was used in this study. Dried leaves of the SAM cultivar also contain monoterpenes (e.g., 0.21% (w/w) camphor, 0.007% eucalyptol, 0.037% α -pinene) and flavonoids (0.37% total) (unpublished). Plants were grown under controlled conditions, harvested, dried, and leaves sieved and pulverized as previously described (Elfawal et al., 2012). Homogenized dried leaf biomass was assayed for artemisinin and deoxyartemisinin using GCMS as described in Elfawal et al. (2012). Identification was via NIST library and purchased standards of artemisinin (Sigma-Aldrich Chemical, St. Louis, MO) and deoxyartemisinin (Toronto Research Chemicals Inc.).

2.2 Mouse infection, feeding and drug delivery

Plasmodium chabaudi ASS (MRA-429) was obtained through the Malaria Research and Reference Reagent Resource Center (MR4) as a part of the BEI Resources Repository, NIAID, NIH. Infection of 20 inbred male C57BL/6 mice weighing an average of 23 g was established by intraperitoneal (i.p.) injection with 10^7 *P. chabaudi*-infected red blood cells as detailed in Elfawal et al. (2012). A second group of 20 of these same mice served as healthy controls. Infected (n=20) and healthy (n=20) mice received oral-gastric gavage of pACT treatment consisting of dried, powdered *A. annua* leaves in a water slurry, prepared fresh, and administered 72 hours post-infection (when parasitemia reached 2–3%) at an amount corresponding to 100 mg AN kg⁻¹ live body weight as detailed in Elfawal et al. (2012). Blood was collected by cardiac puncture from five infected and five healthy mice at each of 4 time intervals (15, 30, 60, and 120 min post-gavage). For additional serum comparisons, two groups of three healthy mice were individually treated with 100 mg kg⁻¹ of pure AN in DMSO and water per kg live body weight with or without mouse chow (Elfawal et al., 2012) and blood collected at 60 min post gavage. Because a preliminary

study showed that serum AN was at best barely detectable when mice were fed the pure drug, blood from each group of mice was pooled prior to extraction and analysis.

2.3 Blood extraction and analysis

Serum and red blood cells were separated by centrifugation at $5,000 \times g$ for 10 min and both fractions were immediately stored at -80°C until extraction. Samples were extracted overnight at room temperature with an equal volume of methylene chloride. Where necessary, extracts were filtered through a $0.45 \mu\text{m}$ filter to remove particulates. Extracts were dried under nitrogen gas at room temperature and the dried residue resuspended in a known volume of methylene chloride for analysis of artemisinin and deoxyartemisinin by GCMS. Analysis and identification using authentic standards was as detailed above for analysis of plant extracts.

2.4 Ethics Statement

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts (Protocol# 2011-0015). All efforts were made to minimize suffering of animals during experimental procedures.

2.5 Statistical analysis

The locally weighted scatterplot smoothing (LOWESS) function was employed to generate a polynomial regression trend line for each of the kinetics plots (Cleveland, 1981). Similar results were obtained using the R PK package (Jaki and Wolfsegger, 2011), which was used to compute the area under the serum concentration versus time curve (AUC). Specifically, functions relevant to the serial sampling design employed in this study were applied. Based on mean concentrations at the various time points, the method of residuals was used to estimate a first order elimination rate constant (and half-life) as well as a first order absorption rate constant.

3.0 THEORY

Oral consumption of dried leaves of *A. annua* is showing therapeutic promise (ICIPE, 2005; Elfawal et al., 2013; Onimus et al., 2013). In contrast to what is known for pure artemisinin, little is known about how the drug moves from orally ingested plant leaves (e.g. pACT) into the blood stream. Comparing the pharmacokinetics of the plant-delivered drug and one of its most common metabolic products in the serum of healthy and infected animals will improve our understanding of artemisinin metabolism and help clarify how such a therapeutic could eventually be implemented.

4.0 RESULTS AND DISCUSSION

4.1 Artemisinin pharmacokinetics differs between healthy and infected mice

Similar to a preliminary study that compared oral gavage of either pure artemisinin or dried *A. annua* leaves (pACT) in healthy mice (Weathers et al., 2011), we fed pACT to healthy or

P. chabaudi-infected mice and measured artemisinin and deoxyartemisinin in the serum for two hours post gavage (Fig. 2). The same data are replotted in Figure 3 to depict the independent kinetics of artemisinin and deoxyartemisinin in healthy vs. infected mice. The first order elimination rate constant for artemisinin in pACT-treated healthy mice was estimated to be 0.80 hr^{-1} , which corresponds to an elimination half-life ($T_{1/2}$) of 51.6 min as determined by the method of residuals (45 ± 5.75 via the PK package of Jaki and Wolfsegger, 2011), similar to that observed in rats by Niu et al. (1985). The first order absorption rate constant was estimated at 1.39 hr^{-1} . C_{max} and T_{max} were 4.33 mg L^{-1} and 60 min, respectively (Table 1). The area under the curve (AUC) for the length of this study was $299.5 \text{ } \mu\text{g} \cdot \text{min mL}^{-1}$. In contrast, the AUC for pACT-treated infected mice was significantly greater at $435.6 \text{ mg} \cdot \text{min L}^{-1}$ (Table 1). Serum levels of artemisinin in the infected mice continued to rise over the 120 min of the study period (Fig. 2), and as a result, the $T_{1/2}$ was not determined; C_{max} and T_{max} were estimated at 6.64 mg L^{-1} and 120 min, respectively. The key pharmacokinetic descriptors are shown in Table 1 and are consistent with those of an earlier preliminary study using only healthy mice (Weathers et al., 2011) and with other studies (Niu et al., 1985; Du et al., 2012) using rats fed pure artemisinin.

Batty et al. (2008) compared the pharmacokinetics of dihydroartemisinic acid (DHA) in healthy and *Plasmodium*-infected mice that were administered a single intraperitoneal dose of DHA (100 mg kg^{-1}) and found no significant difference in elimination half-lives. Since we were unable to determine $T_{1/2}$ for infected mice in our experiment, we cannot compare it to healthy mice. Although a direct comparison cannot be made between the pharmacokinetics of DHA and artemisinin, in contrast to Batty et al. (2008), healthy mice in our study had a significantly lower AUC than infected mice.

4.2 Deoxyartemisinin metabolism differs between healthy and infected mice

Because it is one of several liver metabolic products of artemisinin, we also measured deoxyartemisinin (Svensson and Ashton, 1999). Like many other drugs, artemisinin is metabolized in the liver by cytochrome P450s including CYP2B6 and CYP3A4 (Svensson and Ashton, 1999), yielding a number of compounds including deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and a metabolite named crystal 7 that are found in human urine (Lee and Hufford, 1990). Each of these degradation products lacks the endoperoxide moiety present in artemisinin, and is therefore therapeutically inactive (Lee and Hufford, 1990). In contrast to its metabolism in healthy mice (Fig. 2), metabolism of artemisinin to deoxyartemisinin appeared to be suppressed in the infected mice (Fig. 2); infection retarded the capacity of the mice to form deoxyartemisinin from artemisinin over the two-hour study period. Overall, artemisinin concentrations decreased with a concomitant rise in deoxyartemisinin levels, but only in healthy subjects. In contrast, in infected mice artemisinin levels continued to rise over the study period whilst deoxyartemisinin levels fell and then remained constant. *A. annua* contains a diverse group of compounds (Bhakuni et al., 2001) including flavonoids and monoterpenes that have been shown to inhibit *P. falciparum* (Liu et al., 1992; Elford et al., 1987; Lehane and Saliba, 2008; van Zyl et al., 2006); many also have been shown to inhibit CYP3A4 (Sergent et al., 2009; Choi et al., 2011; de Magalhães et al., 2012).

Results showed an initial dip in artemisinin levels at 15 to 30 minutes in healthy subjects, which we attributed at least partially to conversion of artemisinin to deoxyartemisinin (Fig. 2). The metabolism of artemisinin to deoxyartemisinin appeared to be biphasic, declining slightly between 30 and 60 minutes, and then rising thereafter. The rising arm of the artemisinin pharmacokinetic profile in healthy mice occurred during this period of decreased metabolism to deoxyartemisinin; here, absorption exceeded elimination. When Du et al. (2012) measured deoxyartemisinin in healthy rats given a single oral dose of pure artemisinin (40 mg kg⁻¹), the T_{max} of deoxyartemisinin was 30–60 min longer than that of artemisinin (60 min). Their artemisinin metabolism kinetics were the opposite of our results for a single oral dose of pACT where the peak of artemisinin was later than the first peak for deoxyartemisinin, but earlier than the second deoxyartemisinin peak (Fig. 2). The pharmacokinetics in rats is unlikely the same as in mice, so this may account for the differential response between our two sets of results.

4.3 The presence of a plant matrix enhances artemisinin serum levels

We also examined the effects of mouse chow on pure artemisinin entry into the serum. Mouse chow contains a variety of plant materials including soy, oats, wheat, alfalfa, beet pulp, corn, etc. No artemisinin was detected in the serum of mice fed 100 mg AN kg⁻¹ body weight (Table 2), a result consistent with the reported poor availability of the pure drug (Meshnick et al., 1996). When plant material was present either as mouse chow or *A. annua* pACT, the level of artemisinin in the serum rose to 2.44 and 4.32 mg L⁻¹, respectively, indicating that the presence of plant material, even that in mouse chow, had a positive impact on the appearance of artemisinin in the blood (Table 2). These results were consistent with our preliminary study (Weathers et al., 2011). *A. annua* leaves can contain as much as 1.4% (w/w) essential oils (Bhakuni et al., 2001), which may enhance solubility and thus bioavailability of the nonpolar artemisinin molecule.

4.4 Dosage considerations

For a human comparison, Giao et al. (2001) showed that when humans were given 500 mg oral doses of pure artemisinin (twice on day 1, then once thereafter for days 2–7), the level of recrudescence was 25%. In a summary of early human trials, oral delivery of at least 600 mg per patient for 5 days was required to achieve 10% recrudescence (Meshnick et al., 1996). More recently a Kenyan clinical trial with four cohorts (4 × 12 subjects) of malaria patients (ICIPE, 2005) showed that patients receiving dried *A. annua* leaf tablets containing 11.1 mg artemisinin (twice on day 1, so 22.1 mg d⁻¹) followed by 7.4 mg artemisinin (twice a day for days 2–6, so 14.8 mg d⁻¹) had 9.1% recrudescence (see review by Weathers et al., 2014b).

Furthermore, increasing the amount of dried leaf tablets did not improve therapeutic response, suggesting that maximum effective dose had been achieved. Thus, although the patients treated with dried leaf tablets received about 3–4% the equivalent amount of pure artemisinin used in earlier studies (Meshnick et al., 1996; Giao et al., 2001), they had an equal or better therapeutic outcome than patients treated with pure artemisinin. Those results are consistent with the results of this study.

5.0 CONCLUSIONS

To our knowledge this is the first comparison between healthy and diseased animals of the pharmacokinetics of artemisinin and one of its liver metabolites, deoxyartemisinin, from orally delivered dried leaves of *A. annua*. When accompanied by plant material, more artemisinin enters the blood stream, demonstrating a beneficial effect of the plant matrix on the bioavailability of artemisinin. These results have implications for possible therapeutic use of pACT in treating malaria and other artemisinin-susceptible diseases.

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Glossary

ACT	artemisinin combination therapy
AN	artemisinin
deoxyAN	deoxyartemisinin
AUC	area under the curve
pACT	plant-based artemisinin combination therapy

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Figure 1.
Artemisinin (left) and deoxyartemisinin (right).

Figure 2.

Pharmacokinetics of artemisinin and one of its metabolites, deoxyartemisinin in serum of (left) healthy and (right) *P. chabaudi*-infected mice. Mice ($n = 4 - 6$ per time point per treatment group) were orally gavaged with dried leaves of *A. annua* to deliver 100 mg kg^{-1} body weight dose of artemisinin; bars show SE and * $p < 0.05$, *** $p < 0.001$ via Student's t-test.

Figure 3.

Pharmacokinetics of artemisinin (left) and one of its metabolites, deoxyartemisinin (right) in serum of healthy and *P. chabaudi*-infected mice. Mice ($n = 4 - 6$ per time point per treatment group) were orally gavaged with dried leaves of *A. annua* to deliver 100 mg kg^{-1} body weight dose of artemisinin; bars show SE and * $p < 0.05$, *** $p < 0.001$ via Student's *t*-test.

Table 1
Pharmacokinetic parameters for artemisinin after oral gavage of healthy or *P. chabaudi*-infected mice with pACT.

Dose per animal (mg AN kg ⁻¹)	Disease status	# of subj.	Artemisinin			
			C _{max} (mg L ⁻¹)	T _{max} (min)	T _{1/2} (min)	AUC (mg·min L ⁻¹)
100	healthy	4–6	4.33 ± 0.40	60	51.6 ±	299.5 ^a
100	infected	5	6.64	120	nd	435.6 ^b

Animals were fed *A. annua* dried leaves (pACT) to deliver 100 mg AN kg⁻¹; average body weight was 23 g; nd, not determined.
Letters a, b after AUC values indicate statistical difference at p 0.05; values are ± SE.

Table 2

Artemisinin in serum 60 min post pACT or pure artemisinin gavage.

Mouse treatment	Artemisinin (mg L ⁻¹)
Healthy untreated (3 pooled mice) ^a	0
Healthy + 100 mg AN kg ⁻¹ (3 pooled mice) ^a	0
Healthy + chow + 100 mg AN kg ⁻¹ (3 pooled mice) ^a	2.44
Healthy + pACT at 100 mg AN kg ⁻¹ (n = 5)	4.33 ± 0.40 ^b
Infected + pACT at 100 mg AN kg ⁻¹ (n = 5)	3.78 ± 0.50 ^b

^aThese mice were treated in groups of three; blood was harvested and pooled prior to extraction and measurement of artemisinin, so SE was not calculated.

^bValues are ± SE.