Toxicology 279 (2011) 1–9

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Review

Toxicokinetic and toxicodynamic (TK/TD) evaluation to determine and predict the neurotoxicity of artemisinins

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ARTICLE INFO

Article history:
Received 17 June 2010
Received in revised form 18 August 2010
Accepted 14 September 2010
Available online 21 September 2010

Keywords:
Neurotoxicity
Artemisinin derivatives
Dihydroartemisinin
Drug exposure level
Drug exposure time
Pharmacokinetic profiles
Animal species

ABSTRACT

Studies with laboratory animals have demonstrated fatal neurotoxicity that is associated with administration of artemether (AM) and arteether (AE) intramuscularly or artelinic acid (AL) orally. Toxicokinetic studies showed oil-soluble artemisinins form a depot at the intramuscular injection sites, which is associated with fascia inflammation in muscles. Oral administration of AL induces a gastrointestinal toxicity that is linked with delayed gastric emptying. These effects suggest that the exposure time of artemisinins was extended due to drug accumulation in blood, and this in turn resulted in neurotoxicity. In the present report, the drug exposure time with a neurotoxic outcome (neurotoxic exposure time) was evaluated as a predictor of neurotoxicity *in vivo*. The neurotoxic exposure time represents a total time spent above a lowest observed neurotoxic effect levels (LONEL) in plasma. The dose of AE required to induce minimal neurotoxicity requires a 2–3 fold longer exposure time in rhesus monkeys (179.5 h) than in rats (67.1 h) and dogs (103.7 h) by using a daily dose of 6–12.5 mg/kg for 7–28 days, indicating that the safe dosing duration in monkeys should be longer than 7 days under the exposure. The neurotoxic exposure time of artemisinins could be longer in humans as the comparison of monkeys to humans is likely more relevant than from rodents or dogs. Oral AL required much longer exposure times (8-fold) than intramuscular AE to induce neurotoxicity, suggesting that water-soluble artemisinins appear to be much safer than oil-soluble artemisinins. Due to lower doses (2–4 mg/kg) used with current artemisinins and the more rare use of AE in treating humans the exposure time is much shorter in humans. Therefore, the current regimen of 3–5 days dosing duration should be quite safe. These findings support a recently published WHO guide for malaria treatment with artemisinin regimens, such as artemisinin-based combination therapies and injectable artesunate, to avoid neurotoxicity.

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0300-483X/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2010.09.005

1. Introduction

Artemisinin class compounds act rapidly against drug-resistant *P. falciparum* strains, and are widely used for the treatment of various malarias in humans. The CNS effects from artemisinins that have been observed in laboratory animals are worrying, but difficult to interpret clinically. In the animal toxicity studies of the oil-soluble artemisinin compounds such as artemether (AM) and arteether (AE), the minimal dose of multiple intramuscular injections producing neuropathological findings was 12.5 mg/kg for 7 days in rats, 6 mg/kg for 28 days in dogs, or 8 mg/kg for 14 days in rhesus monkeys (Brewer et al., 1994a, 1998; Classen et al., 1999; Genovese et al., 1995; Kamchonwongpaisan et al., 1997; Li et al., 2007; Petras et al., 1997). A similar finding was observed for oral artelinic acid (AL), which was reported to have similar pathological neurotoxicity in rats following an oral dose at 160 mg/kg daily for 9 days (Si et al., 2007). However, there was little clinical or neuropathological evidence of the neurotoxicity found at high dose levels of over 200 mg/kg/day for 28 days in mice treated with oral AM, artesunate (AS), and dihydroartemisinin (DHA) (Nontprasert et al., 1998, 2000, 2002).

The water-soluble artemisinin derivatives, AS and AL, were designed for intravenous injection. Up to now, no neurotoxicity (pathologic or behavioural) has been observed in animal species following intravenous administration at any repeated dose up to the maximum tolerated dose (MTD). The MTDs of AS and AL have been shown to be 240 and 80 mg/kg, respectively, following intravenous injection daily for 3 days, and neurotoxicity was not detected in these rats (Xie et al., 2005). In another study, intravenous AS had no effect on neurotoxicity scores at 120 mg/kg when administered in sodium carbonate daily for 7 days (our unpublished data). After single intramuscular injection, a high dose of 420 mg/kg of AS or AL did not produce any neuronal necrosis in rats. Up to 200 mg/kg of AS administered orally daily for 5–7 days did not result in neuronal changes or any specific clinical signs (Dayan, 1998).

To date, no systematic toxicity has been reported in humans, despite the use of artemisinin and its derivatives in clinical trials designed with special emphasis on neurological symptoms such as movement, hearing, vestibular, or cerebral abnormalities (Efferth and Kaina, 2010). However, in many of these patients, it is entirely likely that the reason there were no reports from single episodes of CNS events is due to the fact that they were severely ill making it difficult to distinguish between CNS events related to the drug and CNS events related to malaria. Severe malarial infection itself, particularly cerebral malaria in multi-drug resistant strains in the developing world, can leave patients with decreases in neural function. This occurrence has made characterization of potential neurotoxic effects of the artemisinin derivatives very difficult to ascertain, even though these drugs are clinically utilized in ever growing numbers.

Five cases have been reported that suggest episodes of neurotoxicity due to multiple courses of artemisinins therapy (Elias et al., 1999; Franco-Paredes et al., 2005; Haq et al., 2009; Miller and Panosian, 1997; Panossian et al., 2005). These reports may constitute evidence of a causal association that merits further investigation. The possibility exists that repeated and longer duration administration of artemisinin compounds in malaria endemic areas could result in cumulative neuronal damage, especially since these drugs are often freely available for use in tropical countries. Although the authors reported that the toxicity is drug related, there is considerable disagreement regarding these putative episodes of neurotoxicity and the evidence of such a causal association may or may not be only related to artemisinin administration (Gachot et al., 1997; Newton et al., 2005; White et al., 2006).

From the above controversy, it seems necessary to confirm the observations of the neurotoxicity presented in animal species but rarely observed in humans. At least, we need to provide evidence for an indicator to determine whether the possible neurotoxicity noted in animals will occur in humans. Otherwise, the argument over possible artemisinin-associated neurotoxicity will be an endless debate with no substantive answers. In the present review, drug exposure time to induce neurotoxicity from artemisinins in animal species is summarized from the available pharmacological literature. The drug accumulation and prolonged exposure time that accompanies neurological effects should be concluded as a more important marker to determine and predict neurotoxicity than other factors.

2. Prolonged exposure time by accumulation of artemisinins

Previous studies (Li et al., 2006, 2007) have demonstrated that the neurotoxicity of artemisinins does not significantly depend on the conversion rates of artemisinin to DHA (a common active metabolite of artemisinin), the artemisinin distribution in the CNS, or the higher exposure levels (C_{max} and AUC) of artemisinins in plasma. However, the neurotoxicity of artemisinins is highly dependent on the drug exposure time. These studies also demonstrated that the only artemisinin drug accumulation in plasma was found in animals with multiple doses of AE and AM administered intramuscularly and with multiple doses of AL administered orally. Due to a drug induction metabolism, a decline in drug concentration in plasma was observed in repeated dosing of artemisinins (QHS, AS, AM, and DHA) during multiple oral treatments in malaria patients and healthy subjects (van Agtmael et al., 1999; Ashton et al., 1996, 1998; Khanh et al., 1999; Li et al., 2004; Park et al., 1998). The C_{max} and AUC values were markedly reduced from one-third to one-seventh on the last dose day compared with the first day. The decrease in drug exposure levels during treatment is not disease-related, since the artemisinin drug PK of treated patients is similar to that reported in healthy subjects. Similar time-dependent declines (Fig. 1) were also found in animal species treated with intravenous (Li et al., 2005), intramuscular AS (Li et al., 2007), and oral AM (Classen et al., 1999).

2.1. AM and AE accumulation after intramuscular injections

TK data demonstrates that AE and AM accumulation in plasma is observed in rats (Li et al., 1999), beagle dogs (Classen et al., 1999), rhesus monkeys (Li et al., 2007), and humans (Kager et al., 1994) following multiple intramuscular injections. Data collected from rat studies show that the accumulation of AE in plasma is due to a

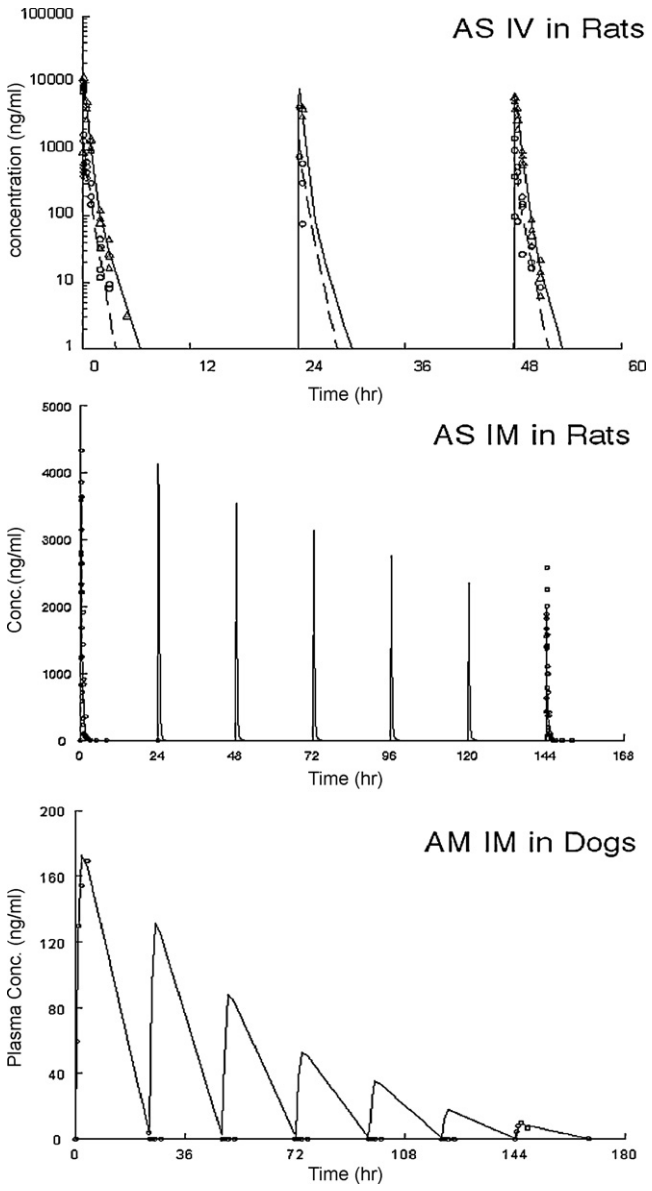

Fig. 1. Pharmacokinetic profiles measured by HPLC-ECD (markers) and computer fitted curves of 36.7 mg/kg of intravenous artesunate (AS, top, dashed line), and its active metabolite DHA (top, solid line) once daily for 3 days (Li et al., 2005). Study of 25 mg/kg of intramuscular artemether (AM, middle) was dosed daily for 7 days in rats (Li et al., 2007). Another PK profiles after oral dose at 600 mg/kg of AM (bottom) daily for 7 days in beagle dogs (Classen et al., 1999).

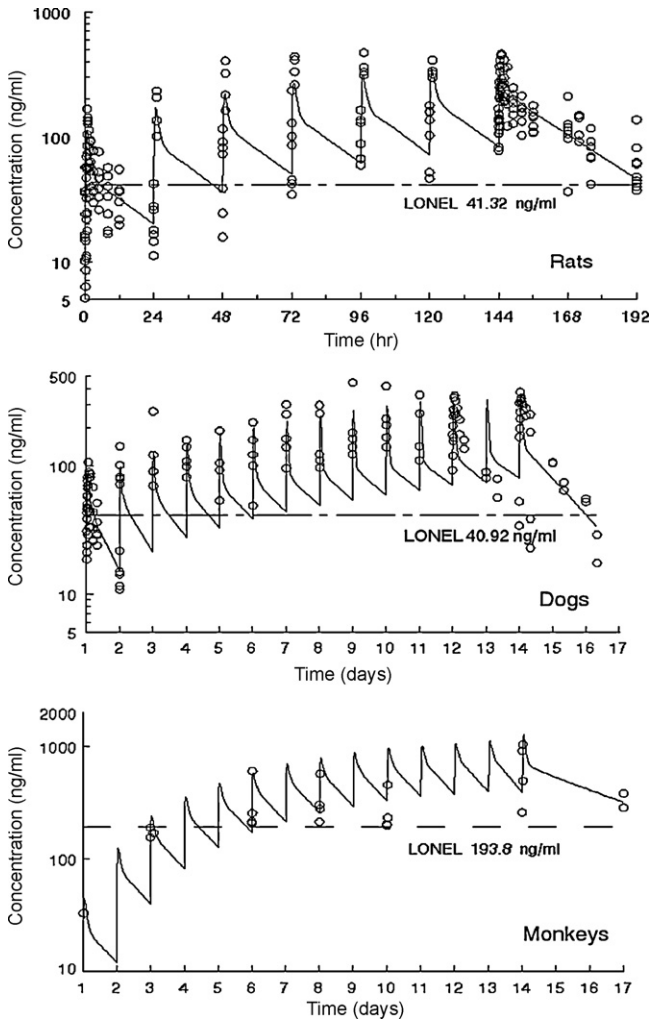

Fig. 2. Pharmacokinetic profiles measured by HPLC-ECD (markers) and computer fitted curves (solid line) of arteether (AE) in sesame oil in rats at daily dose of 25 mg/kg for 7 days (top, Li et al., 1999), in beagle dogs at daily dose of 15 mg/kg for 14 days (middle, Li et al., 2007), and in rhesus monkeys at daily dose of 16 mg/kg for 14 days (bottom, our unpublished data). The lowest observed neurotoxic effect level (LONEL, dashed line) from AE measurement was estimated 41.32 ng/ml in rats, 40.92 ng/ml in dogs, and 193.8 ng/ml in rhesus monkeys, respectively.

slow and prolonged absorption from the injection sites. The elimination $t1/2$ of AE after 7 daily doses was prolonged from 13.7 h on the first day of dosing to 31.2 h on the last dosing day (Li et al., 1999) (Fig. 2, top). AE accumulation was also observed in beagle dogs with severe neurotoxicity and death after daily intramuscular administration of 15 mg/kg for 14 days (Li et al., 2006). The elimination $t1/2$ of AE after dosing for 14 days was prolonged from 11.9 h on day 1 to 21.5 h on day 14 (Fig. 2, middle). Similar accumulation results for AM were reported in dog plasma after daily intramuscular administration of 20, 40, and 80 mg/kg for 7 days (Classen et al., 1999). The analysis of TK parameters on day 2–7 was significantly different, when compared to the parameters estimated on day 1. The elimination $t1/2$ of AM after 7 daily doses increased from 9 h on the first day of dosing day to 37 h on the last day of dosing, suggesting that the exposure time of AM had greatly increased. Moreover, accumulation of AE was shown in the plasma of rhesus monkeys after daily intramuscular administration of 16 mg/kg for 14 doses (Fig. 2, bottom). The elimination $t1/2$ of AE after 14 daily doses increased from 22.6 h on day 1 to 82.1 h on day 14 (Li et al., 2006).

2.2. AL accumulation after intragastric administration

Oral administration of AL also induces a gastrointestinal toxicity that is associated with delayed gastric emptying. Rats dosed with 160 mg/kg of AL daily for 9 days showed moderate neurotoxicity during treatment due to prolonged absorption in the stomach. Comparison of the day 1 and day 9 results revealed that the toxicokinetic parameters were very different. A significantly longer (3.82-fold) elimination half-life was noted on day 9 (10.7 h) in comparison to that observed on day 1 (2.8 h). This prolonged elimination results in drug accumulation. The mean AUC of AL was higher on the last day of dosing (168.01 μg h/ml) than the AUC observed on day 1 (128.38 μg h/ml). Furthermore, progressive delayed gastric emptying and drug accretion were only found in rats treated with 160 mg/kg of AL. These results imply that the observations of delayed gastric emptying in turn resulted in AL accumulation and

Table 1
Muscle injury severity in the injection site areas following repeated arteether (AE) and artemether (AM) injection with vehicle sesame oil by multiple daily intramuscular dosing at 25 mg/kg for 7 days ($n = 3$) in rats (our unpublished data).

Date[a]	Findings	Sesame oil control	AE	AM	Student[b] *t*-test
Day 7	Fascial inflammation, pseudocysts, muscle necrosis, and hemorrhage	1.75 ± 0.43	3.17 ± 0.37	2.50 ± 0.50	$P = 0.0019$
Day 10	Fascial inflammation, pseudocysts, and hemorrhage	1.50 ± 0.50	3.00 ± 0.58	2.00 ± 0.0	$P = 0.0045$

Finding severity were grading as 0: no significant lesions; 1: minimal; 2: mild; 3: moderate; 4: marked; 5: severe.
[a] Day 7 and 10 are one and four days, respectively after last treatment of daily dose for 7 days. The date is given the dosing day as day 0.
[b] The *t*-test was conducted between animals treated both with AE and AM in sesame oil.

the mean half-life of AL was correspondingly extended on the last dosing day compared to day 1 (Si et al., 2007).

2.3. Relationship of drug accumulation to prolonged absorption

The intramuscular administration of AM and AE was associated with a slow absorption because the drugs were dissolved in sesame oil that, when injected, formed a depot from which drug was slowly released (Kager et al., 1994; Li et al., 2004). The slow elimination of AE was recently demonstrated in a rat study, which also found a great deal of accumulation in plasma from injection sites (Li et al., 1999). Following 25 mg/kg of AE in sesame oil daily intramuscular injections for 7 days, the results confirmed and extended the results of earlier studies (Li et al., 1998a) by demonstrating that the absorption of AE from the injection site of the muscle was incomplete. This study also indicated that up to 38% of the total single dose of AE remained in the injection site 24 h after dosing, and 22% of the total single dose still remained in the muscle after 48 h. Following 7 days of multiple IM dosing of AE (25 mg/kg), 91.4% of a single dose (25 mg/kg/day) was still left in the muscles from the injection sites 24 h after last dose (Li et al., 1999).

Our histopathological data demonstrates that the cytotoxicity of the drug on the muscle cell was determined at the injection site (Table 1). The severity of the damage was graded where no significant lesion detected is scored as 0; subacute to minimal damage and mild inflammation is scored as 1–2; damage along the connective tissue and between major skeletal muscle bundles showing subacute to chronic inflammation is scored as 2–3; inflammation observed rarely extending into or between muscle fibers is scored as 3–4; prominent muscle damage with coagulative necrosis surrounded by a reparative response of fibroplasia, fibrosis, skeletal muscle regeneration, and muscle atrophy is scored as 4–5. The distribution of damage observed involves major muscle bundles and occurs in linear tracts, so it likely represents a direct effect of the injected drug and/or vehicle on the muscle.

Overall, the rats in the 7-day group (24 h after last treatment) had more severe lesions than those in the 10-day group (96 h after last dosing), and the animals treated with AE had significantly more severe lesions than those treated with AM (Table 1). The results show that the chronic inflammation of the muscle located at the site of AE injection is more severe (moderate severity 3.00–3.17) than the inflammation noted at the AM injection site (mild severity 2.00–2.50). The inflammation of muscles induced by AE and AM injection may, therefore, be a factor prolonging the absorption from muscles.

Another report of drug accumulation is illustrated in rats dosed with 160 mg/kg of AL daily for 9 days. These rats showed moderate neurotoxicity due to prolonged oral absorption in the stomach (Si et al., 2007). This reservoir is similar to the depot effect seen with the oil-soluble artemisinins (AM and AE) at the intramuscular injection sites. The stomach contents were examined in rats in the daily 160 mg/kg dosing regimen for AL at 8 and 24 h post dosing at different time points during the study. On days 3, 5, 7, and 9, we detected 0, 46.52, 178.59, and 486.21 μg of AL/g in the stomach contents at 8 h after the last dose. The increasing amount of drug observed remaining in the stomach over the course of dosing shows that gastric emptying is inhibited (Kaplan et al., 1992; Li et al., 1998b; van der Velde et al., 1999). The inhibition of gastric emptying progresses from mild on day 5 to severe inhibition on day 9. This inhibition persisted even at 24 h post dosing. At twenty-four hour post dosing on days 5, 7, and 9, we found 5.74, 25.50, and 29.11 μg of AL/g in the stomach contents, respectively. It is postulated that this decrease in GI motility could result from a decrease in vagal tone due to a decrease in sympathetic outflow (Hull and Maher, 1990).

3. Lowest observed neurotoxic effect level (LONEL)

Drug accumulation extends the drug exposure time, which is a major factor inducing neurotoxicity (Li et al., 2006). The current methodology for assessing no observed adverse effect level (NOAEL) involves identifying the highest concentration or dose administered that does not cause a statistically significant or biologically significant response to treatment in comparison to the control group. In the present report, the lowest observed neurotoxic effect level (LONEL) represents the minimal plasma concentration associate with the lowest dose that is found to cause a neurologically and/or statistically significant response to treatment in comparison to the control group. Therefore, determining the neurotoxic exposure time (drug exposure time spent above the LONEL) is critical, and the LONEL should also be determined before evaluating the drug neurotoxic exposure time for each drug in various animal species.

3.1. LONEL of intramuscular AE and oral AL in SD rats

Genovese et al. (1998) estimated that a minimal daily dose of AE at 6.25 mg/kg for 7 days resulted in no observed neurotoxic effect dose (NONED) defined by AE dosing not resulting in histopathological findings in rats. Statistically significant neuropathology in brain stem nuclei, however, was observed in a group of rats treated with 12.5 mg/kg for 7 days. These results demonstrate that AE-induced brainstem neuropathology in rats can occur at the relatively high dose of 12.5 mg/kg (double the NONED dose) for 7 days. The lowest plasma AE concentration of 41.32 ng/ml has been determined following intramuscular dose at 12.5 mg/kg by the previous studies (Li et al., 1999, 2002). Based on the NONED value of 6.25 mg/kg obtained, it was possible to correctly identify the minimal neurotoxic effect level at 41.32 ng/ml, which has been defined as a LONEL in the rat plasma. Administration above this concentration with a certain exposure time should result in neuropathological effects (Dayan, 1998; van Agtmael et al., 1999; Brewer et al., 1994b).

In a TK simulation, 7 multiple AE treatments using 12.5 mg/kg doses were administered intramuscularly in rats (Li et al., 2002), and the exposure time of 67.1 h in plasma was estimated as the minimum time spent above the LONEL concentration of 41.32 ng/ml. This treatment was successful in inducing positive neurotoxicity, confirmed by histopathological examination (Genovese et al.,

Table 2
Drug accumulation, exposure level and neurotoxic exposure time of arteether (AE), artemether (AM), artesunate (AS) and artelinic acid (AL) at over the lowest observed neurotoxic effect level (LONEL) in rats, dogs, and rhesus monkeys concerning with histopathological neurotoxicity after various dose regimens (Li et al., 2007; Si et al., 2007).

Sprague–Dawley rats	Arteether, IM (sesame oil)	Arteether, IM (1:2 CRM/saline)	Arteether IM[a] (sesame oil)	Artesunate, IM (5% $NaHCO_3$)
Neurotoxicity	Severe and death	Moderate	Minimal	No
Dose	25 mg/kg × 7	25 mg/kg × 7	12.5 mg/kg × 7	25 mg/kg × 7
AUC $_{1-7\,D}$ (μg h/ml)	16.92 ± 4.04	46.29 ± 2.06	8.64 ± 4.11	8.57 ± 3.34
$C_{max\ day\ 1}$ (ng/ml)	130 ± 39	1227 ± 171	56 ± 16	3981 ± 992
$C_{max\ day\ 7}$ (ng/ml)	410 ± 91	1826 ± 118	173 ± 48	2078 ± 416
LONEL (ng/ml)	41.32	41.32	41.32	41.32 (from AE)
Exposure time (h)[b]	164.3 ± 7.9	103.0 ± 5.3	67.1 ± 5.8	22.3 ± 4.9

Sprague–Dawley rats	Artelinic acid oral (suspension)	Artelinic acid oral (suspension)
Neurotoxicity	Moderate	Minimal
Dose	160 mg/kg × 9	288 mg/kg × 5
AUC $_{1-9\,D}$ (μg h/ml)	1420 ± 454	1302 ± 905
$C_{max\ day\ 1}$ (ng/ml)	113 ± 83	149 ± 109
$C_{max\ day\ 9}$ (ng/ml)	122 ± 71	143 ± 93
LONEL (ng/ml)	346.26	346.26
Exposure time (h)[b]	186.0 ± 28.0	75.0 ± 12.0

Beagle dogs	Arteether, IM (sesame oil)	Artemether, IM (peanut oil)	Arteether IM[a] (sesame oil)	Artelinic acid oral (capsules)
Neurotoxicity	Severe and death	Moderate	Minimal	No
Dose	15 mg/kg × 14	20 mg/kg × 7	6 mg/kg × 28	25 mg/kg × 14
AUC $_{1-7,\ 14,\ or\ 28\,D}$ (μg h/ml)	24.39 ± 18.04	32.13	9.86 ± 6.55	191.14 ± 90.80
$C_{max\ day\ 1}$ (ng/ml)	90 ± 15	190	38 ± 5	6279 ± 2995
$C_{max\ day\ 7,\ 14,\ or\ 28}$ (ng/ml)	352 ± 203	346	145 ± 83	7177 ± 6248
LONEL (ng/ml)	40.92	40.92	40.92	40.92 (from AE)
Exposure time (h)[b]	277.6 ± 10.7	174.0	103.7 ± 8.6	47.4 ± 11.8

Rhesus monkeys[c]	Arteether, IM (sesame oil)	Arteether IM[a] (sesame oil)
Neurotoxicity	Moderate	Minimal
Dose	16 mg/kg14	8 mg/kg × 14
AUC $_{1-14\,D}$ (μg h/ml)	70962 ± 4588	31245 ± 2614
$C_{max\ day\ 1}$ (ng/ml)	63 ± 9	38 ± 5
$C_{max\ day\ 14}$ (ng/ml)	1038 ± 622	527 ± 306
LONEL (ng/ml)	193.80	193.80
Exposure time (h)[b]	307.4	179.5

DHA: dihydroartemisinin; CRM: cremophor; AUC: area under the curve; IM: intramuscular.
[a] PK data were simulated from our previous studies.
[b] The exposure times above LONEL concentration were calculated with neurotoxic outcome.
[c] The monkey results are from our unpublished data.

1998) (Table 2). The result demonstrated that AE-induced brainstem injury occurred during a minimal period of 67.1 h in plasma exposure above LONEL. In other words, the LONEL (41.32 ng/ml) for neurotoxicity of AE was estimated based on 100% positive findings of neuropathology following dosing at 12.5 mg/kg day for 7 days in rats (Li et al., 1999, 2002).

Recently, AL showed a moderate neurotoxicity confirmed by histopathology after treatment with 160 mg/kg daily for 9 days, but a minimal neuronal degeneration was observed following 5 doses of 288 mg/kg every other day in rats. However, the total dose (1440 mg/kg) and duration (9 days) were identical (Si et al., 2007). The different neurotoxic results could be due to a prolonged exposure time (186 h) in the 160 mg/kg group in comparison to the shorter time (75 h) spent above the LONEL in the 288 mg/kg group (Table 2). Based on the minimal inhibition of gastric emptying on the day 5 and histopathological test in animals treated with oral AL daily for 9 days, the LONEL was calculated as 346.26 ng/ml in this study (Fig. 3). This data demonstrates that AL induced moderate brainstem injury at a severity of 3.25 during a neurotoxic exposure time of 186.0 h in rats. After reducing the exposure time from 186.0 to 75.0 h with the dose regimen at 288 mg/kg every other day for 5 doses, AL could induce toxicity to a minimal brainstem wound at a severity of 1.17.

This study also showed that the LONEL value (346.26 ng/ml) following oral AL administration in rats is approximately 8-fold higher than that (41.32 ng/ml) after intramuscular AE. Intramuscular injection of ^{14}C-arteether was compared to intravenous administration of ^{14}C-artelinic acid in rats. The results of this study showed 0.89% total radioactivity in brain after ^{14}C-arteether administration while the administration of ^{14}C-artelinic acid in rats showed 0.1% of total radioactivity in the brain (Li et al., 2005). This result suggests that ^{14}C-labelled AL is less capable of penetrating through the blood–brain barrier than ^{14}C-arteether.

3.2. LONEL of intramuscular AE in beagle dogs

Davidson (1994) showed that a minimal daily dose of AE at 3 mg/kg for 28 days is a NONED that does not cause clinical neurotoxicity or pathology in beagle dogs. AE-induced brainstem neuropathology has been detected with a dose as low as 5 mg/kg/day as shown by Brewer et al. (1994a,b, 1998), 6.25 mg/kg observed by Dayan (1998), and 6.75 mg/kg observed by Davidson (1994) in dogs based on a daily dose for 28 days. Based on these three findings, the calculated average of the minimal dose necessary to produce neurotoxicity by histopathology in dogs is 6 mg/kg daily for 28. The minimal plasma concentration of AE with a dose of 6.0 mg/kg daily for 28 days has been estimated at 40.92 ng/ml and this value could, therefore, be defined as a LONEL in plasma. The LONEL of 40.92 ng/ml should be the first "at risk" level for causing neurotoxicity in dogs at a daily dose of 6 mg/kg for 28 days (Li et al., 2006). As a result, the LONEL for toxicity of AE in dogs was estimated based on all positive findings of neuropathology in these animals.

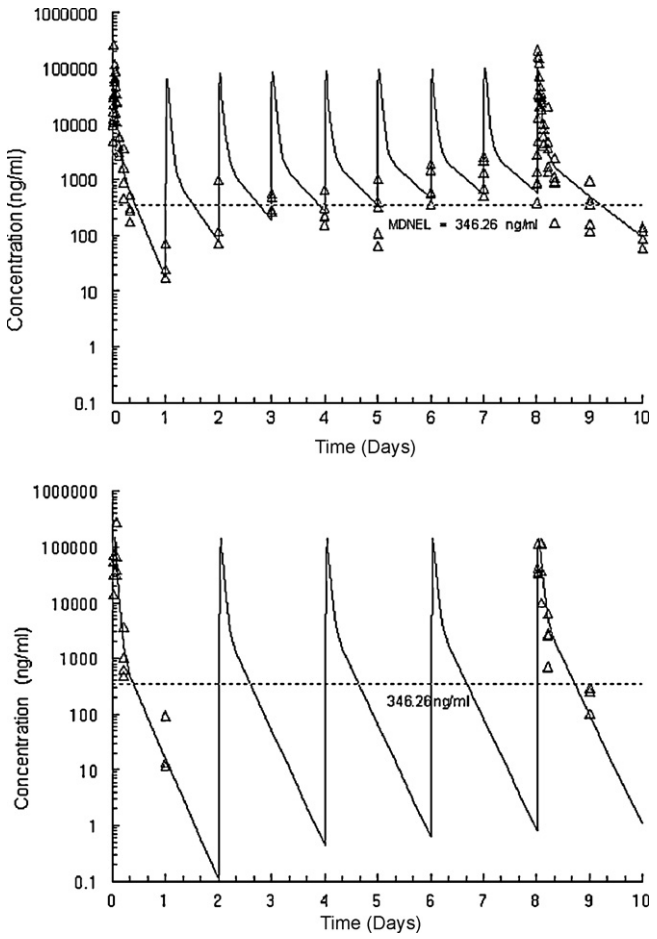

Fig. 3. Pharmacokinetic profiles measured by HPLC-ECD (markers) and computer fitted curves (solid line) of oral artelinic acid (AL) in suspension at 160 mg/kg daily for 9 dosages (top, $n = 5$), and oral AL at 288 mg/kg every other daily for 5 dosages in rats (bottom, $n = 4$) (Si et al., 2007). The two regimens have same total dose with 1440 mg/kg and same treatment period for 9 days. The lowest observed neurotoxic effect level (LONEL) was defined as the plasma level of 346.26 ng/ml, at which anorectic and neuropathological toxicities were noted in daily dosing cohort of rats.

In the TK simulation of 28 repeated AE treatments at 6 mg/kg dosed intramuscularly in beagle dogs, the exposure time of 103.7 h in plasma was estimated as a minimum time above the LONEL (40.92 ng/ml) to induce positive neurotoxicity, which was confirmed by histopathological examination (Brewer et al., 1994b; Davidson, 1994; Dayan, 1998) (Table 2). The result demonstrated that AE-induced brainstem injury occurred during a minimal period of 103.7 h at a dog plasma exposure above this LONEL. Therefore, the LONEL for neurotoxicity of AE was simulated based on 100% positive finding in neuropathology at daily 6 mg/kg for 28 days in beagle dogs (Li et al., 2006, 2007).

3.3. LONEL of intramuscular AE in rhesus monkeys

Since AE-induced brainstem neuropathology in rhesus monkeys occurred at a minimal dose of 8 mg/kg daily doses for 14 days (Petras et al., 1997, 2000), the LONEL of AE was estimated at 193.8 ng/ml. This estimate is based on our TK analysis at 16 mg/kg obtained from 14 days repeated dose studies that should result in a neuropathological determination (Petras et al., 1997; Li et al., 2006, 2007). In the TK simulation of 14 repeated AE treatments at 8 mg/kg intramuscularly in monkeys, the neurotoxic exposure time of 179.5 h in plasma was also calculated as a minimum time spent above the LONEL (193.8 ng/ml) to necessarily induce pathological neurotoxicity. The result demonstrated that AE-induced brainstem injury occurred during a minimal duration of 179.5 h in the neurotoxic exposure time of AE (Li et al., 2006, 2007). The result also shows that the LONEL value of 193.8 ng/ml in rhesus monkeys is 4-fold greater than that of rats (41.32 ng/ml) and dogs (40.92 ng/ml), indicating that rats and dogs seem to be more vulnerable than rhesus monkeys to neurotoxic artemisinin-induced toxicity.

4. Artemisinin exposure time with neurotoxic effects

To determine the relationship between drug exposure time and neurotoxic effects after artemisinin administration at various dose regimens, we calculated the neurotoxic time that the drug plasma level was spent above the LONEL. Although the LONEL is a minimum observed neurotoxic effect level, a certain exposure time was necessary to determine the neurotoxic effects in our previous studies where artemisinins were shown to produce neurotoxicity. This process gave us an initial estimation of drug LONEL and exposure time and allowed us to correlate these data with observed histopathology or neurotoxic effects from artemisinins with various regimens in animal species.

4.1. TK/TD analysis of AE and AL in Sprague–Dawley rats

A neurotoxicity study was conducted to compare AE in two vehicles, sesame oil and cremophore (Li et al., 2002). We calculated what the exposure time of AE with sesame oil would be after dosing over the LONEL (41.32 ng/ml) in rats, and the neurotoxic exposure time we determined was 164.3 h in a 7-day treatment period after daily 25 mg/kg intramuscular injections. The total neurotoxic exposure time of AE with a formulation of 1:2 cremophore/saline at the same dose regimen was 103.0 h, which was over one-third less than with AE in sesame oil. The neurotoxicity outcomes in those rats were reduced from severe to moderate. After simulating the TK data on daily dosing at 12.5 mg/kg for 7 days (Li et al., 2007), the drug exposure time spent over the LONEL was only 67.1 h and the neuropathological toxicity was further reduced to a minimum (Genovese et al., 1998), demonstrating that drug exposure time plays a key role in the development of neurotoxicity (Table 2).

The drug exposure periods of intramuscular AL and AS at the same dose regimen above the concentration of LONEL, which was calculated from the AE study, were 42.48 and 22.3 h (Table 2), respectively, and were significantly shorter (4–7 fold) without any neurotoxic outcomes in comparison to that (164.3 h) observed with intramuscular AE treatments at same dose in rats (Li et al., 2007). It is important to note that the exposure times of AL and AS were also much shorter than a minimal exposure time of 67.1 h (Table 2). The animals treated with AL and AS would have avoided the risk and, therefore, likely avoided the neurotoxic outcome expected due to a reduction of drug exposure times. This result suggests that the shorter exposure times of AL and AS spent above the LONEL seems to be a major factor for avoiding clinical neurotoxicity.

In a similar case, the neurotoxic exposure time for oral AL was 186 h which defines the exposure period threshold to achieve neurotoxicity (Fig. 3, top). These results indicate a correlation between the neuropathology observed in rats and a prolonged AL exposure time. The AL exposure time was related to an accumulation of drug in the plasma resulting from delayed gastric emptying, which induced prolonged absorption of the drug from the stomach (Si et al., 2007). When rats were treated intermittently with 5 doses of AL at 288 mg/kg every other day for 9 days, neuronal degeneration was minimal (significantly different from that of vehicle control group) until day 7 after the last treatment (Table 2). The minimal exposure time required to induce neurotoxicity in these animals was calculated to be 75 h (Fig. 3, bottom). The data, therefore, indicates that

by shortening the drug exposure time at LONEL level, it is possible to reduce the risk of neurotoxicity.

4.2. TK/TD analysis of AE and AL in beagle dogs

AE accumulation was also observed in plasma of beagle dogs after daily intramuscular administration of 15 mg/kg for 14 days (Li et al., 2006). The mean concentration–time profile of AE in the beagle dog is shown in Fig. 2 (middle). The significant changes were found to involve different parameters of AUC and drug half-lives. The elimination $t1/2$ of AE was prolonged from 11.9 h on the first dosing day to 22.5 h on the last dosing day, suggesting that the exposure time of AE had been nearly doubled. Also, the LONEL (40.92 ng/ml) of AE in beagle dogs was first reached in that study on day 6–7 (Li et al., 2006). Therefore, day 6–7 would be the earliest time to cause neurotoxicity in these beagle dogs with the high dose level, based on the TK data analysis.

When estimating the exposure time of AE at concentrations above the LONEL (40.92 ng/ml), the exposure period calculated was 277.59 h in this 14 day study after a daily intramuscular injection of 15 mg/kg in dogs which resulted in severe neurotoxicity and death (Table 2). Compared with AL dosing of suspension or capsules by oral administration, the neurotoxic exposure times were calculated to be 57.9 h for AL suspension, and 47.4 h for AL capsules (Table 2). The animals treated with oral AL showed no neurotoxic outcomes due to the shorter drug exposure times with LONEL, which are even shorter than the minimal neurotoxic exposure time of 103.7 h required to induce a minimal pathological neurotoxicity in beagle dogs (Table 2).

4.3. TK/TD analysis of AE in rhesus monkeys

Substantial plasma accumulation of AE was shown in the plasma of rhesus monkeys after daily intramuscular administration of AE at 16 mg/kg for 14 days (Fig. 2, bottom). Based on a minimal injury to the neuronal population in the monkeys treated with 8 mg/kg daily for 14 days, it was possible to correctly identify 193.8 ng/ml as the LONEL (Li et al., 2006, 2007). The neurotoxic exposure period of AE was calculated as 307.4 h in the study following the daily intramuscular injection of 16 mg/kg for 14 days in the monkeys with moderate neurotoxicity (Petras et al., 1997, 2000) (Table 2). This calculation was based on the estimated exposure time of AE above the LONEL concentration (193.8 ng/ml). The longer neurotoxic exposure time is likely the key factor involved in the induction of neurotoxicity in rhesus monkeys (Li et al., 2007).

The rhesus monkeys treated with intramuscular AE at 8 mg/kg daily for 14 days showed that the drug exposure time spent above LONEL with minimal pathological toxicity was estimated at 179.5 h, which was shorter than the exposure time (307.4 h) from a previous study following daily intramuscular administration of 16 mg/kg for 14 days (Li et al., 2006).

5. Artemisinins exposure time can assess and predict neurotoxicity

Even though no animal species exists with which the neurotoxicity of artemisinins in man can be completely mimicked, the comparison of monkeys to humans is the closest that can be achieved. With animal experiments, only certain aspects of the whole complex TK/TD environment can be analyzed. In order to achieve a prediction of neurotoxicity based on TK/TD parameters successfully, the choice of animal species and the experimental set-up have to be chosen carefully to represent the conditions existing in humans in as suitable a model as possible. The more the model deviates from human TK/TD conditions, the less likely the prediction will be relevant. Today more information is available on the TK/TD properties of artemisinins in animal species. This body of literature will provide data on the neurotoxic doses of artemisinins and on their non-neurotoxic doses relevant to man.

Since toxicity is dependent on chemical/drug exposure level and time (Rangan et al., 1997; Rozman and Doull, 2000), the neurotoxicity of artemisinins has been demonstrated to occur through continued drug exposure over a longer period of time rather than through an elevated drug exposure level over a shorter period of time (Jorgensen, 1980; Li et al., 2002, 2006; Rozman, 1998). The drug exposure times above the LONEL required to cause minimal neurotoxicity in animals for AE, which is the strongest toxicant among the artemisinins, are 67.1, 103.7, and 179.5 h for rats, dogs and rhesus monkeys, respectively, with a 6–12.5 mg/kg daily dose (Table 2). The neurotoxic exposure time of artemisinins could be longer in humans as the comparison of rhesus monkeys to humans is likely more relevant than from rodents or dogs. We predict the safe dosing duration in monkeys should be longer than 7 days (168 h), and this estimate may also be relevant for humans. In addition, due to much lower doses (2–4 mg/kg) used in treatment and the infrequent use of AE in the current antimalarial therapies, the neurotoxic exposure time for humans is predicted to be even longer than in rhesus monkeys with the higher doses. Given this key fact, the safety duration in humans should be much longer than 7 days. Therefore, the 3–5 days dosing duration in current antimalarial regimens should provide an excellent safety margin.

The current clinical dose regimens of three-day artemisinin combined therapies (ACTs) for uncomplicated cases of malaria, and the dose regimens recommended for intravenous AS treatments for severe malaria, which include a few days of a loading dose, may be too short of a drug exposure time to induce neurotoxicity in humans. Also, with regard to acute toxicity, humans appear to be less sensitive than animals (Geyer et al., 1990; Kimbrough, 1990), and humans have much better repair capabilities than animals to respond to such toxicity (Culotta and Koshland, 1994).

In addition, the different artemisinins clearly show different neurotoxic activities at different exposure times. As reported in Table 2, the LONEL of oral AL is 346.26 ng/ml which is 8-fold higher than the LONEL observed after intramuscular AE dosing (41.32 ng/ml) in rats, demonstrating that oral AL treatment seems to be much less likely to induce neurotoxicity than intramuscular AE. Water soluble AL cannot easily penetrate the blood–brain barrier, and this may be a possible mechanism to explain the lower neurotoxicity observed compared to the oil-soluble AE which may cross the blood–brain barrier with greater ease (Li et al., 2005). That may be a reason why another water-soluble artemisinin, AS, has not shown any neurotoxicity when used to treat malaria infections in humans. Currently, oral administration is the most common means of administering artemisinin compounds and combination therapies to malaria patients. Oral administration results in lower peak concentrations and shorter exposure times, which is less likely to induce neurotoxicity. Since more than 99% of malaria patients have been treated with oral artemisinins or intravenous AS, this may be the reason for the lack of neurotoxicity observed in malaria patients. When relating the animal and human neurotoxicity of artemisinins, the different neurotoxic exposure times may possibly provide a greater margin of safety in humans.

6. Conclusion

Studies with laboratory animals have demonstrated neurotoxicity associated with a number of adverse effects including movement disturbances, spasticity, balance deficits, brainstem tissue damage, and even death following administration of some intramuscular doses of oil-soluble AM and AE, or intragastric water-soluble AL. There are significant differences in neurotoxicity

observed between rats, dogs and rhesus monkeys after treatment with different artemisinins suggesting that the exposure time required to induce neurotoxicity after dosing with artemisinins is likely to be longer in humans. TK/TD analysis of neurotoxicity after artemisinin treatment of rats, beagles, and rhesus monkeys has provided a wealth of data to provide a means of predicting the neurotoxic exposure time of artemisinins in humans. Based on this data, we predict the safe dosing duration of artemisinins should be longer than 7 days (168 h). Accordingly, the 3–5 days dosing duration currently used in artemisinin antimalarial therapy should be quite safe. However, neurotoxicity may be caused in humans with inappropriate dose regimens, and therefore, sustained drug exposure times appear to be the critical factor to assess and prevent neurotoxicity. Advances in our knowledge of artemisinin-induced neurotoxicity can help refine the treatment regimens used to treat malaria with ACTs as well as injectable AS products to avoid the risk of neurotoxicity.

Conflict of interest

The author declare no conflict of interest with the study or preparation of the manuscript.

Acknowledgments

This report was supported by the United States Army Research and Materiel Command. The opinions or assertions contained herein are the private views of the author and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2010.09.005.

References

Ashton, M., Sy, N.D., Gordi, T., Hai, T.N., Thach, D.C., Huong, N.V., Johansson, M., Coeng, L.D., 1996. Evidence for time-dependence artemisinin kinetics in adults with uncomplicated malaria. Pharm. Pharmacol. Lett. 6, 127–130.

Ashton, M., Hai, T.N., Sy, N.D., Huong, D.X., Van Huong, N., Nieu, N.T., Cong, L.D., 1998. Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. Drug Metab. Dispos. 26, 25–27.

Brewer, T.G., Grate, S.J., Peggins, J.O., Weina, P.J., Petras, J.M., Levine, B.S., Heiffer, M.H., Schuster, B.G., 1994a. Fatal neurotoxicity of arteether and artemether. Am. J. Trop. Med. Hyg. 51, 251–259.

Brewer, T.G., Peggins, J.O., Grate, S.J., Petras, J.M., Levine, B.S., Weina, P.J., Swearengen, J., Heiffer, M.H., Schuster, B.G., 1994b. Neurotoxicity in animals due to arteether and artemether. Trans. R. Soc. Trop. Med. Hyg. 88 (Suppl. 1), S33–36.

Brewer, T.G., Genovese, R.F., Newman, D.B., Li, Q.G., 1998. Factors relating to neurotoxicity of artemisinin antimalarial drugs "listening to arteether". Med. Trop. (Mars) 58 (Suppl. 3), 22–27.

Classen, W., Altmann, B., Gretener, P., Souppart, C., Skelton-Stroud, P., Krinke, G., 1999. Differential effects of orally versus parenterally administered qinghaosu derivatives artemether in dogs. Exp. Toxicol. Pathol. 51, 507–516.

Culotta, E., Koshland Jr., D.E., 1994. DNA repair works it works its way to the top. Science 266 (5193), 1926–1929.

Davidson, D.E., 1994. Role of arteether in the treatment of malaria and plans for further development. Trans. R. Soc. Trop. Med. Hyg. 88, S51–S52.

Dayan, A.D., 1998. Neurotoxicity and artemisinin compounds do the observations in animals justify limitation of clinical use? Med. Trop. 58, 32S–37S.

Efferth, T., Kaina, B., 2010. Toxicity of the antimalarial artemisinin and its derivatives. Crit. Rev. Toxicol. 16, 1–17 [Epub ahead of print].

Elias, Z., Bonnet, E., Marchou, B., Massip, P., 1999. Neurotoxicity of artemisinin: possible counseling and treatment of side effects. Clin. Infect. Dis. 28, 1330–1331.

Franco-Paredes, C., Dismukes, R., Nicolls, D., Kozarsky, P.E., 2005. Neurotoxicity due to antimalarial therapy associated with misdiagnosis of malaria. Clin. Infect. Dis. 40, 1710–1711.

Gachot, B., Eliaszewicz, M., Dupont, B., 1997. Artesunate and cerebellar dysfunction in falciparum malaria. N. Engl. J. Med. 337, 792–793.

Genovese, R.F., Petras, J.M., Brewer, T.G., 1995. Arteether neurotoxicity in the absence of deficits in behavioural performance in rats. Ann. Trop. Med. Parasitol. 89, 447–449.

Genovese, R.F., Newman, D.B., Li, Q.G., Peggins, J.O., Brewer, T.G., 1998. Dose-dependent brainstem neuropathology following repeated arteether administration in rats. Brain Res. Bull. 45, 199–202.

Geyer, H.J., Scheuntert, I., Rapp, K., Kettrup, A., Korte, F., Greim, H., Rozman, K., 1990. Correlation between acute toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and total body fat content in mammals. Toxicology 65, 97–107.

Haq, M.Z., Mishra, B.R., Goyal, N., Sinha, V.K., 2009. Alpha/beta-arteether-induced mania in a predisposed adolescent. Gen. Hosp. Psychiatry 31, 391–393.

Hull, K.M., Maher, T.J., 1990. L-Tyrosine potentiates the anorexia induced by mixed-acting sympathomimetic drugs in hyperphagic rats. J. Pharmacol. Exp. Ther. 255, 403–409.

Jorgensen, R.J., 1980. Dictyocaulus viviparous: Migration in agar of larvae subjected to a variety of physicochemical exposure. Exp. Parasitol. 49, 106–115.

Kager, P.A., Schultz, M.J., Zijlstra, E.E., van Berg, B., van Boxtel, C.J., 1994. Arteether administration in humans: preliminary studies of pharmacokinetics, safety and tolerance. Trans. R. Soc. Trop. Med. Hyg. 88 (Suppl. 1), S53–54.

Kamchonwongpaisan, S., McKeever, P., Hossler, P., Ziffer, H., Meshnick, S.R., 1997. Artemisinin neurotoxicity: Neuropathology in rats and mechanistic studies in vitro. Am. J. Trop. Med. Hyg. 56, 7–12.

Kaplan, J.M., Spector, A.C., Grill, H.J., 1992. Dynamics of gastric emptying during and after stomach fill. Am. J. Physiol. 263, R813–819.

Khanh, N.X., de Vries, P.J., Ha, L.D., van Boxtel, C.J., Koopmans, R., Kager, P.A., 1999. Declining concentrations of dihydroartemisinin in plasma during 5-day oral treatment with artesunate for falciparum malaria. Antimicrob. Agents Chemother. 43, 690–692.

Kimbrough, R.D., 1990. How toxic is 2,3,7,8-tetrachlorodibenzodioxin to humans? J. Toxicol. Environ. Health 30, 261–271.

Li, Q.G., Peggins, J.O., Fleckenstein, L.L., Masonic, K., Heiffer, M.H., Brewer, T.G., 1998a. The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. J. Pharm. Pharmacol. 50, 173–182.

Li, Q.G., Brewer, T.G., Peggins, J.O., 1998b. Anorectic toxicity of dihydroartemisinin, arteether and artemether in rats following multiple intramuscular doses. Int. J. Toxicol. 17, 663–676.

Li, Q.G., Brueckner, R.P., Peggins, J.O., Trotman, K.M., Brewer, T.G., 1999. Arteether toxicokinetics and pharmacokinetics in rats after 25 mg/kg/day single and multiple doses. Eur. J. Drug Metab. Pharmacokinet. 24, 213–223.

Li, Q.G., Mog, S.R., Si, Y.Z., Kyle, D.E., Gettayacamin, M., Milhous, W.K., 2002. Neurotoxicity and efficacy of arteether related to its exposure times and exposure levels in rodents. Am. J. Trop. Med. Hyg. 66, 516–525.

Li, Q., Lugt, C.B., Looareesuwan, S., Krudsood, S., Wilairatana, P., Vannaphan, S., Chalearmrult, K., Milhous, W.K., 2004. Pharmacokinetic investigation on the therapeutic potential of artemotil (beta-arteether) in Thai patients with severe *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg. 71, 723–731.

Li, Q.G., Xie, L.H., Si, Y.Z., Wong, E., Upadhyay, R., Yanez, D., Weina, P.J., 2005. Toxicokinetics and hydrolysis of artelinate and artesunate in malaria-infected rats. Int. J. Toxicol. 24, 241–250.

Li, Q., Milhous, W.K., Weina, P.J., 2006. Fatal neurotoxicity of the artemisinin derivatives is related to drug pharmacokinetic profiles in animal species. Curr. Topics Toxicol. 3, 1–16.

Li, Q., Milhous, W.K., Weina, P.J., 2007. Antimalarial in Malaria Therapy, 1st ed. Nova Science Publishers Inc., New York, pp. 1–133.

Miller, L.G., Panosian, C.B., 1997. Ataxia and slurred speech after artesunate treatment for falciparum malaria. N. Engl. J. Med. 336, 1328.

Newton, P.N., Day, N.P., White, N.J., 2005. Misattribution of central nervous system dysfunction to artesunate. Clin. Infect. Dis. 41, 1687–1688.

Nontprasert, A., Nosten-Bertrand, M., Pukrittayakamee, S., Vanijanonta, S., Angus, B.J., White, N.J., 1998. Assessment of the neurotoxicity of parenteral artemisinin derivatives in mice. Am. J. Trop. Med. Hyg. 59, 519–522.

Nontprasert, A., Pukrittayakamee, S., Nosten-Bertrand, M., Vanijanonta, S., White, N.J., 2000. Studies of the neurotoxicity of oral artemisinin derivatives in mice. Am. J. Trop. Med. Hyg. 62, 409–412.

Nontprasert, A., Pukrittayakamee, S., Prakongpan, S., Supanaranond, W., Looareesuwan, S., White, N., 2002. Assessment of the neurotoxicity of oral dihydroartemisinin in mice. Trans. R. Soc. Trop. Med. Hyg. 96, 99–101.

Panossian, L.A., Garga, N.I., Pelletierm, D., 2005. Toxic brainstem encephalopathy after artemisinin treatment for breast cancer. Ann. Neurol. 58, 812–813.

Park, B.K., O'Neill, P.N., Maggs, J.L., Pirmohamed, M., 1998. Safety assessment of peroxide antimalarials: clinical and chemical perspectives. Br. J. Clin. Pharmacol. 46, 521–529.

Petras, J.M., Kyle, D.E., Ngampochjana, M., Young, G.D., Bauman, R.A., Webster, H.K., Corcoran, J.D., Peggins, J.O., Vane, M.A., Brewer, T.G., 1997. Arteether: risks of two-week administration in *Macaca mulatta*. Am. J. Trop. Med. Hyg. 57, 390–396.

Petras, J.M., Young, G.D., Bauman, R.A., Kyle, D.E., Gettayacamin, M., Webster, H.K., Corcoran, K.D., Peggins, J.O., Vane, M.A., Brewer, T.G., 2000. Arteether-induced brain injury in *Macaca mulatta* I. The precerebellar nuclei: the lateral reticular nuclei, paramedian reticular nuclei, and perihypoglossal nuclei. Anat. Embryol. (Berl.). 201, 383–397.

Rangan, U., Hedli, C., Gallo, M., Lioy, P., Snyder, R., 1997. Exposure and risk assessment with respect to contaminated soil: significance of biomarkers and bioavailability. Int. J. Toxicol. 16, 419–432.

Rozman, K.K., 1998. Quantitative definition of toxicity: a mathematical description of life and death with dose and time as variables. Med. Hypotheses 51, 175–178.

Rozman, K.K., Doull, J., 2000. Dose and time as variable of toxicity. Toxicology 144, 169–178.
Si, Y., Li, Q., Xie, L., Bennett, K., Weina, P.J., Mog, S., Johnson, T.O., 2007. Neurotoxicity and toxicokinetics of artelinic acid following repeated oral administration in rats. Int. J. Toxicol. 26, 401–410.
van Agtmael, M.A., Cheng-Qi, S., Qing, J.X., Mull, R., van Boxtel, C.J., 1999. Multiple dose pharmacokinetics of artemether in Chinese patients with uncomplicated falciparum malaria. Int. J. Antimicrob. Agents 12, 151–158.
van der Velde, P., Koslowsky, I., Koopmans, H.S., 1999. Measurement of gastric emptying during and between meal intake in free-feeding Lewis rats. Am. J. Physiol. 276, R597–605.
White, N.J., Ashley, E.A., Nosten, F., 2006. Toxic brainstem encephalopathy after artemisinin treatment for breast cancer. Ann. Neurol. 59, 725–726.
Xie, L.H., Johnson, T.O., Weina, P.J., Si, Y.Z., Haeberte, A., Upadhyay, R., Wong, E., Li, Q.G., 2005. Risk assessment and therapeutic indices of artesunate and artelinate in *Plasmodium berghei*-infected rats. Int. J. Toxicol. 24, 251–264.