

Artemisinin Derivatives: Summary of Nonclinical Safety Data Introductory Remarks

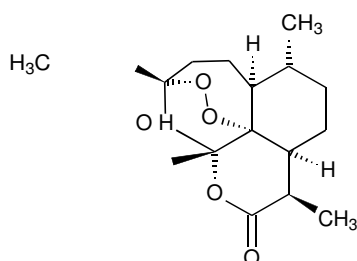
This summary of nonclinical safety data on artemisinin derivatives used in the treatment of malaria has been prepared for use in the assessment of the safety of active ingredients and drug products submitted by manufacturers of such products for approval in the WHO Prequalification Scheme. It follows as closely as possible the layout provided by the ICH Common Technical Document (CTD), with one notable exception: Since the WHO Prequalification Scheme, in contrast to regulatory submissions for marketing permits, does not require the applicant to provide a complete documentation containing all relevant nonclinical study reports, the Nonclinical Written Summaries for pharmacodynamics, pharmacokinetics and toxicology could not be prepared. For the same reasons, the Nonclinical Tabulated Summaries were not prepared as would be usual for a CTD, since they could only consist of a more general compilation of the information utilized in this summary, i.e. of the publications cited, rather than of a concrete, tabular description of studies and their results.

The present document is therefore written in the form of the CTD Nonclinical Overview and provides a general summary of the nonclinical pharmacological, pharmacokinetic and toxicological properties of artemisinin derivatives. It deals with those derivatives that have been submitted as active ingredients to the WHO prequalification scheme so far, either as single compounds or for their use in combinations with other antimalarials. It primarily utilizes publicly available information, but takes also into account information submitted to the WHO prequalification scheme on nonclinical investigations that have been performed by some of the applicants. This review therefore does rely also in part on the confidential data contained in the various submissions for drawing some general conclusions about the potential for target organ toxicity-related, adverse effects. Although these latter data have, in principle, to be considered as proprietary and confidential, they have been used in this document in such a way as to provide the necessary information, but so as not to violate confidentiality.

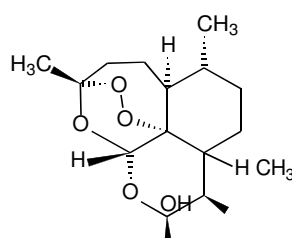
Nonclinical Overview for Artemisinin Derivatives

1. INTRODUCTION

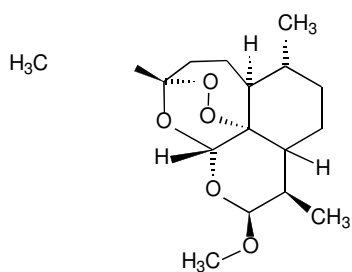
Artemisinin is a natural component of the plant *Artemisia annua*, concoctions of which have been used for a very long time in traditional Chinese medicine for the treatment of fever. In 1972, the component responsible for the pharmacodynamic action, *qinghaosu* or artemisinin, has been isolated from the leaves of this plant, and its activity against the malaria parasite *Plasmodium falciparum* was subsequently demonstrated. A number of semi-synthetic derivatives were also prepared for use in malaria combat programmes. Their properties and therapeutic usefulness have been repeatedly reviewed (Meshnick et al., 1996; van Agtmael et al., 1999; Meshnick, 2002; Olliaro and Taylor, 2003; Sriram et al., 2004). Best known among the different derivatives are artemether, arteether (artemotil), artesunate and arteminol (β -dihydro- artemisinin, DHA). The biological activity of artemisinin and its derivatives is based on the reactivity of the endoperoxide bridge, the common structural feature of artemisinin and all of its derivatives (see structural formulae below).



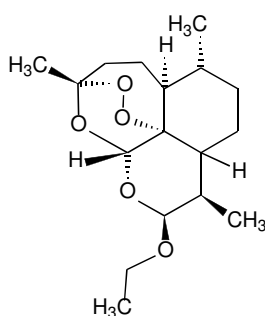
Artemisinin (Qinghaosu)



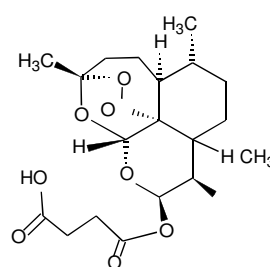
Arteminol (Dihydroartemisinin)



Artemether



Arteether



Artesunate

Artemisinin derivatives are fast acting substances, leading to a rapid clearance of the malaria parasites from the blood, while the short biological half-life precludes a long-lasting activity. Therefore, artemisinins are preferably used not in monotherapy, but in combination with longer-acting drugs that have a slower onset of activity. The WHO Roll Back Malaria programme has advocated such a strategy, recommending the use of artemisinin-containing therapies (ACT) in areas of emerging, high resistance to the most commonly used antimalarials (WHO, 2003). Also, a recent publication has reviewed the use of artemisinin-based combination therapies in uncomplicated malaria (Davis et al., 2005). Another asset of artemisinins is their apparently excellent human safety and tolerability (Price et al., 1999).

In order to secure the supply of artemisinin-derived products of sufficiently high quality, the WHO has invited manufacturers of such drugs to apply for approval in its prequalification scheme. Although the main emphasis in the prequalification scheme is put on the satisfactory demonstration of a sufficient product quality, the safety and efficacy of the submitted products have to be documented as well. This latter requirement, however, has proven to be somewhat difficult for applicants submitting proposals for generic-type drugs without access to the pertinent, primary information, especially for those without in-house generated nonclinical data. In general terms, publicly available information is more or less restricted to either basic research (e.g., mode of action, resistance, etc.) or to clinical, efficacy trials. In the nonclinical safety area specific topics have been extensively discussed in the published literature, but information on more conventional toxicology studies is only sparsely to be found in publications or toxicology databases. Furthermore, whether or not the principles of Good Laboratory Practice have been complied with in such published studies cannot be ascertained.

This overview summarizes nonclinical data on artemisinin (qinghaosu), and on artemimol (dihydroartemisinin, DHA, or dihydroqinghaosu, DQHS), artemether, arteether and artesunate, substances which are the artemisinin-related active ingredients in products submitted to the Prequalification Scheme of WHO. Nonclinical data on combination products with lumefantrine, mefloquine, amodiaquine and sulfadoxine/pyrimethamine are also included, where available.

2. PHARMACODYNAMICS

2.1 Introduction

Artemisinin and its derivatives have been shown to act very efficiently against the asexual, erythrocytic forms of *Plasmodium falciparum* (from the early ring stages to the schizontes); activity has also been demonstrated against *P. vivax*. The generally recommended effective doses in humans with uncomplicated *falciparum* malaria range between 10 mg/kg/day for artemisinin and 2 - 5 mg/kg/day for artemisinin derivatives. They are however, inactive against extra-erythrocytic forms (sporozoites, liver schizontes or merozoites), and gametocytes seem also to be less sensitive, as judged from *in vitro* data, although a decreased transmission of malaria has been observed in areas with extensive use of artemisinins, pointing to some inhibitory influence on gametocyte development. The various derivatives do have antimalarial activity of their own, but the main therapeutic efficacy is due to the (rapidly occurring) biotransformation into the primary metabolite, artemimol (β -dihydroartemisinin, DHA), which is considered to be the ultimate active agent. Activity of artemisinins can be investigated *in vitro* as well as *in vivo*. The *in vitro* systems generally use *P. falciparum* infected human erythrocytes, while the *in vivo* models mainly use rodents infected with *P. berghei*.

Since, when artemisinins are used as monotherapy, a minimum of a seven day course of therapy is required to prevent recrudescence, for the favoured regimens of less than seven days a combination with another effective blood schizonticide is necessary. Therefore, also the data concerned with the *in vitro* effects of various combinations of artemisinins with other antimalarial compounds are of great interest. Investigations have thus been conducted in nonclinical models as well as in clinical studies on the efficacy of various fixed-dose combinations of artemisinins with other antimalarials. In these combinations, the short-lived and fast acting artemisinin derivative is generally complemented by a component with a markedly longer biological half-life which serves to suppress the recrudescence of the disease when plasma levels of the artemisinin component drop below the minimal inhibitory concentrations.

Another issue of importance is the investigation of the antimalarial mode of action, since this question bears on the possibility of the use of artemisinin derivatives in areas where the parasite is resistant against the

conventionally employed drugs, such as chloroquine. Probably because their mode of action is different from the one of other antimalarials, artemisinins are active against chloroquine-, mefloquine- and pyrimethamine-resistant strains of *P. falciparum*. On the other hand, the potential for the emergence of resistance to artemisinins is also of tantamount importance for the final utility of these substances in the combat against malaria.

Furthermore, artemisinins have shown some activity in schistosomiasis; these compounds, however, have not found clinical use in this indication, although they have been proposed as promising candidates for combination therapies with praziquantel (Utzinger et al., 2003). Artemisinins have also been reported to exhibit some additional pharmacodynamic activities, among which immunomodulatory, cytotoxic/cytostatic, and anti-viral properties have been described in various publications. Finally, the important area of safety pharmacology is also covered by some published information.

2.2 Primary Pharmacodynamics

In vitro effects

Data on the *in vitro* activity of artemisinin and its derivatives against *P. falciparum* have been reported in a number of studies. The reported EC₅₀ values depend on the compound and lie generally around or below 10 nM; they thus demonstrate the high sensitivity of the parasite towards these compounds (see Table 1). Artesimol, an antimalarial in its own right, but also the primary metabolite of all other artemisinin derivatives, proved to be the most active of the tested substances, followed by artemether and artesunate.

The *Plasmodium* strains used in these investigations were either laboratory adapted strains, or isolates obtained from malaria patients. Furthermore, also parasite samples were tested for their sensitivity towards artemisinins that had been proven to be resistant against other antimalarial drugs, notably against chloroquine. The results generally demonstrated nearly equal sensitivity of drug-resistant isolates as compared to drug-sensitive ones.

As mentioned in the Introduction, artemisinins are recommended to be used in combination with other antimalarials of longer duration of action, and therefore the investigation of pharmacodynamic interactions of artemisinins with other compounds forms an important part of the information about the potential usefulness of such combinations. A number of published investigations is consequently available, and these have generally shown additive or synergistic effects. The observed inhibitory concentrations of a combination of artemisinin and tafenoquine, applied in a 1:1 molar ratio, were lower than the expected values, calculated from the concentration- response curves of the individual compounds; this effect, which became more pronounced with increasing concentrations, thus indicated synergism (Ramharter et al., 2002); analogous results were obtained for a combination of artesimol with clindamycin (molar ratio 1:10'000) with additive to synergistic effects observed (Ramharter et al., 2003). When artemisinin was investigated in different strains of *P. falciparum* for the interactions of with quinine, atovaquone and mefloquine, the mean values for the fractional inhibitory concentrations (FICs) of the combinations decreased between the EC₅₀ and the EC₉₉, again indicating a synergistic action (Gupta et al., 2002). Synergism was also demonstrated for the combination of artemether with lumefantrine (Hassan Alin et al., 1999), and amodiaquine and its major metabolite, desethylamodiaquine, were shown to exhibit additive to markedly synergistic interactions with artemisinin (Mariga et al., 2005). Artesimol and atovaquone, on the other hand, did show indifference in the combination activities towards various *P. falciparum* strains, as demonstrated by FIC₅₀ and FIC₉₀ values of slightly higher than unity (Fivelman et al., 2004).

Table 1 *In vitro* activities of artemisinin derivatives against *P. falciparum*
(different, chloroquine-sensitive and -resistant strains)

Compound	EC ₅₀ (nM)	EC ₉₀ (nM)	EC ₉₉ (nM)	Reference
Artemisinin	3.2 - 108			Hassan Alin et. al., 1990
	20	112	300	Congpuong et al., 1998
	9	60	368	Gupta et al., 2002
	2.1 - 3.0	36 - 54	306 - 367	Mariga et al., 2005
	12.1	88.5	447.2	Ramharter et al., 2002
	5.4 - 26			Sharma et al., 2000
	1 - 7			Skinner et al., 1996
	11.3	52.1	181.2	Sponer et al., 2002
Arteminol (DHA)	4.7 - 23			Hassan Alin et. al., 1990
	1.16 - 3.83			Fivelman et al., 2004
	0.61	5.61	34.33	Ramharter et al., 2003
	0.98 - 1.25			Ringwald et al., 1999
	0.8 - 3			Skinner et al., 1996
Artemether	0.98 - 6.1			Hassan Alin et. al., 1990
	0.28	35.45		Hassan Alin et al., 1999
	0.32 - 4.7			Sharma et al., 2000
	2.8 - 3.46			Pradines et al., 1998
	0.006 - 0.1			Skinner et al., 1996
Arteether	3.3 - 7			Sharma et al., 2000
Artesunate	4.2			Brockman et al., 2000
	0.2 - 0.6			Skinner et al., 1996

Stage sensitivity has also been investigated, since this property may be of clinical importance in terms of parasite clearance kinetics and recrudescence rates. While, *in vitro*, artemisinin and artemether, in their own right, do not act on trophozoites (see Figure 1), the primary metabolite arteminol demonstrated a very fast action on all three asexual stages tested (Skinner et al., 1996)

In vivo effects

Mice were treated with different antimalarial drugs for three consecutive days following the injection of erythrocytes infected with a drug-sensitive strain of *P. berghei* (K173). Artemisinin, given as a subcutaneous injection of an aqueous suspension, yielded an ED₅₀ of 5.6 mg/kg and an ED₉₀ of 10.5 mg/kg (Chawira et al., 1987). In the same study, artemisinin was found to be equally effective against a primaquine-resistant strain

(ED₉₀ of 7.0 mg/kg) as well as against a mefloquine-resistant strain (ED₉₀ of 14.5 mg/kg). Synergistic effects were seen for the combinations of artemisinin with mefloquine, primaquine, clindamycin and spiramycin, while antagonistic effects were obtained with pyrimethamine, sulphadiazine, sulfadoxine, cycloguanil and dapsone, while chloroquine effects

Figure 1 *In vitro* stage-specificity of inhibition of *P. falciparum* (from Skinner et al., 1996)

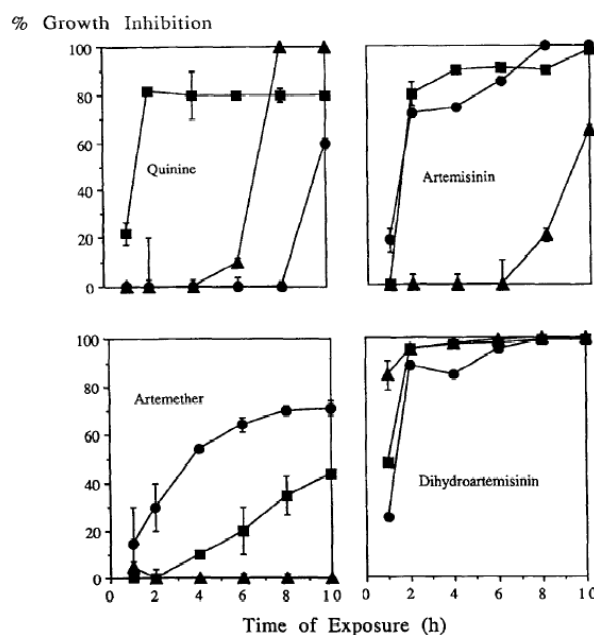


Fig. 1. Stage-specific activities of quinine, artemisinin, artemether and dihydroartemisinin on culture-adapted parasites of *Plasmodium falciparum*. Synchronized cultures of rings (0 h: ●), trophozoites (20–24 h: ▲) and schizonts (36 h: ■) were exposed to the EC₅₀ concentration of each drug for 0–10 h as described in Materials and Methods. The data are presented as percentage growth inhibition $\pm$ S.D. with time of drug exposure for the 3 asexual stages compared with untreated controls. Quinine, artemisinin and artemether were tested 6 times on 5 isolates, whereas dihydroartemisinin was tested twice on 2 different isolates. Because all 6 parasite isolates demonstrated the same stage-specific sensitivity to the individual drugs, a representative figure is presented for each drug.

were considered to be additive (Chawira et al., 1987). Sprague-Dawley rats, infected with *P. berghei*, were treated with 3 consecutive daily intravenous injections of artesunate at dose levels of 2.3, 4.6, 9.2, 18.35, 36.7 and 73.4 mg/kg. The three lower doses were inactive, while at the highest dose a complete parasite clearance was obtained; at the doses of 18.35 and 36.7 mg/kg four of six animals exhibited clearance. Recrudescence, however, was observed in all cases, with time to recrudescence rising from 5.0 to 8.2 days with increasing doses (Li et al., 2003). Artesunate given orally as an aqueous suspension for 4 consecutive days to *P. berghei* infected mice exhibited ED₅₀ and ED₉₀ values of 6.33 and 23 mg/kg against the chloroquine-sensitive strain K173 N, and 6.4 and 32 mg/kg against a highly chloroquine-resistant subline derived from this strain, respectively. Rats, infected with *P. berghei* were treated i.v. with daily doses of 2.3 – 480 mg/kg artesunate, and the doses with which either a 50 % reduction of parasitaemia (SD₅₀), or a complete parasite clearance in 50% (CD₅₀) or 100% (CD₁₀₀) of the animals was obtained, were calculated. The CD₅₀ was estimated to be 7.4 mg/kg, and the CD₁₀₀ was determined at 60 mg/kg. Compared with the maximum tolerated dose of 240 mg/kg, therapeutic indices of 32.6 and 4.0, respectively, were estimated (Xie et al., 2005). Doses of 140.4 mg/kg and above had a complete curative effect when tested over a period of 28 days. In the same study, monkeys (*Macaca mulatta*) were infected with *P. knowlesi* and treated with 3.16, 10 and 31.6 mg/kg artesunate in comparison with the same doses of chloroquine. Artesunate showed faster clearance than, and equal curative efficacy with, chloroquine (Shi et al., 1999). Mice infected with chloroquine-sensitive and -resistant strains of *P. berghei* were treated for 3 consecutive days with intravenous injections of either artemisinin or artesunate. The ED₅₀ and ED₉₀ values for artemisinin were determined to be 2.87 and 5.89 mg/kg for the sensitive, and 2.87 and 5.98 mg/kg for the

resistant strain, while the respective values for artesunate were found to be 0.3 and 1.14, and 0.5 and 1.14 mg/kg, respectively. Rhesus monkeys, infected with *P. knowlesi*, received three consecutive iv doses of 6, 8 or 12 mg/kg; time to parasite clearance was shown to be in the order of 16 - 20 hours (Yang et al., 1982).

The use of different *Plasmodium* species, other than the human malaria parasite *P. falciparum*, may be seen, however, to constitute an assessment problem with regard to the extrapolation of the results obtained from nonclinical studies to human patients and to human efficacy of antimalarial treatments. Although *P. falciparum* infections can be induced and sustained in primates, such models are expensive and not easily available. In order to circumvent this problem, a mouse model has been developed that utilizes immunocompromised animals which then are susceptible to infection with *P. falciparum*. The mouse strain used, immunodeficient BXN mice, lacks T-cell and T-independent B-cell functions, and by chemical immunomodulation (i.e., reduction of tissue macrophage numbers by treatment with dichloromethylene diphosphonate and control of the increase in the number of polymorphnuclear neutrophils by a monoclonal antibody) a medium grade parasitaemia can be obtained by infusion of *P. falciparum* infected human blood cells, which then allows for investigating parasite biology and drug efficacy. Infection of these mice with a chloroquine-sensitive strain of *P. falciparum* and subsequent treatment with chloroquine, mefloquine or arteminol provided evidence for the validity of the model in the sense that the results for parasite time-to-clearance, and for percent clearance at 48 hours corresponded to the respective human values. Arteminol was also active against the chloroquine-resistant strain in this model, and against all stages of the parasite (Moreno et al., 2001).

Activity against gametocytes

P. falciparum gametocytes are the essential link between humans and the Anophelid vector and are thus mainly responsible for maintenance of transmission. Therefore, any gametocytocidal activity of antimalarials is of importance, and interest in such potential activities of artemisinins has resulted in a small number of investigations.

In an *in vitro* study gametocytes of *P. falciparum* were found to be sensitive to artemisinin at concentrations of 100 - 1000 ng/ml, while exposure to 10 ng/ml did not reduce the number of gametocytes in culture. It was furthermore shown that only younger stages of gametocytes were susceptible to the inhibitory effect, while mature gametocytes (stages IV and V) were unaffected (Kumar and Zheng, 1990). In another *in vitro* study, artemisinin and a fluorescence-tagged derivative of arteminol (code name NIHO2) were found to exhibit 100% gametocytocidal activity at concentrations of around 100 ng/ml, a concentration which was about 12 - 15 times higher than the EC₉₀ for the asexual, erythrocytic stages. While the EC₅₀ and EC₉₀ for the latter stages could be determined as 1.7 and 6.6 ng/ml for artemisinin and 2.7 and 8.4 for NIHO2, gametocytocidal activity was obtained with IC₅₀ and IC₉₀ values of 1.3 and 22 ng/ml for artemisinin, and 6.6 and 19 ng/ml for NIHO2, respectively (Mehra and Bhasin, 1993).

An *in vivo* study has furthermore been conducted in Rhesus monkeys infected with *P. cynomolgi* and which were subsequently treated orally with artemether. The endpoint in this study was the infection of *Anopheles stephensi* feeding at various times after drug treatment on the monkeys, either as percentage of mosquitoes infected or as mean number of oocysts. While after single doses of 1.25 and 2.5 mg/kg did not significantly affect infection rate and oocyst count in the mosquitoes, a dose of 5 mg/kg was able to block oocyst development temporarily, i.e., for 24 - 48 hours, while 96 hours after treatment, oocysts appeared again. At the dose of 10 mg/kg no oocysts were found at feeding intervals of up to 96 hours, and mosquitoes kept for 14 days after feeding did not harbour infective sporozoites in their salivary glands (Tripathi et al., 1996).

There are a few results that indicate the possibility of a reduction in transmission of malaria in areas where artemisinin derivatives are extensively used. A clinical study in *P. vivax* malaria patients showed that treatment with artesunate lowered the risk for, and the duration and magnitude of, gametocyte carriage significantly, and to a greater extent than treatment with chloroquine (Nacher et al., 2004). Another clinical study demonstrated also a decreased gametocytaemia in patients treated with a combination of mefloquine and artesunate, but not with mefloquine and primaquine (Suputtamongkol et al., 2003). Finally, artesunate

has been demonstrated to reduce gametocyte carriage in *P. falciparum* infections in Thailand (Pukrittayakamee et al., 2004).

In summary, the schizontocidal and antimalarial efficacy of artemisinin and its derivatives, can be considered as clearly proven in nonclinical studies. Furthermore, the *in vitro* as well as *in vivo* investigations on the primary pharmacodynamic properties and nonclinical efficacy that were specifically conducted in the context of regulatory submissions by manufacturers have generally yielded results with EC- and ED-values that can be considered as equivalent to the published values, and they thus unequivocally support the excellent efficacy of artemisinins.

2.3 Mode of Action

The structural determinant of the activity of artemisinins is the endoperoxide bridge which is a specific feature of this type of compounds. It has been suggested that the parasiticidal activity starts with the reaction of artemisinins with haem iron, leading to the generation of activated oxygen species, such as oxygen radicals, or of a C-centred radical of artemisinin itself, which are then further producing lethal damage to the parasite (Meshnick et al., 1993; Olliaro et al., 2001a). Chemically, such a redox-cycling reaction between artemisinin and ferrous/ferric ions has been described *in vitro* in an aqueous buffer solution (Sibmooh et al., 2001). Artesimol, at concentrations of 0.5 and 1.0 ng/ml, has also been demonstrated to increase oxidative stress in *P. falciparum*-infected erythrocytes, but not in uninfected cells, and depleting in consequence intraerythrocytic levels of antioxidants, especially glutathione (Ittarat et al., 2003). The reductive scission of the endoperoxide bond may be catalyzed by ferrous ion in the form of haemin, produced by metabolic breakdown of haemoglobin in the food vacuole of the parasite, and binding affinities of artemisinins with ferrous haem have indeed been found to be correlated to the respective antimalarial activities (Paitayatat et al., 1997). In support of the involvement of alkylating molecular species derived from artemisinins, it has been shown *in vitro* that arteether and arteminol do react with specific (minor) plasmodial proteins under conditions where no reaction occurred with erythrocyte proteins (Asawamahasakda et al., 1994). An early explanation of the schizontocidal activity of artemisinins suggested an interference with haemoglobin catabolism in the food vacuole of the parasite, inhibiting haemozoin formation and increasing haemozoin breakdown, both resulting in an accumulation of toxic haem, as well as in the generation of membrane damage and alkylation of parasite proteins by the free radicals produced by the catalytic action of haem upon the artemisinin endoperoxide (Pandey et al., 1999). These effects might effectively be brought about through the alkylation of haem by artemisinin which has been demonstrated *in vitro* as well as *in vivo* (Kannan et al., 2005; Robert et al., 2005). Another explanation for the activity of artemisinins may be their observed inhibitory actions on parasite proteases, a possibility corroborated by the recent finding that protease inhibitors used in HIV treatment have also some anti-malarial effect (Senior, 2005).

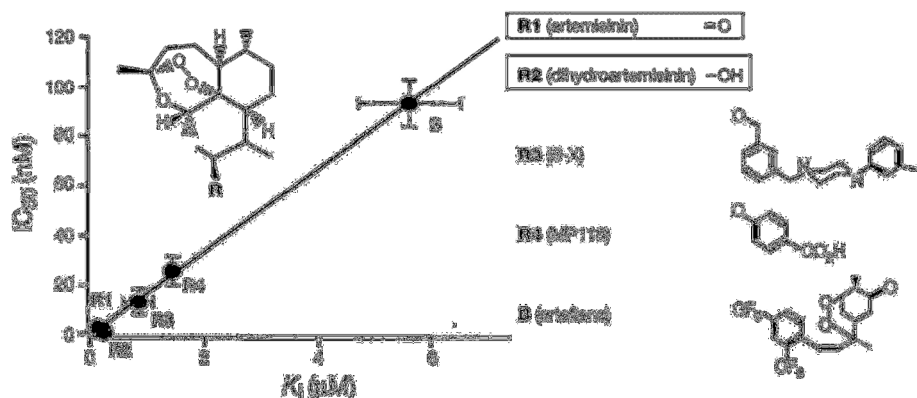
On the other hand, various more recent investigations have provided evidence that neither haemozoin formation can be considered as target of the antimalarial activity of artemisinins (Haynes et al., 2003), nor that the presence of haem is necessary for the schistosomicidal activity (Parapini et al., 2004).

As a very specific target for the activity of artemisinins, the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) has recently been suggested (Eckstein-Ludwig et al., 2003). The only plasmodial enzyme of the SERCA-type, PfATP6, was clearly inhibited in a concentration-related manner by artemisinin, and K_i values for this inhibition by a number of artemisinin derivatives correlated linearly to their *in vitro* IC_{50} values (see Figure 2). In addition, three-dimensional molecular modeling has provided evidence for a correlation between the fit of docking to PfATP6 of artemisinins and their activity; the binding was suggested to proceed by hydrophobic interaction, and the resulting structure would leave the endoperoxide bridge exposed to the outside of the binding pocket. In this way, activation by ferrous iron could proceed, and the resulting radical might then inactivate the enzyme (Jung et al., 2005). This mode of action has received some support from the observation that a specific mutation in the gene for PfATP6 confers a decreased sensitivity to the parasite towards artemether. Isolates of *P. falciparum* from French Guiana and Senegal with the mutation S769N were shown to have IC_{50} values of up to 117 and 45 nM, respectively, as compared to the normal sensitivity of about 0.2 nM (Jambou et al., 2005).

Also, a proteomic approach to the investigation of cellular changes elicited by artemisinins has been suggested, and several possibilities of investigations by this method as applied to artemisinins and the problems of their mode of action have been outlined (Cooper and Carucci, 2003). In a recent proteomic study, artemether treatment showed clearly an upregulation of membrane-associated calcium-binding protein in the parasite (besides the upregulation of a number of stress-related heat-shock proteins, a proteinase and two proteins related to protein metabolism) and may thus point to the involvement of changed calcium homeostasis in the parasite (Makanga et al., 2005).

Furthermore, other targets for the schizonticidal activity of artemisinins have been suggested as well: Artemisinins have been shown to reduce the erythrocyte membrane fluidity which is increased upon infection with *P. falciparum* (Sibmoo et al., 2000), to inhibit endocytosis (Hoppe et al., 2004), and to damage the mitochondrial electron transport chain (Li et al., 2005b). Whether these effects could in the first instance be responsible for the schizonticidal activity of artemisinins, or just appear as side-effects of the general properties of these substances, cannot be judged at present.

Figure 2 Inhibition of PfATP6 by different artemisinin derivatives (from Eckstein- Ludwig et al., 2003)



2.4 Secondary Pharmacodynamics

Artemisinins have been reported to exhibit activities in pharmacodynamic areas other than malaria, too. Publications have described antiviral, anticancer, immuno-modulating and antiangiogenic activities.

Influences of artemisinin and its derivatives on immune functions have been reported especially in a number of early Chinese publications. The results of these investigations have been rather complex and did not lend themselves to any easy interpretation. More recently, artemisinin, artemimol and arteether were tested in mice for possible immuno-modulating properties. No antiinflammatory properties were detected for the three compounds, as an intramuscular dose of 600 mg/kg did not affect the carrageenan-induced oedema. Also, doses of up to 600 mg/kg were without modulatory effect on the delayed-type hypersensitivity reaction. On the other hand, a significant and dose-dependent reduction of haemolytic plaque formation was observed with all three agents, applied in intramuscular doses of 400 and 600 mg/kg, indicating an influence on the humoral response in mice immunized with sheep red blood cells (Tawfik et al., 1990). Humoral immune functions were also reduced in another study in mice with artesunate, applied intramuscularly at a dose of 75 mg/kg. In contrast to the former study, also cell-mediated immune functions were elevated by this treatment. Other observed changes in immune functions consisted of increased rate of PHA-stimulated lymphocyte transformation, increased spleen weight, and increased serum complement (C3) levels, but decreased thymus weight and decreased phagocytosis of peritoneal macrophages (Lin et al., 1995). Again, it is rather difficult to draw any conclusions with respect to immunomodulating activities and their relevance to the human therapeutic situation.

More recently, cytotoxic effects of artemisinins on cancer cells *in vitro* have been described. An investigation on a panel of 55 representative human tumour cell lines compared the growth inhibitory influence of artesunate with conventional anticancer drugs (Efferth et al., 2001). Depending on the origin of the cell line, growth inhibitory concentrations (GI_{50}) ranged from 1.11 and 2.13 μ M for leukaemia and colon cancer cells, respectively, to 25.62 μ M for non-small cell lung cancer cells. These values compared well with the respective figures for agents such as cisplatin, ifosfamide, cyclophosphamide, 5-fluorouracil, doxorubicin, mitomycin C, vincristine, etoposide, methotrexate, etc. (see Figure 3, from Efferth et al., 2001). It has to be noticed, however, that the logarithmic scale used for indicating the concentrations tends to visually belittle differences, and that thus in most cases the artesunate concentrations needed to induce an effect are orders of magnitude greater than those of the most active cytostatics. Furthermore, it has also to be taken into account that not all of the comparator cytostatics are indeed indicated for all of the cancer types utilized in this screen.

Figure 3 In vitro activity of artesunate against 55 tumour cell lines in comparison with conventional cytostatics (from Efferth et al., 2001)

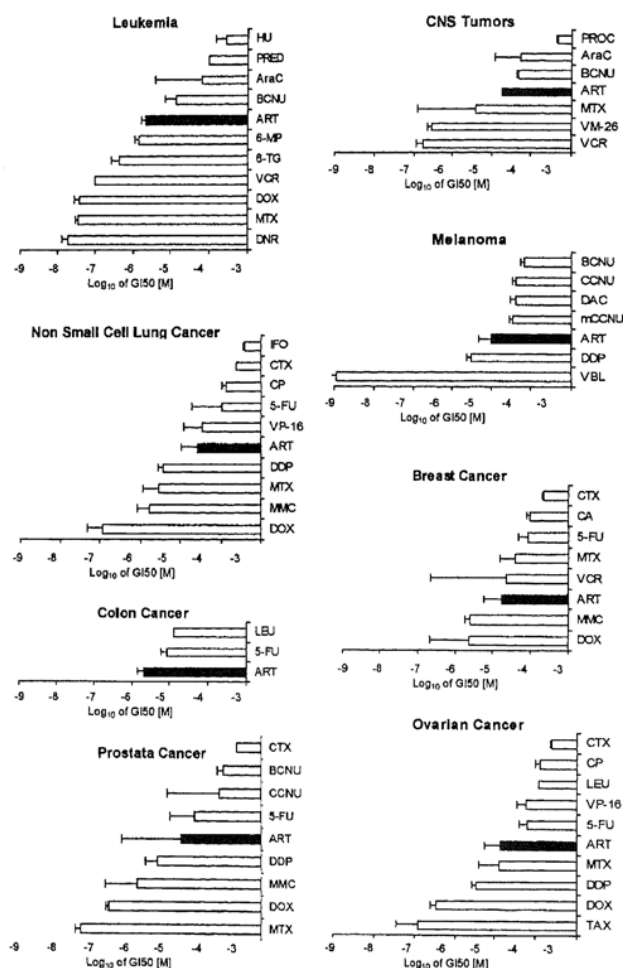


Figure 3. GI_{50} values of artesunate in comparison to GI_{50} values of common cytostatic drugs determined in 55 cell lines of the N.C.I. tumor panel. AraC, cytosine-arabinoside; ART, artesunate; BCNU, carmustine; CA, chlorambucil; CCNU, lomustine; CP, carboplatin; CTX, cyclophosphamide; DAC, dacarbazine; DDP, cisplatin; DNR, daunorubicin; DOX, doxorubicin; 5-FU, 5-fluorouracil; HU, hydroxyurea; IFO, ifosfamide; LEU, leucovorin; mCCNU, semustine; MMC, mitomycin C; Mitox, mitoxantrone; 6-MP, 6-mercaptopurine; MTX, methotrexate; PROC, procarbazine; PRED, prednisone; TAX, paclitaxel; 6-TG, 6-thioguanine; VBL, vinblastine; VCR, vincristine; VP-16, etoposide; VM-26, teniposide.

A further investigation into the mechanism of the observed cytostatic effects of artesunate correlated the expression of a number of genes with its activity; the genes were clustered into groups relevant to cancer, such as drug resistance, DNA repair, apoptosis regulating and tumour suppressor genes. The inhibitory activity of artesunate was demonstrated to correlate with the expression of most of these genes, except the drug resistance genes, indicating that its activity was not specifically directed towards any one specific group of genes, in contrast to many of the comparator cytostatics. Concomitant with these analyses, the activity of artesunate was shown not to be influenced by the drug-resistance status of the cell line investigated (Efferth et al., 2003). Recently, the induction of apoptosis has been suggested as mechanism of the cytotoxicity specifically directed to cancer cells. In an *in vitro* study, arteminol was able to induce apoptosis in Molt-4 cells at a concentration of 12 μM in a time-dependent manner (Singh and Lai, 2004). The concentration used does, however, far exceed therapeutically attainable levels, and it is thus doubtful, whether this effect is indeed of biological importance.

Two trioxane dimers have been prepared by chemical synthesis from artesunate, one an isonicotinate N-oxide and the other an isobutyrate dimer. When compared to artesunate in the use against *P. falciparum*, they were shown to have about equivalent activity *in vitro* (EC_{50} of 0.53 and 2.4 nM, respectively, as compared to 1.5 nM for artesunate) and higher activity *in vivo* with ED_{50} values of 0.8 and 1.5 mg/kg iv (artesunate: 5.5 mg/kg), and 2.0 and 4.5 mg/kg po (artesunate: 8.0 mg/kg), respectively. Four prostate cancer cell lines were subsequently tested with respect to their sensitivity towards these two compounds. They were both shown to exhibit a clear growth inhibitory action, with IC_{50} values between 36.1 and 84.6 nM of the isonicotinate N-oxide dimer, and IC_{50} values between 116.0 and 231.4 nM of the somewhat less active isobutyrate dimer (Posner et al., 2004). Both of these dimer derivatives were less acutely toxic than artesunate in a limited test in mice.

An antiviral activity of artesunate has also been described, where the replication of human cytomegalovirus, propagated in various cell lines, could be inhibited. The sensitivity of both, ganciclovir-sensitive and -resistant virus variants, was in the same range, as demonstrated by mean IC_{50} values of 5.8 and 6.9 μM , respectively. The antiviral activity of artesunate extended also to the human immunodeficiency virus (HIV) and the herpes simplex virus, although these exhibited lower sensitivities. The mechanism of antiviral activity involved cellular activation pathways, with marked reductions in protein levels and DNA binding activities of the virus-induced transcription factors Sp1 and NF- κB ; furthermore, the activation of phosphoinositide 3-kinase was also inhibited through the reduced phosphorylation of two downstream effectors, Akt and P70S6K, at an artesunate concentration of 5 μM . In consequence of these inhibitory actions of artesunate, the production of viral proteins was significantly reduced and viral replication inhibited (Efferth et al., 2002).

Finally, angiogenesis is also an important area in which the activity of artemisinins has been extensively investigated. These studies showed that artemisinin, artesunate and arteminol caused downregulation of the expression of vascular endothelial growth factor (VEGF) and associated factors and consequently inhibited angiogenesis. Vascularization of embryoid bodies, generated from mouse embryonic stem cells, was significantly inhibited by artemisinin at concentrations of 0.5 μM and higher (see Figure 4). Since the effect could be counteracted by radical scavengers such as mannitol or vitamin E, it was suggested that again the generation of reactive oxygen species through activating reactions at the endoperoxide bridge could be the underlying mechanism (Wartenberg et al., 2003).

Human microvascular dermal endothelial cells seemed to be less responsive to the effects of artesunate, since inhibition of growth as well as of differentiation was obtained only at concentrations above 12.5 μM . The expression of the two VEGF receptors, Flt-1 and KDR/Flk-1, was shown to be inhibited by 27%, however, already at a concentration of 0.5 μM . Additionally, the neovascularization of chicken chorioallantoic membrane was inhibited significantly by local treatment with a concentration as low as 10 nM/100 μl /egg (Chen et al., 2004a). These results were also supported by findings in human umbilical vein epithelial cells (HUVEC), in which arteminol caused a significant decrease in the binding of VEGF at concentrations of 0.1 μM and above, and a concomitant reduction in the expression levels of the two VEGF receptors (already at a concentration of 0.05 μM). Arteminol did also reduce the extent of neovascularization in chicken chorioallantoic membrane similar to the effect observed with artesunate, and finally, a concentration- and time-related increase in HUVEC apoptosis was observed (Chen et al., 2004b).

Figure 4 Inhibition of angiogenesis by measurement of the endothelial cell- specific marker platelet endothelial cell adhesion molecule-1 (PECAM-1) in mouse embryoid bodies (from Wartenberg et al., 2003)

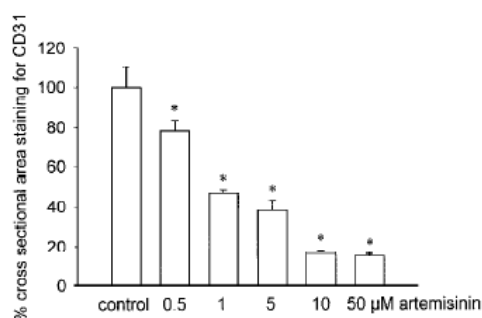


Figure 1.

Dose-response curve of the antiangiogenic effect of artemisinin. Embryoid bodies were treated from Day 3 through Day 8 with artemisinin in concentrations of 0.5, 1, 5, 10, and 50 µM artemisinin. Cell areas positive for platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31) were evaluated in optical sections of 100-µm thickness by confocal laser scanning microscopy. Note that artemisinin exerted a dose-dependent antiangiogenic effect.

In an *in vivo* system, involving human tumour cells xenografted into nude mice and the investigation of their growth and vascularization, subcutaneously administered artesunate was found to significantly increase the number of animals with VEGF-negative tumours (4/10 at 50 mg/kg, and 8/10 at 100 mg/kg). In addition, microvessel density decreased in tumours of animals treated with 50 and 100 mg/kg (Chen et al., 2004c). In another *in vivo* experiment, nude mice were implanted subcutaneously with a Matrigel pellet containing VEGF, TNFα and heparin as angiogenic stimuli. The extent of blood vessels growing into the pellet was estimated after 4 days by measuring the haemoglobin content of the pellet. Artesunate, whether added directly to the pellet at a concentration of 7.2 µM or given orally in the drinking water at a dose of 100 mg/kg/day, nearly completely suppressed the vascularization of these pellets (Dell'Eva et al., 2004).

The various activities of artemisinins, different from their antimalarial activities, can be regarded as scientifically interesting and in part potentially useful, in view of the relative non-toxicity of these derivatives. However, there are differences in the importance and relevance of these findings. The immunomodulating properties that have been described are difficult to judge with respect to their human relevance, as has already been mentioned. In addition, the doses needed to elicit such effects may be regarded as rather high and not relevant to human exposures. Also the cytostatic effects of artesunate, which were observed *in vitro*, may not be relevant to the clinical situation, as it is well known that even for conventional cytostatics the correlation between *in vitro* activity and *in vivo* efficacy is far from simple. It has to be mentioned, however, that in a recently published case study the application of artesunate in addition to conventional chemotherapy was described to have resulted in a certain tumour regression and prolongation of the patient's life span (Berger et al., 2005).

Additionally, since the investigations with artesunate for its anti-cancer as well as for its anti-viral activities have been conducted *in vitro*, i.e. without any notable conversion to arteminol, it is not known, whether, and how, the *in vivo* transformation to arteminol would influence these activities. Finally, kinetic considerations may also cast some doubt on the relevance of these data for the *in vivo* situation. *In vitro* concentrations needed to elicit such effects have invariably been in the (low) micromolar range. Although looking promising, these concentrations may prove to be too high for eliciting any relevant *in vivo* activity, since exposure to artemisinins has been shown to be below 1 µg/ml even after repeated application in a dosing schedule that resulted in marked accumulation of the applied drug. Arteether, e.g., after intramuscular application for 7 consecutive days in a dose of 25 mg/kg, resulted in mean plasma levels of around 300 ng/ml (or about 1 µM), and only peak concentrations reached levels necessary for, e.g., inhibition of viral replication or cytostatic activity (see section 3.1, Figure 5). Also in humans, pharmacokinetic data show that the level of 1 µM is not easily reached in the plasma upon administration of artemisinins. An oral dose of 2.3 mg/kg artesunate given to healthy volunteers yielded a mean artesunate C_{max} of 290 nM (range 242 - 607 nM),

concomitant with an arteminol $C_{\max}$ of 1.9 μM (range 1.46 - 2.58 μM); thus, the IC_{50} for an antiviral or cytotoxic effect would most certainly not be reached in humans with conventionally used doses (Batty et al., 2002). Furthermore, the mean plasma levels would be substantially lower than the $C_{\max}$ levels for most of the time because of the short half-lives of artesunate and arteminol in the organism, which further decreases the potential of artemisinins for inducing relevant activities.

It has to be remarked, however, that the most recently investigated influence of artemisinins on angiogenesis may have a bearing on the, also recently discovered, teratogenic effects of these substances (see section 4.5, Reproductive Toxicity). In addition to the suggested effects on the formation of erythroid precursor cells in the embryo, an inhibitory influence on vascularization of the embryo could also play a role in the induction of these effects. Since there are no toxicokinetic data available on the exposure of embryos in the early stages of pregnancy, it is difficult to judge whether this property could really have an impact on the development of the embryo and contribute to the observed effects, although this possibility might not be dismissed completely.

2.5 Safety Pharmacology

Safety pharmacology investigations on artesunate and on arteether have been reported as “general” or “systemic” pharmacology in two publications. Furthermore, information on certain safety pharmacology endpoints can additionally be extracted from other publications.

An extensive study of the effects of artesunate on the central and peripheral nervous system, the cardiovascular, respiratory and gastrointestinal systems of various animal species (mice, rabbits, guinea pigs, dogs, monkeys) was conducted with artesunate. The compound was administered as intravenous, intramuscular or intraperitoneal injections at doses of up to 640 mg/kg. In general, no relevant effects were noted in these species at doses of up to 100 mg/kg. Mice exhibited some effects on the CNS at doses of 200 mg/kg and above (sedation, increased barbiturate sleeping time, analgesia), and lowered body temperature was observed in rats at a dose of 450 mg/kg, and in rabbits and dogs at doses of >160 mg/kg. One anesthetized dog was used to estimate the influence on artesunate on the cardiovascular system and exhibited decreased heart rate and blood pressure, and changes in the ECG, at a dose of 320 mg/kg; respiratory depression occurred in the one anesthetized dog used for this part at a dose of 640 mg/kg. In rabbits, respiratory depression was observed at doses of 320 mg/kg and above, without any changes in the blood gases and blood pH. Finally, an *in vitro* experiment demonstrated an antagonizing effect on barium-induced contractions of intestinal smooth muscle (concentration not specified), but was without influence on acetylcholine-induced contractions (Zhao, 1985).

The general pharmacological effects of arteether were studied in mice, rats and cats and comprised investigations on the central nervous, cardiovascular and renal systems. The substance was applied in an oil formulation mainly by intramuscular injection. There were no CNS (behavioural, analgesic, or anticonvulsant) effects in mice at doses of up to 200 mg/kg, and no antiinflammatory activity was detected in rats treated with 50 mg/kg. In anesthetized cats, intramuscular injection of doses of 2.5 - 10 mg/kg did not result in any cardiovascular effects, since the increase in blood pressure and the decrease of heart rate was shown to be caused by the vehicle. On the other hand, a minor diuretic effect was observed in rats at a dose of 100 mg/kg (about one fifth of the response to furosemide). Finally, there was some inhibition of the passive cutaneous anaphylactic reaction in mice and rats elicited by egg albumin (Kar et al., 1989). The diuretic effect observed in this latter study was confirmed in a further study with intravenous application of artesunate in rats *in vivo* and in an *in vitro* model involving the isolated thick ascending limb of the loop of Henle. Inulin clearance was decreased, despite an increase in renal blood flow, indicating a decreased glomerular filtration rate; urinary excretion of Na, K and Cl was increased, probably mediated by local induction of nitric oxide production and inhibition of chloride transport (Campos et al., 2001).

Artemisinin has been investigated *in vitro* for its potential to interact with potassium ion currents in Guinea pig ventricular myocytes. At concentrations of 5, 50 and 100 μM , artemisinin inhibited concentration-dependently the potassium current, and no selectivity could be observed for the inhibition of I_{Kr} and I_{Ks} , respectively.

Although inhibition of I_{Kr} is related to the prolongation of the QT-interval in the ECG and is thus considered to be an indication of a certain potential for proarrhythmic effects, the concentration range for, as well as the

magnitude of, the blocking effect do not seem to indicate any serious problems for the clinical application (Yang et al., 1998). Analogous results have been obtained by measuring the influence of artemisinin on action potentials elicited in isolated C-type nodose ganglion neurons by a patch-clamp technique. Artemisinin was used at concentrations of 10, 30 and 100 μM and was shown to inhibit depolarizing as well as repolarizing action potentials due to the blockade of Na^+ and K^+ ion channels (Qiao et al., 2003).

Within a toxicity study on the effects of multiple intramuscular injections of artemimol, artemether and arteether in rats, gastric motility was also investigated. Gastric transit of amaranth dye given p.o. 40 minutes before sacrifice on the last day of treatment, i.e., after the 7th dose, was severely inhibited to about 50% of the control value at an artemether dose of 25 mg/kg; arteether at the same dose inhibited transit to >80%, and doses of 50 and 100 mg/kg of these two compounds completely blocked gastric transit, as did all three doses of artemimol. Retention ratios of the stomach contents were increased concomitantly (Li et al., 1998b).

Where effectively mentioned in the respective submissions, and additionally supported by the manufacturer's own studies, the safety pharmacology data provided by the manufacturers corroborate these findings.

In summary, artemisinin, artemimol, artesunate, artemether and arteether have been shown to exhibit some effects on the central nervous, the cardiovascular and the renal systems, but the effects have been relatively minor and occurred at high doses only; they can therefore be considered as negligible for the human therapeutic situation. The observed safety pharmacology hazards of artemisinins can therefore be judged not to be relevant to humans, and no special risks for the function of major organ systems of patients, or concerns for serious side effects, through the treatment with these compounds can be derived from these data. It has to be considered, however, that artemisinins, under specific circumstances, exhibit neurotoxicological effects in nonclinical animal models, and that therefore the single-dose safety pharmacology experiments may not reflect the full potential for serious adverse effects in humans. These neurotoxicological effects and the potential consequences resulting from the data will be discussed in more detail below (see section 4.6, Neurotoxicity).

2.6 Resistance

Resistance of *P. falciparum* against artemisinin derivatives has not yet been described, even in areas where artemisinin-based drugs have been extensively used (White, 2004; Chaijaroenkul et al., 2005). The observation that a specific mutation in the gene for PfATP6 confers a decreased sensitivity to the parasite towards artemether (Jambou et al., 2005) may also not be interpreted to indicate the development of "resistance to treatment", since the reported maximal IC_{50} values of up to 117 and 45 nM, respectively, from those isolates of *P. falciparum* that exhibit the PfATP6 mutation S769N still lie within the range of achievable maximal human plasma concentrations, and together with the large accumulation of artemisinin derivatives into infected erythrocytes (see 3.3) these reduced sensitivities would not be expected to lead to treatment failure. Furthermore, a study on the relationship between drug response in *P. falciparum* isolates and polymorphisms in drug transporter genes showed such an association only for the ABC transporter G7 with the activity of artesunate, and thus drug transporters might not be a target for development of resistance, although these data certainly need confirmation and extension (Anderson et al., 2005).

It may be speculated that the mode of action, involving the generation of reactive molecular species with the potential to harm specific steps or parts in the biology and biochemistry of the parasite (see section 2.3) by an indiscriminately damaging action such as protein alkylation, should hypothetically be less prone to evasive responses by the parasite. However, the use of artemisinins in combination with other malaria drugs of different modes of action will tend to further reduce the probability for the emergence of resistance against these compounds.

2.7 Pharmacodynamics Assessment

The different artemisinin derivatives have been investigated in nonclinical models *in vitro* as well as *in vivo* and have been proven to show excellent activity against erythrocytic stages of malaria parasites. The possible gametocytocidal activity could additionally tend to reduce transmission between human and insect hosts, leading to a further decrease in disease burden. All artemisinin derivatives have activity of their own

as demonstrated *in vitro*, and their metabolic transformation to the first and major metabolite arteminol (dihydroartemisinin, DHA), which exhibits even better activity than most of the parent compounds, tends to increase the schizontocidal activity *in vivo* even further. Artemisinins exhibit a fast onset of activity, but the short half-life of the active compounds in plasma leads to an increased potential for recrudescence, if artemisinins are used in monotherapy and for a short course of treatment only. Therefore, WHO has recommended that artemisinins should be used in combination with antimalarials of a longer duration of activity, preferably also with a different mode of action. Resistance of *P. falciparum* towards artemisinins has not yet been described to occur to a major extent.

The mechanism of action has not been fully elucidated. Earlier investigations suggested an inhibition of the detoxification reactions in the food vacuole of the parasite, especially interference with haemozoin formation, by artemisinins leading to increased concentrations of haemin and consequently increased oxidative stress.

More recently, the *P. falciparum* Ca^{2+} -dependent ATPase (PfATP6) has been identified as a potential, specific target for the schizontocidal activity, and proteomic analyses have also provided evidence for an involvement of oxidative stress in the antimalarial effects of artemisinins.

Although some functional effects on major organ systems have been described in safety pharmacology studies, the doses needed to elicit these effects are much higher than therapeutic doses, and most of the effects themselves can be considered to be minor and not representing potential risks to patients for inducing serious side effects. The exception could be the prolongation of the QT interval that has rather consistently been observed during toxicity studies; since the dose levels where this effect could be observed provided exposures in some excess of the probable human ones, the potential for serious cardiovascular side effects in patients may not be high. Although the possibility should not be disregarded, it has to be kept in mind that some QT prolongation might also be disease-related (Riamet SmPC, 2005).

Artemisinins have been shown in nonclinical investigations to possess additional pharmacodynamic properties that become evident at concentrations or doses, higher than those needed to treat plasmodial infections. These properties may therefore be of limited importance, although some of them might be considered as potentially interesting possibilities.

3. PHARMACOKINETICS

Pharmacokinetic properties of arteminol, artesunate, artemether and arteether have been investigated in rats, and those of arteether also in dogs. The very early investigations were conducted with analytical methods that were of less than sufficient sensitivity, and were not designed to provide full information on metabolites. More recently, kinetic data have been obtained with better sensitivity and specificity, and also toxicokinetic data are now available in some instances.

The pertinent plasma kinetic parameters of various artemisinin derivatives are summarized in Table 2, while the compilation of human data by Kyle et al. (1998) is presented for comparative purposes in Table 3.

The distribution, metabolism and excretion of some of these compounds has been studied in rats, and some *in vitro* data are additionally available. The respective information, reported in a small number of publications, does not provide, however, a full picture of the relevant properties of artemisinin derivatives.

3.1 Plasma pharmacokinetics

Arteether has been applied by intramuscular injection to Beagle dogs at a dose of 25 mg/kg in an oily solution, and the pharmacokinetic profile in plasma has been determined by HPLC with electrochemical detection. The rapid absorption with a T_{max} of 45 minutes was followed by a distribution phase with a half life of 0.84 hours, and a relatively protracted elimination phase with a half life of 27.95 hours. The metabolite arteminol was not measured in this study because of the analytical method used (Benakis et al., 1991).

In a large comparative study the kinetic parameters were determined for arteminol, artesunate, artemether, arteether and artelinate in the plasma of rats. the substances were administered intravenously, intramuscularly and orally at equal doses of 10 mg/kg. At the same time, the conversion of the primary compounds to their common metabolite, arteminol, was also estimated. Large differences in the conversion rates and the calculated “equivalent arteminol dose” were observed between the different primary compounds. For artemether, the conversion rates on intravenous, intramuscular and oral application, respectively, were calculated to be 3.7, 9.0 and 12.4 %, respectively, with “equivalent arteminol doses” of 0.20, 0.26 and 0.61 mg/kg. For arteether the analogous figures were 3.4, 14.2 and 15.9 %, and 0.09, 0.16 and 0.81 mg/kg. Artesunate provided the highest conversion rates and the respective “equivalent doses”, with values of 38.2, 25.3 and 72.7 %, and 1.49, 0.87 and 9.67 mg/kg. The differences can be explained by the different chemical and physicochemical properties of the various compounds: Artesunate, on the one hand, is a rather simple ester of arteminol and undergoes first-pass metabolism already in the plasma by the action of esterases, whereas the other compounds, on the other hand, have to undergo metabolism by hepatic cytochrome enzymes; additionally, their higher lipophilicity is probably limiting the distribution into the tissues (Li et al., 1998c).

Table 2 Summary of pharmacokinetic parameters of various artemisinin derivatives in plasma of laboratory animals and humans

Compound (analyte)	Species	Dose (mg/kg)	C _{max} (ng/ml)	T _{max} (hrs)	AUC (ng·h/ml)	T _{1/2α} (hrs)	T _{1/2β} (hrs)	V _{Dss} (L)	Cl (ml/min/kg)	F	Reference	
Arteether	dog	25, im	110	0.75	2797	0.84	27.95	0.377	1.69	*/**	nd	Benakis et al., 1991
Arteether	rat	10, iv	3724	na	842	0.1	0.45	0.72	200			Li et al., 1998c
		10, im	160.7	0.68	285.7	0.34	3.62	33.8	199.5			idem
		10, po	324	0.29	298	0.29	1.79	13.8	268.0		35.4	idem
Artenimol	rat	10, iv	6683	na	3184	0.22	0.95	0.50	55.4			Li et al., 1998c
		10, im	1579	0.29	2719	0.73	4.44	4.42	56.2			idem
		10, po	769	0.25	615	0.24	4.94	21.5	63.6		19.3	idem
Artemether	rat	10, iv	6231	na	1857	0.1	0.53	0.67	91.8			Li et al., 1998c
		10, im	692	0.48	1007	0.23	1.78	6.01	91.0			idem
		10, po	381	0.48	366	0.19	2.04	16.1	90.6		19.7	idem
Artesunate	rat	10, iv	2106	na	738	0.15	0.35	0.87	190.9			Li et al., 1998c
		10, im	1650	0.25	773	0.15	0.54	1.77	215.9			idem
		10, po	208	0.5	217	0.26	1.34	14.4	240.1		29.5	idem
Arteether	rat	25, iv	14883	na	5587	0.20	0.67	0.93	75.1			Li et al., 1999
		25, im	119.9	1.14	1302	0.45	16.20	128.2	75.2		23.4	idem
		25, multiple doses, im, day 7	410.0	1.25	11366	0.64	31.24	41.8	157.6			idem
Artesunate	rat	150, po	713.1	0.083	316.1	nd	nd	nd	nd			Olliario et al., 2001b
Artenimol	rat	Artesunate, 150, po	2040.3	0.625	1241.7	nd	nd	nd	nd			idem

Compound	Species	Dose (mg/kg)	C _{max} (ng/ml)	T _{max} (hrs)	AUC (ng·h/ml)	T _{1/2α} (hrs)	T _{1/2β} (hrs)	V _{DSS} (L)	Cl (ml/min/kg)	F	Reference
α-Arteether	rat	2, iv	nd	na	131.7		0.55	3.6	4.5		Sabarinath et al., 2005
		9, po	109.7	0.083	73.8		2.4	122.2	36.0		idem
		17.5, po	99.4	0.083	50.9		1.7	244.3	102.5		idem
		30, po	137.4	0.083	111.5		1.5	176.5	79.9		idem
		9, im	70.4	0.5	1058.5		22.0	76.8	2.4		idem
		17.5, im	183.6	0.5	1247.1		21.7	129.3	4.1		idem
		30, im	109.6	0.33	601.1		11.9	253.3	14.8		idem
β-Arteether	rat	2, iv	nd	na	184.8		0.85	9.3	7.6		Sabarinath et al., 2005
		9, po	10.3	0.083	46.8		4.2	770.8	127.5		idem
		17.5, po	18.9	0.083	33.9		4.5	1965.9	303.8		idem
		30, po	8.1	0.083	25.6		2.8	3024.2	742.7		idem
		9, im	60.6	0.33	1183.3		28.5	213.2	5.2		idem
		17.5, im	136.5	0.5	2414.4		27.5	198.7	5.0		idem
		30, im	27.8	0.33	407.1		25.2	1660.6	45.7		idem
Artenimol	rat	arteether, 2, iv	152.4	0.083	44.7		0.25				Sabarinath et al., 2005
		arteether, 30, im	7.4	0.33	10.8		1.04				idem
		arteether, 9, po	4.7	0.083	6.6		0.3				idem
		arteether, 17.5, po	16.9	0.5	10.2		0.4				idem
		arteether, 30, po	40.5	0.33	50.9		0.53				idem
Artesunate	rat	36.7, iv, day 1	16492	na	1324	0.04	0.36	8.1	474.2	*	Li et al., 2005a
		36.7, iv, day 3	1377	na	490.6	0.12	0.46	76.87	2172	*	idem

Compound	Species	Dose (mg/kg)	C _{max} (ng/ml)	T _{max} (hrs)	AUC (ng·h/ml)	T _{1/2α} (hrs)	T _{1/2β} (hrs)	V _{Dss} (L)	Cl (ml/min/kg)	F	Reference
Artenimol	rat	artesunate, 36.7, iv, d1	10842	0.19	4893	0.20	0.36				idem
		artesunate, 36.7, iv, d3	6485	0.18	3327	0.21	0.65				idem
Artemisinin	rat	150, iv				0.4	0.50	4.1		*	China Coop. Res, Group, 1982
Artemether	rabbit	iv				0.11	0.66	3.0		*	idem
Artesunate	rat	200, iv				0.26		1.1		*	idem

nd: not determined

na: not applicable

T_{1/2α}: Distribution half-lifeT_{1/2β}: Elimination half-lifeV_{Dss}: Distribution volume at steady-state

F: relative bioavailability

* = Volume of distribution expressed as L/kg

** = Clearance expressed as ml/min

Table 3 Pharmacokinetics of artemisinin derivatives in humans (Tables I and II from Kyle et al., 19Figure 598)*Pharmacokinetics of artemisinin and its derivatives after oral dosing.*

Oral drug ⁽¹⁾	Dose (mg/kg)	n ⁽²⁾	C _{max} ⁽⁴⁾ (ng/ml)	Parent drug		Dihydroartemisinin				
				AUC ⁽⁵⁾ (hr/μg/l)	t _{max} ⁽⁶⁾ (hr)	t _{1/2} ⁽⁷⁾ (hr)	C _{max} ⁽⁴⁾ (ng/ml)	AUC ⁽⁵⁾ (hr/μg/l)	t _{max} ⁽⁶⁾ (hr)	t _{1/2} ⁽⁷⁾ (hr)
Artemisinin										
Healthy	10	12	391	2054	1.81	2.59				
	5.3	10	260	819	1.0	1.9				
	10	6	623	2611	2.66	2.51				
	6.3	6	360	1190	1.67	2.61				
	10	8	581	2228	2.5	2.42				
Disease	9.2	19	615	2234		2.2				
	9.2	19	588	2601	2.4	2.3				
	10	11	364		2.88	2.72				
Dihydroartemisinin										
Healthy	1.1	3	130		1.33	1.63				
	2.2	3	710		1.33	1.57				
Artesunate										
Healthy	3.6	6	570	740	0.5-1.0	0.65				
	2.6	6	119	100	0.71	0.27	340	502	1.5	0.87
	3.5	1	577	312	0.5	0.39	1141	1340	1	
Disease	1.64	6					553	1147	1.5	0.8
Artemether										
Healthy	3.6	6	118	1100	3	3.1	379	6600	6	10.6
	5.5	6	474	2170	2	2.8	724	7440	3.6	5.1
	3.5	6	310	671	1.88	2	273	753	1.92	
	5	8	121	491	1.7	2.6	301	1042	1.8	1.9
Disease	3.6	8	231	5800	3	4.2	593	4960	7.4	12.5

⁽¹⁾ Drug administered and types of patients : Healthy = healthy volunteersDisease = patients with acute, uncomplicated *falciparum* malaria.⁽²⁾ n = number of participants in study.⁽⁴⁾ C_{max} = peak concentration.⁽⁵⁾ AUC = area under the curve.⁽⁶⁾ t_{max} = plasmatic peak time.⁽⁷⁾ t_{1/2} = half-life time.

Table 3 (Cont'd)

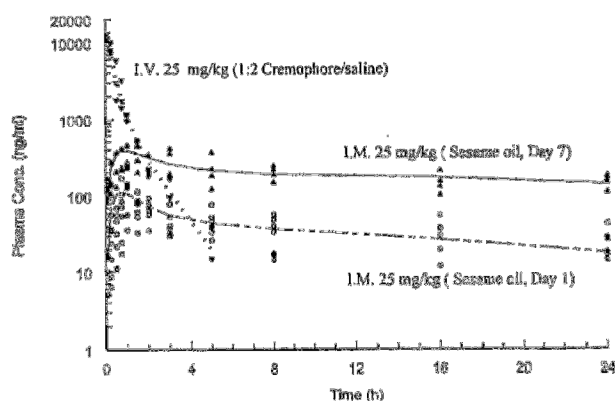
Pharmacokinetics of artemisinin derivatives after intramuscular or intrarectal dosing.

intramuscular drug	Dose (mg/kg)	n ⁽²⁾	C ^{max} (³⁾ (ng/ml)	Parent drug			Dihydroartemisinin			
				AUC ⁽⁵⁾ (hr/μg/l)	t ^{max} (⁶⁾ (hr)	t ^{1/2} (⁷⁾ (hr)	C ^{max} (³⁾ (ng/ml)	AUC ⁽⁵⁾ (hr/μg/l)	t ^{max} (⁶⁾ (hr)	t ^{1/2} (⁷⁾ (hr)
Artemisinin										
Healthy	5.3	10	209	2419	3.4	7.44				
Artesunate										
Severe Disease	2	6		510		0.49		390	0.19	
Artemether										
Healthy	6	6	145	5.2	7.7					
	10	6	224	6.3	11.1					
	5.5	8	540	5200	3.9	2.0	646	8440	4.9	5.4
	5	8	175	2648	8.7		42	607	7.0	
Severe Disease	3.2	16	73	608	2.12					
		10	40.5	245	1.33					
Intrarectal drug										
Artemisinin										
Healthy	15	5	40		4.6	4.8				
	10	9	170		11.3	4.1				
	5.3	10	< 55 ⁽¹⁰⁾							
Disease	10	6	180		9.6	4.0				
Dihydroartemisinin										
Healthy	8	5	100		4.7	4.82				
Artesunate										
Disease	15.6	10	301	871	1.83	1.86	916	6145	1.0	0.67
Artemether										
Healthy	5	8	75	625	3.1		26	248	3.5	

⁽¹⁾ Drug administered and types of patients : Healthy = healthy volunteersDisease = patients with acute, uncomplicated *falciparum* malaria.⁽²⁾ n = number of participants in study.⁽³⁾ C^{max} = peak concentration.⁽⁵⁾ AUC = area under the curve.⁽⁶⁾ t^{max} = plasmatic peak time.⁽⁷⁾ t^{1/2} = half-life time.

In order to provide a kinetic basis to the understanding of the anorectic and neurologic toxicities observed in studies with repeated intramuscular administration of arteether, a pharmacokinetic and toxicokinetic study was performed in rats. Arteether was applied intramuscularly in an oil solution at a dose of 25 mg/kg either in a single dose, or in repeated daily doses for 7 days. Pharmacokinetic parameters were calculated with the plasma values obtained after the first application as well as after the 7th injection (see Table 2). Accumulation of arteether was observed with repeated application: Trough values can be estimated from the respective plasma profiles (see Figure 5) to be around 25 ng/ml after the first dose, while after the 7th dose the trough level is about 10-fold higher, at around 250 ng/ml. This protracted elimination and consequent accumulation was demonstrated to be due to the relatively slow absorption from the injection site in the muscle. It was shown that 24 hours after a single i.m. application, 38 % of the total dose remained in the injection site, and that this amount was reduced to 22 % at 48 hours. Upon multiple i.m. injections, the amount remaining in the injection sites 24 hours after the last dose corresponded to 91.4 % of a single dose. The relative bioavailability of a single i.m. injection was calculated to be 23.4 % (LI et al., 1999).

Figure 5 Plasma profile of arteether in rats after single intravenous, and single or repeated intramuscular application of 25 mg/kg (from Li et al., 1999)



Plasma concentration-time profiles following single i.v. injection in 1:2 cremophore/saline (open triangle and dotted line); single i.m. injection in sesame oil (open circles and solid line) and day 7 after last multiple i.m. dose in sesame oil (closed triangle and dashed line).

Artesunate was given to rats in a single oral dose of 150 mg/kg, and the plasma pharmacokinetic parameters were determined for the parent compound as well as for the primary metabolite, arteminol. Artesunate was shown to be very rapidly absorbed, with a T_{max} of only 5 minutes, but was also rapidly converted into arteminol. The stability of artesunate has been shown to be dependent on the pH of the solution; consequently, in the acid environment of the stomach (pH of 1.2), artesunate is rather short-lived with a $T_{1/2}$ of 10.3 minutes, while at neutral pH, its stability is much higher ($T_{1/2}$ of 7.3 hours). This indicates that in the early part of the plasma profile, arteminol levels are mainly due to absorption of preformed arteminol from the stomach (Olliaro et al., 2001b).

The pharmacokinetics of the two diastereomers of arteether were studied in rats by intravenous, intramuscular and peroral application of a preparation with a fixed ratio of 30:70 (% w/w) of the α and β isomers (Sabarinath et al., 2005). The intravenous dose was 2 mg/kg, while for the i.m. and p.o. applications doses of 9, 17.5 and 30 mg/kg were administered. It was found that the α -isomer was absorbed as rapidly as the β -isomer, but that β -arteether exhibited a longer half-life and a higher volume of distribution. The plasma levels obtained with i.m. and p.o. administration yielded two concentration maxima, one occurring very early after the application (5 minutes after p.o., and 20 - 30 minutes after i.m.) and a second one at 20 - 45 minutes after p.o. and 60 - 120 minutes after i.m. dosing. It is relatively difficult to draw conclusions from this study, since neither C_{max} nor AUC values did show any linear dose dependency. However, certain features of exposure to arteether upon different administration modes are apparent. The rate and extent of conversion of the arteether isomers to arteminol was highest with oral and intravenous application, and plasma levels of arteminol could be measured after intramuscular application only at the highest dose.

In rats infected with *P. berghei* the toxicokinetics of artesunate were compared to the respective parameters obtained from treatment with artelinate. Animals were treated once per day for three days with an intravenous dose of 36.7 mg/kg, and plasma concentrations of artesunate as well as of the metabolite arteminol were determined by LC-MS-MS or HPLC-ECD. Autoinduction of metabolism was evident for artesunate, since the AUC after the third dose was reduced to about one third of the day 1 value; C_{max} was also reduced, while clearance was significantly increased. The ratio of metabolite to parent compound over the three days of treatment was determined to be 5.26 (Li et al., 2005a).

3.2 Absorption

As can be seen from the pharmacokinetic parameters, absorption is fast for all artemisinins and for all application modes. There are, however, only early Chinese nonclinical studies dealing explicitly with the extent of absorption. In mice and rats, tritiated artemisinin (qinghaosu) was rapidly absorbed after oral application, with a $T_{\max}$ of 0.5 - 1 hour (China Cooperative Research Group, 1982a). Furthermore, the amount remaining in the muscle after intramuscular administration of arteether in an oily preparation has been determined after single and multiple doses (Li et al., 1999).

One issue in the area of absorption has been investigated, however. In humans, experience has shown that relative oral bioavailability of artemisinins decreases after multiple doses. A study with an *in situ* intestinal perfusion model in rats was therefore conducted in order to investigate the possibility that this decreased bioavailability might be the result of an increased efflux mediated by P-glycoprotein. In this model, pretreatment of the animals for 5 days with an oral artemisinin dose of 60 mg/kg did not influence the jejunal permeability of artemisinin, and artemisinin was shown to be not a substrate for P-glycoprotein. The observed decrease in relative oral bioavailability of artemisinin in humans should therefore be related rather to an induction of metabolism than to any effect on absorption (Svensson et al., 1999).

3.3 Distribution

In the early Chinese studies organ distribution of artemisinin has been estimated by radioactivity determination or by thin-layer chromatography. The resulting pattern 1 hour after oral administration of 900 mg/kg artemisinin to rats showed highest levels in liver, followed by brain, plasma, and lung, with kidneys, muscle, heart and spleen at somewhat lower levels of exposure. The tissue content of radioactivity in mice after intramuscular administration of ^3H -artemether decreased considerably within 24 hours; again, liver was the most exposed organ, but brain levels were very low in this experiment (see figure 6). In pregnant mice treated with ^3H -artemisinin radioactivity could also be detected by autoradiography in the fetuses (China Cooperative Research Group, 1982a).

In another early study, tissue distribution of artesunate has been investigated in rats after an intravenous injection of 50 mg/kg. Rats were killed either 10 or 60 minutes after drug administration, and tissue concentrations were measured by a radio-immunoassay. Shortly after drug administration, the levels in the intestine were markedly higher than in any other organs; brain levels were second, followed by the concentrations in kidney and liver. One hour after the injection, the levels were markedly reduced and were more similar between organs; artesunate concentrations in brain and fat seemed to be declining at a lower rate than in other organs (Zhao and Song, 1989). The analytical method used did not allow for the determination of arteminol, and the results of this study need therefore be regarded with a certain reservation.

Figure 6 Organ and tissue distribution of radioactivity after intramuscular administration of ^3H -artemether (from China Cooperative Research Group, 1982,a)

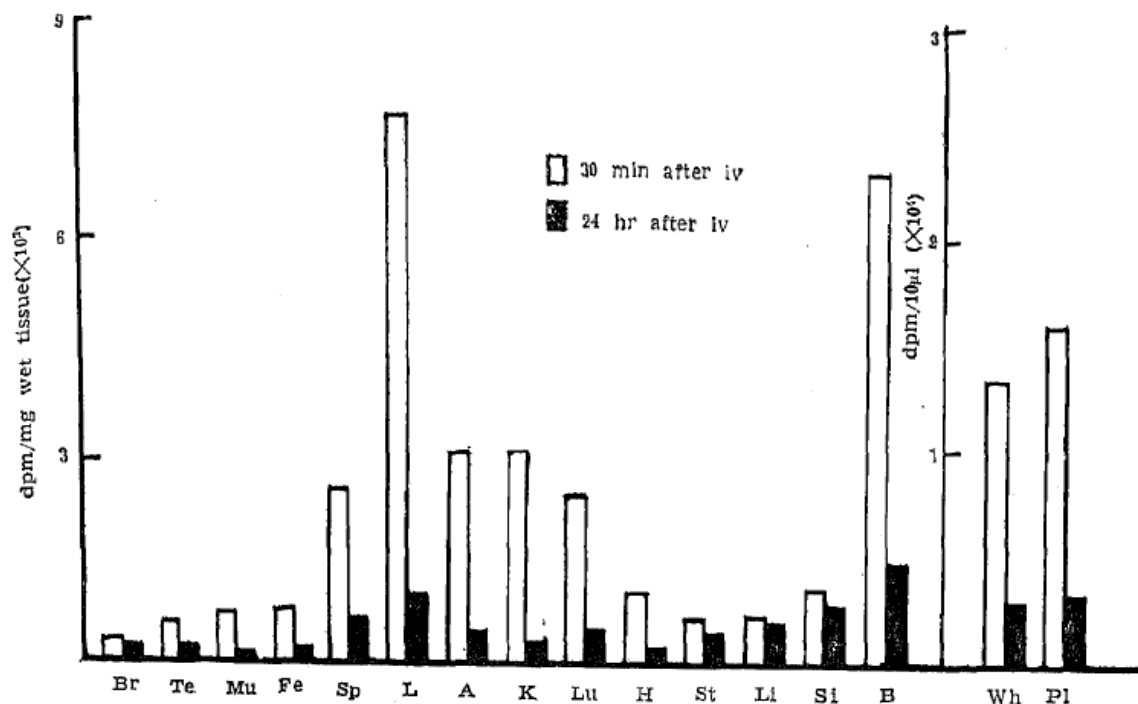


Fig. 5. Distribution of radioactivity in mice after administration of ^3H -artemether (mean of 6 animals)

Br: Brain; Te: Testis; Mu: muscle; Fe: Femur; Sp: Spleen; L: Liver;
 A: Adrenal; K: Kidney; Lu: Lung; H: Heart; St: Stomach; Li: Large intestine;
 Si: Small intestine; B: Bile; Wh: Whole blood; Pl: plasma

An important question related to the potential neurotoxicity of artemisinins is their propensity for penetration into the central nervous system. In the study cited above, the brain concentration at 10 minutes following dosing was found to be 46.3 µg/g, compared with a serum level of 17.2 µg/ml, indicating a rather large exposure of the brain to this substance. In a more recent clinical study on patients with severe malaria, artesunate and arteminol were determined in the cerebrospinal fluid (CSF) at different times after an intravenous dose of 120 mg artesunate. In this study, no artesunate was detected in the CSF, while arteminol was demonstrated to be present in CSF already at the earliest sampling time (15 minutes after dosing). CSF levels of arteminol tended to increase somewhat with time, indicating a lower elimination rate for the brain than for plasma and other organs. Since the mean concentration of about 100 nM in CSF is only about 10% of the concentration required *in vitro* to inhibit neurite outgrowth, a measure of neurotoxicity, it can be concluded that the levels determined for this kind of treatment would not be indicative of a potential for inducing neurotoxicity (Davis et al., 2003).

Protein binding was investigated for artemether. Mean binding to human serum proteins was estimated to be in the range of 97.5 - 98.6 % over a concentration range of 0.32 - 10 µg/ml. Under physiological conditions of protein concentrations, it was observed that artemether was distributed as follows: α1-acid glycoprotein 33 %, albumin 17 %, high density lipoproteins 12 %, low density lipoproteins 9.3 %, and very low density lipoproteins 12 % (Colussi et al., 1999). This high binding to serum proteins may be related to the lipophilic nature of artemether, since protein binding of arteminol has been reported to be only in the range of 47 - 76 % (Riamet SmPC, 2005).

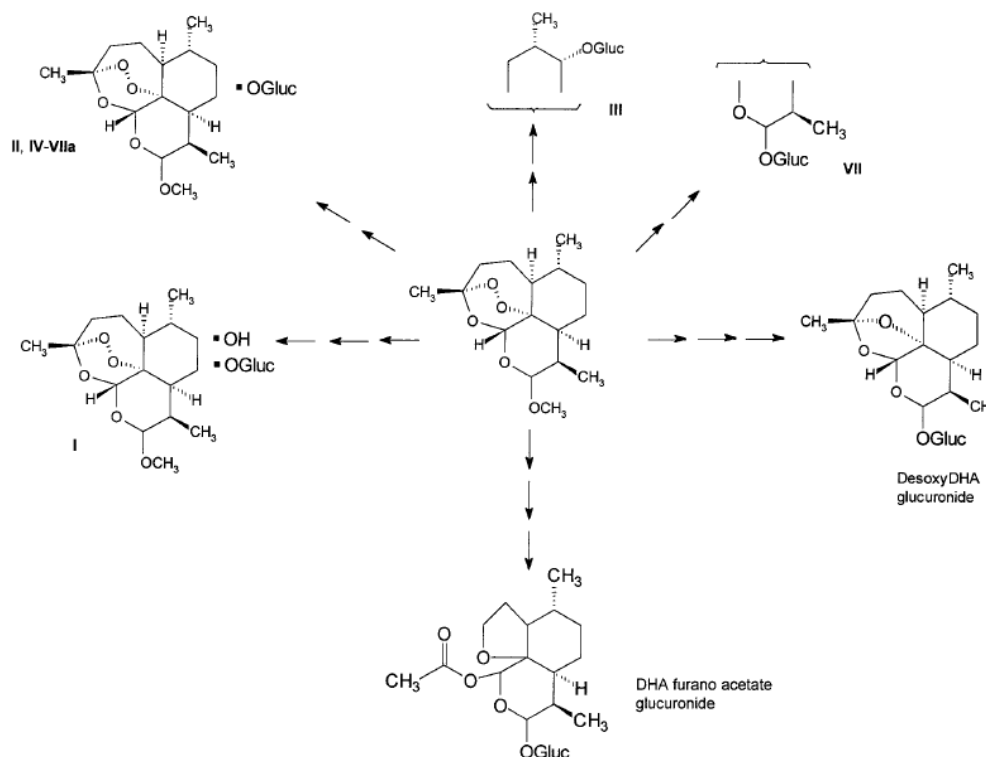
A prominent property of artemisinins is their propensity for selective distribution into infected erythrocytes. Erythrocytes infected with *P. falciparum* have been shown to take up artemisinin derivatives to concentrations greater than 100-fold of those which were found in uninfected erythrocytes under the same treatment conditions (Meshnick et al., 1996). This selective uptake has been demonstrated to involve a carrier-mediated mechanism, in contrast to the simple, passive diffusion of artemisinins into non-infected red blood cells (Vyas et al., 2002).

3.4 Metabolism

The first and most important step in the biotransformation of artemisinin and of all its derivatives consists in the formation of the dihydro-form of artemisinin, artemimol. Further metabolic breakdown has also been investigated and described in some publications.

An early in vitro experiment showed that artemisinin was metabolized by liver slices, but not, or only to a minor extent, by slices of other organs. Inducing mice with phenobarbital resulted in a large increase in the amount of exhaled $^{14}\text{CO}_2$ from intramuscularly applied [methyl- ^{14}C]-artemether, indicating an induction of a demethylation reaction. The respective metabolite was not identified at that time, but it is clear that the CO_2 formed in the course of the biotransformation of artemether to artemimol (China Cooperative Research Group, 1982a)

The hepatic metabolism and biliary excretion of the formed metabolites has been investigated in rats for artemimol and for β -artemether by means of LC-MS and LC-MS-MS analysis. Both compounds were given as intravenous injections at a dose of 35 $\mu\text{M}/\text{kg}$; they were also applied in radioactive form labelled with ^{14}C in position 13 of the molecule. After application of artemimol, 34.8 % of the radioactivity was recovered from the bile within the first hour after application, and 48.4 % were recovered between 0 and 5 hours postdose. The principal metabolite was found to be the glucuronide of artemimol, and minor metabolites were demonstrated to be products of reductive cleavage and rearrangement of the endoperoxide bridge (Maggs et al., 1997). With artemether, 38.6 % of the radiolabel were recovered from bile within the first 3 hours postdose, and 42.3 % were found within the first 5 hours. The major metabolites in bile for the time interval of 0 - 3 hours were artemimol (22.6 % of the dose), the glucuronide of 9 α -hydroxy-artemether (33.4 %) and glucuronides of various other hydroxylated metabolites. Figure 7 presents the proposed metabolic pathways for the conversion of artemether (Maggs et al., 2000). Glucuronidation products were also found to be present in the urine of malaria patients who had received a dose of 120 mg artesunate (Ilett et al., 2002).

Figure 7 Proposed biotransformation pathways of artemether in rat liver (from Maggs et al., 2000)

In section 3.1 it has been mentioned that the reduction of exposure (determined as $C_{\max}$ or AUC) observed after multiple dosing with artemisinin derivatives, as compared with the respective values after a single dose, suggests an autoinduction of metabolism. This issue has been investigated *in vitro* with liver microsomes originating either from “naïve” or from artemisinin-pretreated rats. The pretreatment at a dose of 60 mg/kg/day for five days resulted in significant changes in the kinetics of biotransformation: $V_{\max}$ increased by a factor of 2, while K_m decreased by a factor of 3, compared with control kinetics (Gupta et al., 2001). The responsible cytochrome P450 isoenzyme was subsequently identified as CYP 2B6 (Simonsson et al., 2003). Further *in vitro* studies with human and rat liver, and with recombinant enzyme preparations have elucidated the role of various cytochrome P450 isoenzymes in the metabolism of artemisinins. The disappearance rate for artemisinin was highest for CYP 2B6, while CYP 3A4 was less active, and CYP 2A6 judged to be only of minor importance (Svensson and Ashton, 1999). In this assay, only the disappearance of artemisinin was determined, and thus probably only the conversion of artemisinin to artemimol played a major role. The further metabolism of artemisinin has been investigated with human and rat liver microsomes *in vitro*. In this study, CYP 2A6 and CYP 2B6 were identified as the isoenzymes mainly responsible for the formation of four distinct metabolites. When the rats used for the preparation of the liver microsomes were pretreated with artemisinin, an induction of metabolic activity ranging from 2.5- to 7.2-fold was observed. CYP 3A4 did not seem to play a major role in these biotransformations (Svensson et al., 2003). It is stated in this paper that three of the four metabolites formed by rat liver microsomes were also found in human microsomal incubations; they have not been characterized, but it has to be assumed that the main metabolite should correspond to artemimol.

While for the metabolism of artemisinin, artemether and arteether the cytochrome P450 enzymes play the major role for the transformation into the primary metabolite artemimol, common esterases perform the hydrolytic cleavage of the ester bond in artesunate. This difference has most probably to be regarded as the cause for the more rapid transformation of artesunate into the active metabolite, artemimol, than can be observed with the other artemisinins.

3.5 Excretion

After oral administration of ^3H -artemisinin or ^3H -artenimol to mice, 80% of the radioactivity was excreted in the urine during 24 hours. After an intravenous injection of ^3H -artemether, 68% of radioactivity were recovered in urine and faeces during the first 24 hours, while 95% were excreted within 72 hours (China Cooperative Research Group, 1982a).

There are no further, recent specific studies investigating the excretion of artemisinins and their metabolites. The limited study with artesunate that has already been mentioned in section 3.3, and the liver metabolic studies in the rat are the only other ones from which some excretion data can be extracted. In the former publication, only very little artesunate was found to be present in urine, faeces and bile (0.3, 0.01, and 0.3 % of the applied dose, respectively); however, due to the analytical method used, no information on the excretion of artesunate metabolites can be obtained from these results (Zhao and Song, 1989). On the other hand, in the investigations described in section 3.4, biliary excretion in rats has been quantified for total radioactivity after dosing the animals with ^{14}C -artenimol (Maggs et al., 1997) and ^{14}C -artemether (Maggs et al., 2000), respectively.

3.6 Pharmacokinetics Assessment

The plasma pharmacokinetics of artemisinin derivatives have been relatively well characterized in recent years, when analytical methodology allowed the detection and quantitation of the various substances as well as of their primary metabolite, artemimol, in biological matrices, although this observation does not extend to an as great extent to other metabolites. A striking feature of artemisinin pharmacokinetics is the observation that humans have a much higher exposure to artesunate and artemimol than have animal species at equal doses. On the other hand, doses efficacious in killing the malaria parasite are more or less equal on a mg/kg basis between laboratory animals and human patients. Rodents, with their very active cytochrome metabolizing systems, do produce a large variety of metabolites of unknown or undetermined antimalarial activity, which are less abundant, or cannot be found, in human plasma; if they retain the intact endoperoxide bridge, they may be estimated to be still schizonticidally active and contribute to the therapeutic action in rodents. Under this assumption, a direct comparison between plasma levels needed for therapeutic efficacy should take into account not only parent compound and artemimol, but should encompass all molecular species with an intact endoperoxide bridge.

This apparent discrepancy between animal species and humans in terms of therapeutically efficacious doses as compared to the plasma concentrations obtained does also have important consequences on the safety assessment of artemisinins. In certain, specific instances, toxic effects have been observed to occur at doses (on a mg/kg basis) comparable to the human therapeutic doses, meaning that no “safety window” might be definable with respect to exposure to parent compound and/or the primary metabolite artemimol. This aspect will have to be taken up in the discussion of the various toxicology endpoints, and be considered in the final overall safety assessment.

Only limited information is available on tissue distribution, especially with respect to the exposure of the CNS, as well as to the exposure of embryonic and foetal tissues in pregnancy, and a comprehensive mass balance investigation of artemisinins in animals as well as in humans is also lacking. Both of these issues would need to be addressed for further refining the nonclinical safety assessment.

Two further issues may be considered as resolved and posing no special problems:

Pharmacokinetic interactions with CYP 3A4 inhibitors may be possible, but since the relevant parent compounds (artemisinin, artemether and arteether) have a pharmacodynamic activity of their own and are of equal or lesser toxicity than the primary metabolite, metabolic inhibition is considered not to be able to change to any appreciable extent the activity and safety profile of the relevant derivatives. Likewise, since the metabolite is also pharmacodynamically active, induction of the metabolism by CYP inducers should not influence activity and/or toxicity to any relevant extent.

Autoinduction of metabolism has indeed been observed, but the lowering of plasma concentrations of parent compound and primary metabolite (artemimol) in animal models did not seem to influence the therapeutic efficacy.

Finally, although pharmacokinetic interactions may theoretically be possible with other antimalarials that are also metabolized by CYP enzymes, combinations with other antimalarial drugs showed no clinically relevant tendencies for influencing the pharmacokinetics of either combination component (Giao and de Vries, 2001).

4. TOXICOLOGY

Published reports on results of the more conventional target organ toxicity tests are scarce, and this is also reflected in the information retrievable from databases, such as the Registry of Toxic Effects of Chemical Substances (RTECS, 2005), where the main reliance is on early Chinese data generated for the registration of artemisinin and its derivatives in China, and which have been subsequently published. Most publications in the area of toxicology, however, are dealing with the potential neurotoxicity of artemisinin derivatives. More recently, also reproductive toxicity, specifically the potential of artemisinins for eliciting teratogenic effects, has received enhanced attention, and has been discussed in more detail. This review therefore has to rely also in part on the confidential data contained in the various submissions for drawing some general conclusions about the potential for target organ toxicity-related, adverse effects.

4.1 Single dose toxicity

Artemisinin derivatives are of relatively low acute toxicity. A number of published investigations mention results obtained with single doses of various compounds and by different modes of administration. Although they mostly report exact values for the LD₅₀, the results may have to be regarded with some reservation due to either the incomplete description of the assay, the limited number of animals used or the lack of adherence to generally accepted protocols. Nevertheless, the range of doses in which to expect acutely toxic responses is certainly well above therapeutically active doses, and single dose toxicity should thus not play a relevant, major role in the risk-benefit assessment of the artemisinins. Indeed, in early investigations, therapeutic indices of several 100- to over 1000-fold have been found for artemisinin and its derivatives, irrespective of the way of administration (China Cooperative Research Group, 1982b).

The single dose toxicity of artemisinin (qinghaosu) yielded an LD₅₀ of 4228 mg/kg for oral, and of 3840 mg/kg for intramuscular application in mice; the respective figures for rats were determined as 5576 and 2571 mg/kg. One dog each was given 400 and 800 mg/kg as an i.m. injection; both dogs survived the 10-day observation period. The dog treated with the high dose experienced tonic/clonic convulsions immediately after dosing, but symptoms disappeared after 30 minutes. Decreased reticulocyte counts and slightly increased serum GPT and AP values were observed on the second day, but had also resolved at the end of the assay period (China Cooperative Research Group, 1982b).

Artemether was found to exhibit an i.m. LD₅₀ of 263 mg/kg in mice. One monkey tolerated an i.m. dose of 141 mg/kg, rabbits showed no toxicity at an i.m. dose of 160 mg/kg, and oral administration of 130 mg/kg to one dog did not produce toxic signs either (China Cooperative Research Group, 1982b).

For arteether no mortality was observed in mice within the first 48 hours after the administration of an intramuscular dose of 1000 mg/kg (Kar et al., 1989).

Artesunate was given as intravenous and intramuscular injections to mice; LD₅₀ values were determined as 520 and 475 mg/kg, respectively. Three dogs were treated intravenously in a rising dose schedule with 37.5 mg/kg on day 1, 75 mg/kg on day 2 and 150 mg/kg on day 3. Reduced appetite on day 2, and vomiting and reduced activity on day 3 with recovery after two more days were the only clinical symptoms reported (China Cooperative Research Group, 1982b; Yang et al., 1982). In another study, artesunate exhibited an intravenous LD₅₀ of 699 mg/kg in mice.

Maximum tolerated intravenous doses for Guinea pigs, rabbits, dogs and monkeys were 240, 160, 80 and 60 mg/kg, respectively, and the minimum lethal doses for these species were found to be 480, 640, 480 and 160 mg/kg, respectively. Toxicity symptoms observed were CNS effects (depression, unstable gait, tremor, convulsion) and respiratory suppression (Zhao, 1985).

In the various submissions to WHO single dose toxicity of artesunate has also been described as low: Oral administration yielded approximate LD₅₀ values of 1000 - 1300 mg/kg in mice, and of 600 - 900 mg/kg in rats, while dogs tolerated doses of up to 120 mg/kg. In this latter species, reticulocyte count was decreased at doses of 80 mg/kg and above, while histopathology showed some liver damage at 120 mg/kg and above. Intravenous, intramuscular and intraperitoneal toxicities described in these submissions correspond also closely to the published values.

In two studies conducted for regulatory submission oral administration of arteminol to mice has resulted in LD₅₀ values in the range of 700 - 900 mg/kg, while in another study a much lower toxicity (LD₅₀ of about 1500 mg/kg) has been reported, with a wide difference between the genders (males: 1100 mg/kg; females: 2000 mg/kg). Rats seem to be much more sensitive, since in this latter study, oral LD₅₀ values of about 350 mg/kg have been noted; an analogous gender difference in sensitivity was also observed for rats. Analogous figures for intraperitoneal and (presumably) intramuscular application of arteminol are in the order of 300 and 700 mg/kg, respectively.

Single dose toxicity has also been investigated with artemether and reported in submitted documentation. There were no toxic effects in dogs treated orally with doses of up to 1000 mg/kg, and the transient clinical symptoms observed in rats given 800 mg/kg p.o. resolved without consequences. Intramuscular LD₅₀ values were reported in two additional submissions as either about 300 mg/kg or about 1000 mg/kg, respectively.

4.2 Repeated dose toxicity

There is a small number of publications on artemisinins with application of repeated doses that are containing also some toxicology information. Although mainly focused on neurotoxicity endpoints a few general toxicity parameters are described therein.

Artemisinin (qinghaosu)

Repeated dose toxicity data have been reported for artemisinin (qinghaosu) in rats, dogs and monkeys. Daily intramuscular dosing of rats with 200, 400 and 600 mg/kg for 7 days did not produce mortality, and only slight congestion was observed for some organs in the mid- and high-dose groups. Daily oral administration of 250, 500 and 1000 mg/kg for 14 days was without toxic effects on the treated animals. Oral application of a daily dose of 100 mg/kg to four dogs for 5 days did not produce any clinical symptoms or changes in respiration or heart rate. Finally, Rhesus monkeys were treated with daily intramuscular doses of 24, 48, 96 and 192 mg/kg for 14 days. Three of four monkeys in the high-dose group died within 3 days after the last dose with severe clinical symptoms (apathy, reduced appetite, bradycardia), haematological disorders (absence of reticulocytes in peripheral blood, and reduced erythrocyte counts, haemoglobin content and haematocrit) and cardiac toxicity; one animal in the 96 mg/kg group also died, and the six monkeys in this group exhibited the same symptoms as those of the high-dose group. At the two lower doses, toxic findings were confined to the haematological effects which were found to be reversible within 22 days after the last dose (China Cooperative Research Group, 1982b).

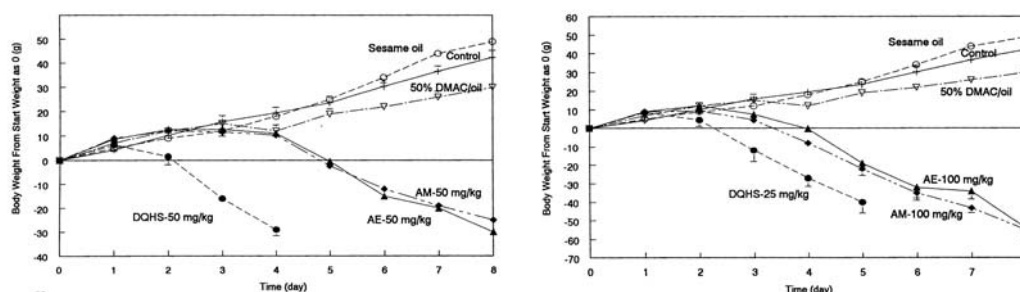
Artesunate

Intravenous application of artesunate to dogs in doses of 10 and 40 mg/kg for 14 days did not produce clinical symptoms, or haematological, biochemical or histopathological changes. The same result was obtained in monkeys given 10 and 32 mg/kg as daily intravenous injections for 14 days (China Cooperative Research Group, 1982b).

Intravenous application of artesunate for three consecutive days to rats in doses of up to 240 mg/kg induced a dose-dependent decrease in reticulocyte numbers, as well as decreases in erythrocytes, haematocrit and haemoglobin; the decrease in reticulocyte numbers was generally the most sensitive measure of artesunate effects, since statistical significance of the difference to controls was reached in females at 7.5 and in males at 15 mg/kg. At and above a dose of 60 mg/kg, also body weight was depressed (Xie et al., 2005).

Artesunate was administered intramuscularly to rats in a daily dose of 50 mg/kg for 7 days. Food and water consumption was observed to decline from the first or second dose, and body weight showed also a tendency to decrease over the treatment time; after the end of treatment, all parameters started to recover (Li et al., 1998a). This study was extended to include the respective effects induced by arteether and arteminol as well. The three compounds were administered i.m. in doses of 25, 50 and 100 mg/kg for a duration of 7 days. Rats receiving 100 mg/kg arteminol exhibited neurotoxicity (ataxia, tremors, convulsion) and died within 2 days. Neurologic symptoms also developed in animals of the lower arteminol dose groups, as well as in animals of the two higher artemether and arteether dose groups, although only after 4 - 8 days. Food and water intake, and body weights were continually reduced for all treatment groups as compared with the vehicle-treated controls (see figure 8, Li et al., 1998b). The effect on gastric transit time (see section 2.5, Safety Pharmacology) was held partly responsible for the lower food intake and body weights.

Figure 8 Body weight development of rats treated i.m. with arteminol (DQHS), artemether (AM), and arteether (AE) (from Li et al., 1998b).



Effects of DQHS, AM, and AE on body weight reduction following 50 mg/kg and 25 mg/kg for DQHS, and 100 mg/kg for AM and AE daily intramuscular administration for 7 days in rats ($n = 6$).

Artemether

Rats receiving total intramuscular doses of 40 to 360 mg/kg over a 9 to 14 day period exhibited some body weight loss and a slight hepatic fatty degeneration in the high dose group (China Cooperative Research Group, 1982b).

Rats were treated orally with artemether at doses of 80 and 400 mg/kg once every two weeks for five months; at the end of the treatment period, 6/10 animals were killed, while the other 4 rats per group were kept untreated for recovery for another month. Haematology data showed only a reversible reduction of reticulocyte count and a reversible increase in haemoglobin levels at both doses. Liver and kidney function and histopathological examination showed no differences between treated and untreated rats with the exception of a decreased activity of alanine transaminase at the end of treatment in the high dose group. There were no treatment-related effects in the ECG; especially no changes in the QT interval were observed. At the high dose of 400 mg/kg transient focal vesicle degeneration of the liver or slight damage to the lamina propria of the intestinal villi was observed, but there was no evidence of neurohistopathological alterations (Xiao et al., 2002).

Artemether was orally administered to rats once every two weeks for a total of 10 doses at 80 and 400 mg/kg. Haematology and clinical chemistry investigations, ECG recordings, and histopathology were performed. At both doses, reticulocyte counts were significantly reduced at 1 day after the last treatment,

an effect that was shown to be reversible after the recovery period of 4 weeks. Liver and kidney functions were also reversibly affected at the high dose, as alanine transaminase activity was significantly decreased and blood urea nitrogen was increased. No effects could be observed in the ECGs, especially no differences in QT intervals were found.

Histopathological effects, finally, were confined to the liver in 2 of 6 rats, but no influence of the treatment was observed on the CNS (Shuhua et al., 2002).

Obviously in an attempt to mimic the clinical situation, dogs were given two three- day courses of i.m. injections with an interval of 7 days; a second group was given these three-day courses over a period of 3 months. Total doses applied were 24 and 972 mg/kg, respectively (daily doses not stated). There were no abnormal findings reported, with the exception of body weight loss and slight hepatic fatty degeneration in the high-dose group (China Cooperative Research Group, 1982b).

In a pilot study dogs were treated intramuscularly with artemether at a daily dose of 20 mg/kg for 5 or 30 days. Clinical signs of neurotoxicity were absent in dogs treated for 5 days, but were noted in some dogs from day 23 on. Histopathologic changes were present only in animals treated for 30 days. Neurotoxicity, increased haemosiderosis and extramedullary hematopoiesis in the spleen, and haemosiderin- like pigment in the hepatic Kupffer cells were most frequently detected. Haematologic findings indicated a hypochromic, microcytic anaemia, and electrocardiographic examinations indicated a trend toward prolonged QT intervals. In the main study, artemether was administered to dogs intramuscularly at doses of 20, 40 or 80 mg/kg/day for 8 days; this treatment resulted in clinical signs (reduced activity, salivation, vomiting) in some dogs of the 40 and 80 mg/kg/day groups. Compared with untreated dogs the mean body weights were reduced in all dose groups and this reduction was statistically significant for the high-dose groups. At day 7 mean QT intervals were prolonged in all dose groups. Additionally, artemether was administered orally at doses of 50, 150 or 600 mg/kg/day for 8 days. Mean body weights were reduced in the high- and mid-dose groups and feed consumption decreased in females of the high-dose group, while prolongation of QT intervals reached relevant levels (i.e., >25 %) in the high-dose group only. Neuronal damage was evident in the brains of animals treated intramuscularly with a clear dose- dependence, but no such effects could be observed for the orally treated groups (Classen et al., 1999).

In a limited dog study, artemether was administered orally to dogs as a suspension in corn oil three times per day at a dose of 45 mg/kg (total daily dose 135 mg/kg) for two weeks. Since no concurrent control group (untreated or vehicle- treated) was used, parameter values were compared with historical control figures.

Food consumption of the animals was lower in the treatment period compared to the pre-treatment period. With the exception of occasional vomiting and soft or liquid faeces, no adverse clinical symptoms were apparent during the whole study. The only remarkable deviation from “controls” was found for the liver weights of two animals (1/4 male; 1/4 female) with relative weights above the upper limit of the historical control values. Concomitant with the increases in the weights histopathological changes were seen, consisting of slight to moderate diffuse hepatocellular hypertrophy, and which were observed in most of the treated dogs.

Other histological findings can be considered as of no toxicological relevance. There were no behavioural and histopathological signs of neurotoxicity (Peys et al., in press).

No abnormal findings were reported for artemether-treated monkeys to which total intramuscular doses of 97 and 292 mg/kg (no daily doses given) were administered over a period of 1 to 3 months (China Cooperative Research Group, 1982b).

4.3 Genotoxicity

The only published data on genotoxicity assays are an *in vitro* bacterial mutagenicity test and an *in vivo* bone marrow micronucleus test in mice, both conducted on artemisinin (qinghaosu). Concentrations of up to 300 µg per plate in the bacterial assay, and doses of up to 845.6 mg/kg in the micronucleus test were shown to be without effect, and the compound can thus be regarded as devoid of genotoxic activity (China

Cooperative Research Group, 1982b). Also in regulatory submissions, studies have not shown genotoxic effects for any of the artemisinin derivatives tested, and in any of the assay systems used in conformity with ICH Guideline S2B "Genotoxicity : A Standard Battery for Genotoxicity Testing for Pharmaceuticals".

4.4 Carcinogenicity

No carcinogenicity studies have been performed with artemisinins. In view of the short duration of treatment, and in the absence of any indications of a potential carcinogenic hazard, this lack of experimental information can be accepted, also from a regulatory point of view. Indeed, the ICH Guideline S1A "Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals" does endorse this view completely.

4.5 Reproductive toxicity

Published information on reproductive toxicity of artemisinin derivatives has been scarce, but it has been generally acknowledged that artemisinins have a distinctive embryotoxicity exhibiting a very steep dose-response relationship. The Chinese investigations for the registration documentation have been conducted on artemisinin and artemether in rats with relatively high doses. Doses are presented as fractions of the LD₅₀, i.e., 1/400, 1/200 and 1/25 corresponding to 14, 28 and 223 mg/kg (LD₅₀ equal to 5576 mg/kg), and application was either in the first 6 days of pregnancy, or on days 7 to 12, or 13 to 19, respectively. Dosing in the very early stage had no effect on the development of the fetuses, while treatment at the later stages resulted in complete embryonic loss. When treatment was administered between pregnancy days 6 and 15 (comparable to current protocols), an about 50% loss of embryos was observed at the lowest dose, while at the higher doses total loss was observed again. Umbilical hernia was the only malformation detected in 6.1% of the fetuses, when 14 mg/kg was administered on days 6 to 8 of pregnancy. Results in mice were described as comparable (China Cooperative Research Group, 1982b). Artemether has also been tested in mice and rats by intramuscular injection, and analogous results are reported. In no instances, teratogenic effects were reported (China Cooperative Research Group, 1982b). Further studies conducted with artemether in mice, rats and rabbits, and of artesunate in rats have been summarized by Longo et al. (2006); they have generally provided an analogous picture, with large embryonic losses at relatively modest doses. In mice, 100% resorptions were observed at a dose of 21.4 mg/kg, while in rats and rabbits >90% resorptions were seen at 10.7 and 2.7 mg/kg, respectively. The complete information on reproductive toxicity that was available up till 2002 has subsequently been discussed by two informal consultation meetings convened by WHO, and the relevant conclusions were summarized in a report (WHO, 2003) as follows:

- 1.1 *It was agreed that artesunate and all the other artemisinin analogues are developmental toxicants in the rat and rabbit. The dose-response relationships for the compounds are unusually steep. The effects are dominated by embryo-lethality and late resorptions and a few instances of morphological abnormalities, all under circumstances not related to maternal toxicity.*
- 1.2 *All the artemisinin analogues have been associated with embryo-lethality over a narrow dose range. General experience has shown that morphological abnormalities and embryo-lethality are part of a continuous spectrum. A small change in dose or other factors may favour the occurrence of one over the other.*
- 1.3 *There have been minor indications in some experiments of pre-implantation loss and possibly of some change in sperm counts, but neither has been systematically studied.*
- 1.4 *Fertility has not been affected.*
- 1.5 *The main effect of embryo-lethality, as seen in many embryo toxicity studies, is not necessarily predictive of the same effect in humans. If there were a similar action in pregnant women it might become apparent during the first trimester of pregnancy.*
- 1.6 *The mechanism of the developmental toxicity in animals is not known. It is not clear whether the initial site of action is in the mother, the fetus, or the placenta. In addition to the predominant*

embryo-lethality, there are instances of effects on development of the cardiovascular system, the axial skeleton, and the limbs, but these do not suggest the nature of the toxic action.

- 1.7 There is decreased sensitivity to the effect on fetal growth or survival following dosing in later stages of pregnancy. Maternal dosing during lactation, post-natal development and maturation has not led to any convincing toxic effects.*
- 1.8 The limited experimental results available about various combination therapies of artemisinin analogues and other antimalarial drugs (chlorproguanil and dapsone) do not show increased toxicity in comparison with the artemisinin compounds alone.*
- 1.9 Doses in animals associated with effects on the embryo are similar to those used in clinical practice, but as there are no relevant pharmacokinetic data it is impossible to interpret this information in terms of systemic exposure, or to estimate a safety margin in a conventional manner.*

In a recent study the effects of artesunate, as well as of the combination of artesunate with chlorproguanil and dapsone (CDA), on the embryonal development and on teratogenic effects (such as listed above under point 1.6) have been further investigated in rats and rabbits (Clark et al., 2004). In rats, artesunate alone was administered orally in doses of 6, 10 and 16.7 mg/kg between days 6 to 17 of pregnancy and the animals were killed on day 20. Toxicokinetic parameters for artesunate and its metabolite arteminol were recorded on the first and last days of treatment. Effects observed in the foetuses consisted of a low incidence of cardiovascular defects (ventricular septal and vessel defects) and of a syndrome of skeletal defects, especially with long bone shortening and/or bending. In the experiments with CDA, doses of 12.5, 25 and 50 mg/kg were applied, corresponding to artesunate doses of 5.9, 11.8 and 23.5 mg/kg. Analogous observations were made in this part of the study, and it was concluded that the effects were due to artesunate only, while addition of the other two components to the combination did not influence toxicity.

The summary of the results obtained is given in the following table, compiled from the paper by Clark et al. (2004). The known embryotoxic effects are apparent as reduced numbers of foetuses and litters at the high dose of CDA and the two higher doses of artesunate alone.

The toxicokinetic data showed the known phenomenon of the autoinduction of metabolism in terms of decreased $C_{\max}$ values on day 17 as compared to day 6 for both analytes, artesunate and arteminol; AUCs were determined only on day 6 and could only be estimated for arteminol. The $C_{\max}$ values for arteminol at the doses of 6, 10 and 16.7 mg/kg were 17.6, 49.8 and 119 ng/ml, respectively, for day 6, and 0 (below limit of analysis), 35.1 and 26.3 ng/ml, respectively, for day 17. The AUCs for arteminol at the two higher doses of artesunate were 49.9 and 116 ng·h/ml, respectively, on day 6.

Table 4 Selected teratogenic effects in rat fetuses treated with CDA or artesunate (compiled from Clark et al., 2004)

Incidences of Selected Cardiovascular, Long Bone and Scapular Malformations and Associated Skeletal Defects in Rats - Fetuses (Litters)								
	CDA				Dose (mg/kg/ day)			
	0	12.5	25	50	0	6	10	16.7
<i>External/visceral</i>								
No. Examined	276 (23)	240 (21)	191 (18)	18 (3)	284 (23)	250 (20)	73 (9)	33 (3)
Ventricular septal defect	0	0	0	2 (2)	2 (1)	6 (5)	1 (1)	0
Abnormal origin subclavian artery	0	2 (2)	2 (1)	0	0	1 (1)	1 (1)	0
<i>Skeletal</i>								
No. examined	140 (23)	123 (21)	96 (18)	10 (3)	142 (23)	125 (20)	36 (9)	18 (3)
Shortened and/or bent long bones and shortened and bent scapulae	1 (1)	0	4 (3)	2 (1)	5 (1)	2 (2)	3 (3)	0
Scapula bent (with no long bone findings)	1 (1)	0	1 (1)	0	0	1 (1)	1 (1)	0
Rib bent	0	0	0	2 (1)	1 (1)	0	1 (1)	0
Rib kinked	2 (2)	0	8 (5)	2 (1)	6 (2)	1 (1)	2 (1)	0
Rib nodulated	7 (4)	3 (3)	20 (8)	1 (1)	9 (3)	7 (4)	8 (6)	0
Rib incompletely ossified	1 (1)	2 (2)	9 (6)	1 (1)	4 (1)	1 (1)	2 (1)	0
Stemebrae cleft	0	0	0	1	0	1	0	0

In rabbits, artesunate was applied alone in doses of 5, 7 and 12 mg/kg between days 6 and 19 of pregnancy, and CDA doses amounted to 10, 15 and 25 mg/kg, corresponding to artesunate doses of 4.7, 7.1 and 11.8 mg/kg, respectively.

Sampling for toxicokinetics was performed on days 6 and 19, and dams were killed on day 29 of pregnancy. Rabbits exhibited an analogous pattern of cardiovascular and skeletal defects. Cardiovascular defects were especially prominent in artesunate- alone treated litters; the lack of a clear dose-response relationship was attributed to the intergroup variability associated with the low incidences observed. Also in rabbits, C_{max} values of arteminol decreased between day 6 and 19 from 45.5, 66.2 and 180 ng/ml for artesunate doses of 5, 7 and 12 mg/kg to 30.8, 42.6 and 97 ng/ml, respectively (Clark et al., 2004).

The mechanism of these teratogenic effects has been investigated and results have first been reported in three meeting abstracts. To begin with, it had been established that the most sensitive period for teratogenic effects of artemisinins in rats is between days 10 to 11 of pregnancy (White et al., 2005); this fact had already been observed also in early Chinese experiments for general embryotoxicity of artemether (China Cooperative Research Group, 1982b). Artesunate was shown to produce a marked reduction in the numbers of embryonic erythroid cells around 18 – 24 hours after a single dose, with the effect persisting for several days. It was furthermore shown that embryonic erythroid cells were more sensitive to the cytotoxic and lytic action of artesunate than adult erythrocytes (Laffan et al., 2005). These results suggest an inhibitory action on the development of early erythroid precursor cells, leading to anaemia and hypoxia, and resulting finally in damage to growing tissues and embryonic death. Attention should be drawn in this respect also to the anti-angiogenic effects ascribed to artemisinins which might have an additional (negative) influence on the development of the cardiovascular system (see section 2.4). Such effects have been observed *in vitro* at concentrations of 0.1 to 1 μ M, i.e. at around 30 - 300 ng/ml, concentrations which may correspond to C_{max} values obtainable in humans after therapeutic doses.

Further investigations have been conducted with rat embryos in culture (Longo et al., 2006). Embryos at gestation day 9.5 (GD 9.5, 1 - 3 somites) were excised and treated *in vitro* with arteminol at concentrations ranging from 0.01 - 2.0 μ g/ml for 2 days (GD 9.5 - 11.5), with concentrations from 0.05 - 2.0 μ g/ml for 90 minutes on GD 9.5 with further incubation without drug until GD 11.5, or with 1.0 and 2.0 μ g/ml for 90 minutes at the end of the culture period on GD 11.5, after which embryos were observed for heart beat, yolk sac circulation, morphological abnormalities, number of somites and various other parameters. The primary

event leading to developmental damage was considered to consist of a specific toxicity to red blood cells during yolk sac haematopoiesis, while at higher concentrations also angiogenesis seemed to be affected. Tissue damage and effects on embryo morphology and malformations were attributed to these effects. The tables 5A - 5C, reproduced from the paper of Longo et al. (2006), provide a compilation of the major findings.

From the findings, it becomes evident that the primary target are the embryonic erythroid precursor cells forming in the blood islands of the yolk sac. It is furthermore suggested, that the mechanistic basis of this damage to erythroid precursor cells is the same as, or at least analogous to, the mechanism for the schizonticidal activity of artemisinins. On the one hand, haemoglobin is actively synthesized in these cells, which furthermore are deficient in antioxidant defence possibilities, and consequently production of reactive oxygen species and increased oxidative stress may damage specifically these cells. On the other hand, the conditions in the embryo culture *in vitro* would point to a rather minor role of radical generation, and the inhibition of the SERCA-ATPase could be another important target, with consequences for an impaired calcium-ion homeostasis and cell death.

It remains to be established to what extent this mechanism of embryotoxicity and teratogenicity could play a role in humans, and what could be the practical consequences for the recommendations for use of artemisinins in pregnancy, which are now based on current knowledge.

Table 5A Effects induced by *in vitro* exposure of rat embryos from GD 9.5 to 11.5 to arteminol (from Longo et al., 2006)

Developmental characteristics of rat embryos exposed to DHA for 48 h: embryonic viability, morphometric parameters, total morphological scores and abnormalities							
Observations	Control DMSO 0.1%	DHA concentrations (µg/mL)					
		0.01	0.05	0.1	0.5	1	2
Embryos assessed (no.)	51	15	15	15	15	15	15
Embryos with heart-beat	51	15	15	15	15	15	15
Normal	51	15	15	15	15	0	0
Arrhythmic	0	0	0	0	0	15	15
Embryos without heart-beat	0	0	0	0	0	0	0
Yolk sac circulation							
Normal ^a	51	15	0	0	0	0	0
Anemic ^b	0	0	15 (+)	15 (++)	15 (+++)	15 (+++)	15 (+++)
Reduced ^c	0	0	0	0	15	15	15
Allantois							
Normal ^d	51	15	0	0	0	0	0
Anemic ^e	0	0	15	15	15	14	–
Ectopic ^f	0	0	0	0	0	2	–
Absent ^g	0	0	0	0	0	1	15
Yolk sac diameter (mm)	4.62	4.58	4.52	4.58	4.14**	4.18**	3.19**
Crown-rump length (mm)	3.93	3.86	3.87 ^h	3.84 ⁱ	3.62 ^j **	3.63 ^k	– ^j
Head length (mm)	1.84	1.84	1.76	1.79	1.58**	1.44**	1.03**
Somite number	23.94	23.87	23.71 ^h	23.23 ⁱ	21.89 ^j **	23.00 ^k	– ^j
Total morphological score	39.90	39.93	39.53	39.00	36.27**	31.80**	19.87**
Major abnormalities (%)	0.00	0.00	0.00	0.00	93.30***	100.00***	100.00***

^a Full yolk sac plexus of vessels with artery and vein well separated.
^b Pale vessels; (+) slight; (++) moderate; (+++) marked.
^c Corona of blood islands with anastomoses/vitelline vessels with few yolk sac vessels or irregular circulation due to arrhythmic heart-beat.
^d Umbilical vessels.
^e Umbilical vessels present but pale.
^f Allantois fused with chorion but ectopic.
^g Allantois free in the exocoelom.
^h Excluding one embryo that was unable to turn (in torsion); N=14.
ⁱ Excluding two embryos that were unable to turn (in torsion); N=13.
^j Excluding six embryos that were unable to turn (in torsion or ventrally curved); N=9.
^k Excluding thirteen embryos that were unable to turn (in torsion or ventrally curved); N=2.
^l All embryos were unable to turn (in torsion or ventrally curved); data not recorded.
^{**} P < 0.01; one-way ANOVA with Tukey's multiple comparisons.
^{***} P < 0.01; chi-square test.

Table 5B

Developmental abnormalities observed on live embryos exposed to DHA												
	Control DMSO 0.1%	DHA concentrations (µg/mL)										
		48-h exposure						1.5-h exposure at the start of culture				
		0.01	0.05	0.1	0.5	1	2	0.05	0.1	0.5	1	2
Total no. of embryos examined	51	15	15	15	15	15	15	10	10	15	10	10
Total no. of embryos affected ^a	7	1	2	6	14	15	15	1	0	1	3	6
Total no. of embryos without abnormalities ^b	44	14	13	9	1	0	0	9	10	14	7	4
Major abnormalities ^c												
Embryos examined	51	15	15	15	15	15	15	10	10	15	10	10
Embryos affected	0	0	0	0	14	15	15	0	0	0	0	0
Complete embryonic disruption	–	–	–	–	0	6	15	–	–	–	–	–
Unexpanded encephalic vesicles	–	–	–	–	4	2	0	–	–	–	–	–
Unexpanded telencephalic vesicles	–	–	–	–	6	5	0	–	–	–	–	–
Open neural tube	–	–	–	–	4	1	0	–	–	–	–	–
Markedly retarded optic vesicles	–	–	–	–	1	8	0	–	–	–	–	–
Markedly retarded otic vesicles	–	–	–	–	1	8	0	–	–	–	–	–
Misshapen somites	–	–	–	–	0	4	0	–	–	–	–	–
Short caudal extremity	–	–	–	–	7	4	0	–	–	–	–	–
Markedly reduced first branchial arch	–	–	–	–	3	9	0	–	–	–	–	–
Markedly reduced second branchial arch	–	–	–	–	5	9	0	–	–	–	–	–
Minor abnormalities ^c												
Embryos examined ^d	51	15	15	15	1	0	0	10	10	15	10	10
Embryos affected	7	1	2	6	0	–	–	1	0	1	3	4
Situs inversus viscerum	6	1	0	1	0	–	–	1	–	0	1	0
Slight retardation of telencephalic vesicles	1	0	0	1	0	–	–	0	–	1	0	0
Slightly shortened caudal extremity	0	0	1	1	0	–	–	0	–	0	3	4
Slight retardation of first branchial arch	0	0	1	3	0	–	–	0	–	0	1	2
Slight retardation of second branchial arch	0	0	0	0	0	–	–	0	–	0	0	1

^a Embryos showing major and/or minor abnormalities.
^b Normal embryos.
^c Each embryo may have more than one alteration.
^d Excluding embryos with major abnormalities.

Table 5C

Histological changes observed on live embryos exposed to DHA															
Observations	Control DMSO 0.1%	DHA concentrations (μg/mL)													
		48-h exposure						1.5-h exposure at the start of culture					1.5-h exposure at the end of culture		
		0.01	0.05	0.1	0.5	1	2	0.05	0.1	0.5	1	2	1	2	
Tissues (paraffin embedded)															
Normal	+	+	+	–	–	–	–	+	+	+	–	–	+	+	
Cell deaths															
Mesenchyme of caudal region	–	–	–	+	++	+++	–	–	–	–	+	+	–	–	
Mesenchyme of branchial arches	–	–	–	+	++	+++	–	–	–	–	+	+	–	–	
Mesenchyme surrounding aorta	–	–	–	+	++	+++	–	–	–	–	–	–	–	–	
Reduced number of red blood cells in dorsal aorta and heart	–	–	–	+	+	++	–	–	–	+	+	+	–	–	
Complete tissue disorganization	–	–	–	–	–	–	+	–	–	–	–	–	–	–	
Red blood cells (cytospin preparation)															
Normal shape	+	+	+	+	–	–	–	+	+	–	–	–	+	–	
Normal concentration	+	+	+	–	–	–	–	+	+	–	–	–	+	+	
Reduced concentration	–	–	–	+	+	++	+++	–	–	+	++	++	–	–	
Misshapen	–	–	–	–	+	+	+	–	–	–	+	+	–	–	
Misshapen nucleus	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	
Dead	–	–	–	–	+	+	+	–	–	–	+	+	–	–	

(+) Present finding, slight; (++) present finding, moderate; (+++) present finding, marked; (–) absent finding.

4.6 Neurotoxicity

In a pharmacokinetic study with repeated intramuscular injection of arteether at a dose of 20 mg/kg/day for 8 days to dogs a syndrome of progressive clinical neurological defects in conjunction with cardio-respiratory collapse and death was noted, while no such effects were seen to occur at the lower dose of 10 mg/kg. These findings were subsequently investigated further in dogs given i.m. doses of 5, 10, 15 and 20 mg/kg/day for 28 days, and in rats given arteether or artemether in i.m. doses of 12.5, 25 and 50 mg/kg/day for 28 days by ECG and histopathology of the brain.

Lesions were observed in the brains of both rats and dogs, and consisted of scattered neuronal degeneration and necrosis, characterized by swelling and rounding of nerve cell bodies, increased eosinophilia, vacuolization of cytoplasm with loss of Nissl substance (central chromatolysis), swelling and fading of nuclei, and separation and clumping of fibrillar and granular components of nucleoli; similar effects were seen in the rat brains (Brewer et al., 1994a; Brewer et al., 1994b). These findings have been reproduced and confirmed in a number of other studies in mice, rats and monkeys, and the histopathological lesions in the brainstem have been observed either in the presence or the absence of clinically manifest neurotoxicity (Genovese et al., 1995; Petras et al., 1997; Kamchonwongpaisan et al., 1997; Genovese et al., 1998a; Genovese et al., 1998b; Genovese et al., 1999; Petras et al., 2000; Nontprasert et al., 2002), and reviews have also been discussing these results (Brewer et al., 1998; Dayan, 1998).

It was recognized soon that this neurotoxicity, although probably a class effect of artemisinins, was dependent not only on the dose applied but also on the mode of application and the physical-chemical properties of the derivative tested.

Two studies in mice investigated the aspects of compound differences as well as differences due to the application route. Artemether, in doses of 30, 50, 75 and 100 mg/kg, and artesunate, in doses of 30, 50 and 100 mg/kg, were given daily as intramuscular injections for 28 days. Mortality was higher for artemether in the high-dose group compared with artesunate at the same dose; no significant effects on body weight were observed at the lowest dose, while differences in body weight development were apparent between artemether and artesunate at the higher doses. Neurological observations at the high dose showed a clear distinction between artesunate, which had practically no effect, and artemether, where only about 10% of the animals remained neurologically normal (see figure 9). Also, olfactory responses, estimates as the time needed to find food, were significantly impaired in the artemether- but not in the artesunate-treated groups (Nontprasert et al., 1998).

In an analogous setting with treatment for 28 days, mice received intramuscular or oral doses of artemether and artesunate. Artemether was administered i.m. at doses of 150 and 200 mg/kg; artesunate was given either in aqueous solution at doses of 150, 200 and 250 mg/kg, as well as in an oil preparation at 100 mg/kg. Oral doses were also given as different formulations, either as aqueous solution, or in the form of oil-coated food pellets; doses ranged from 100 to 300 mg/kg. Artemether again proved to affect neurological parameters more than artesunate (see figure 10), and intramuscular injection was more toxic than oral application (Nontprasert et al., 2000).

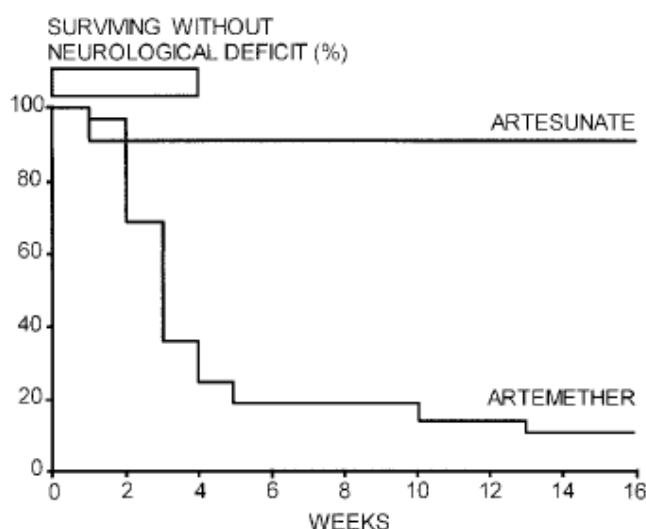
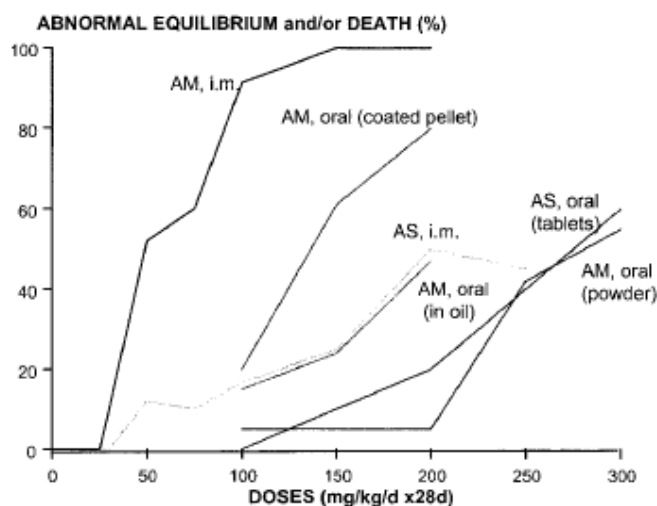
Figure 9 Kaplan-Meier graph for neurological effects in mice (from Nontprasert et al., 1998).

FIGURE 1. Proportion of mice (%) receiving intramuscular artesunate and artemether at a dose of 100 mg/kg/day that survived and remained neurologically normal. The hatched bar represents the time during which injections were given once a day.

Figure 10 Effects of application route and formulation of artemether and artesunate on mortality and neurotoxicity (from Nontprasert et al., 2000).

Additionally, in a study on artemether in dogs, doses of 20, 40 and 80 mg/kg were administered intramuscularly, while 50, 150 and 600 mg/kg were given orally for 8 days. Animals of the i.m. high-dose group presented with reduced activity and salivation on days 7 and 8, while no neurologic symptoms were apparent in the p.o. treated dogs. In corroboration of the clinical findings, the brains of dogs treated intramuscularly showed neuropathic lesions with dose-dependently increased incidence and severity, while no such lesions were observed in orally treated animals. Toxicokinetic profiling revealed major differences between the two modes of application (see figure 11). While accumulation is evident for the repeated intramuscular dosing (as has also been shown elsewhere, see figure 5), oral dosing apparently has the contrary effect, explainable by autoinduction of first-pass metabolism through the enteric route of application in contrast to the parenteral route. Plasma levels of artemether remained measurable after i.m. administration, but were below the limit of detection after oral application (Classen et al., 1999).

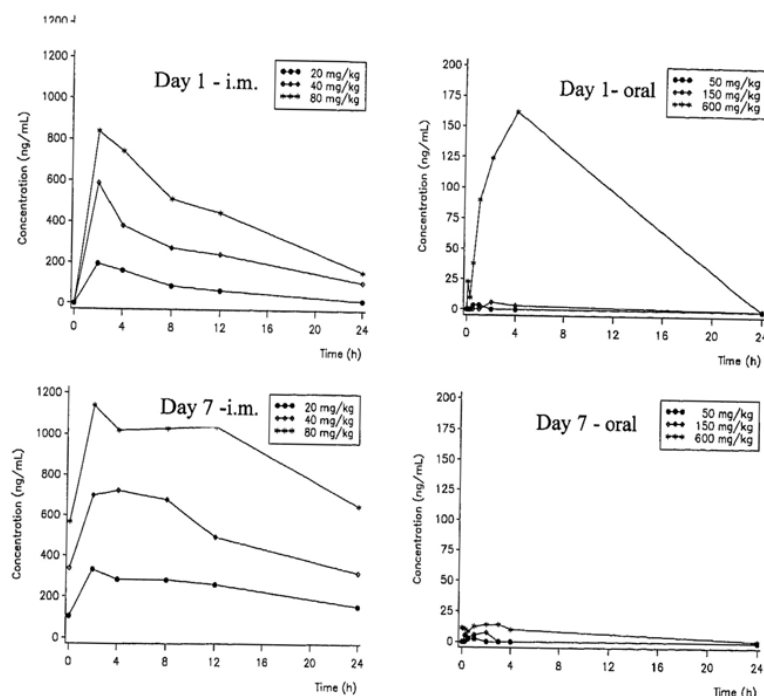
A comparative study with arteether, artesunate and artelinate showed very clearly that artemether, administered intramuscularly in a sesame oil formulation, produced exceedingly higher levels of neuronal damage in brainstem nuclei than did artesunate, administered in an equimolar dose as an aqueous formulation in 2.5 % sodium carbonate (see figure 12). Equally, behavioural performance tests showed significant deficits only for the arteether-treated animals (Genovese et al., 2000).

Since the auditory, vestibular and motor nuclei of the brain stem proved especially vulnerable to the effects of artemisinins, their involvement in the expression of this neurotoxicity has been specially investigated, and the respective regional damage was also used to investigate eventual neurotoxic effects in humans. The auditory component was specifically investigated in rats. Rats were trained in a complex radial-arm maze task with auditory stimuli as the cues for choosing the correct arm of the maze. While training is described as “a considerable process, requiring several shaping procedures”, the performance generated was then very stable for an extended period of time. Intramuscular injections of β -arteether in sesame oil at a daily dose of 25 mg/kg had no influence on the performance after the first few injections; after 7 injections, however, performance estimated as either percentage of correct choices, or as number of choices per session, deteriorated significantly, pointing to a neurotoxic defect in auditory processing (see figure 13).

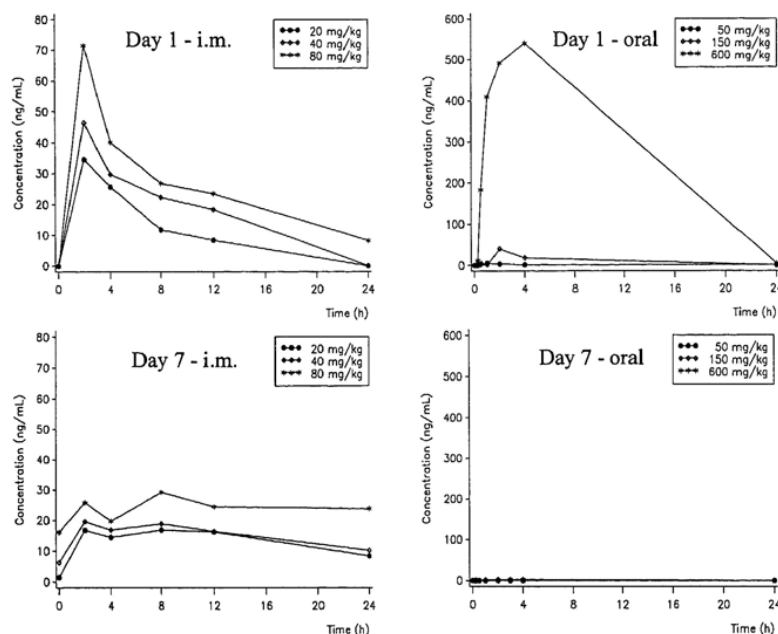
Histopathology finally showed that treated rats - in contrast to vehicle-treated animals - showed marked damage (necrosis, chromatolysis, gliosis) to neurons in the nucleus trapezoides (Genovese et al., 2001).

Not only the administration route and the physical-chemical properties determine the kinetic profile and neurotoxicity but also the formulation, as has been shown in a further investigation. Intramuscular application of artemether in single or multiple doses of 25 mg/kg to rats was either in an sesame oil or in an aqueous cremophor- based formulation. Toxicokinetic profiles were different for the two formulations, with the oil formulation producing lower C_{max} levels than the cremophor formulation, but with higher sustained levels resulting in an accumulation of the compound and in progressively higher trough levels (see figure 14). In line with the kinetic profile, animals receiving the substance in the oil formulation had a higher level of neurotoxic damage with significantly more severe gradings than the cremophor formulation. This finding supports the notion that the induction of the neurotoxic damage in the brain stem is not dependent on C_{max} values reached, but on a sustained exposure the level of which should not fall below a certain concentration for any extended period of time. On the other hand, efficacy, measured as the dose curing 50 % of the mice of a *P. berghei* infection, was higher for the cremophor formulation for both, artemether and arteether, by factors of 1.7 to 2.4, obviously reflecting the higher C_{max} values obtained with the aqueous formulation (Li et al., 2002).

Figure 11 Toxicokinetic profiling of artemether in dogs treated either orally or intramuscularly (from Classen et al., 1999)



Mean plasma profiles of artemether in healthy Beagle dogs after single or repeated intramuscular administration of artemether.



Mean plasma profiles of dihydroartemisinin in healthy Beagle dogs after single or repeated oral administration of artemether.

Figure 12 Neuronal damage in the brainstem, of rats treated intramuscularly with equimolar doses of arteminate, artesunate and arteether (from Genovese et al., 2000)

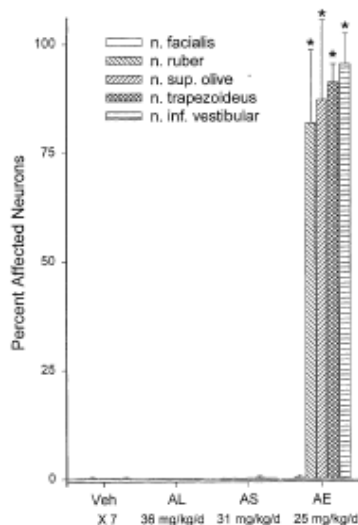
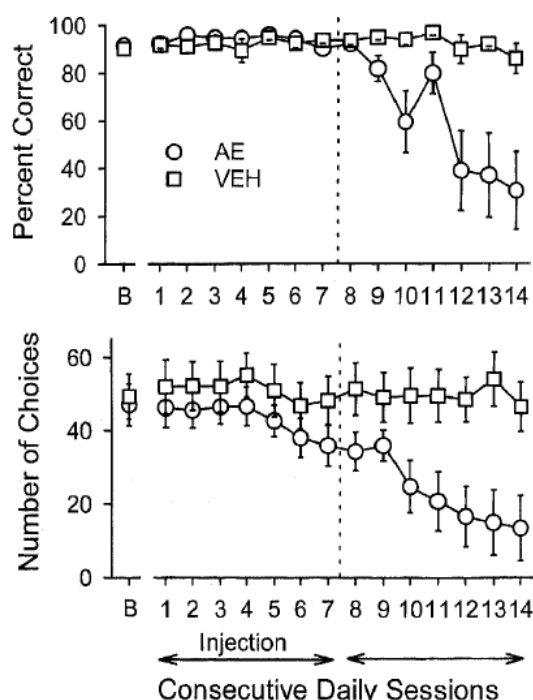


Fig. 3. Average percentage of examined neurons in selected brainstem nuclei judged to be damaged following daily administration of vehicle, AL (36 mg/kg), AS (31 mg/kg), and AE (25 mg/kg), for 7 days. Rats were sacrificed 7 days following the last administration. Each bar represents mean cell counts from six rats. Vertical lines about each bar represent the SEM. * Indicates a statistically significant difference from the vehicle control ($p < 0.05$).

Figure 13 Behavioural effects of arteether (AE) or vehicle (VEH) on the auditory radial-arm maze task. Arteether was injected daily for seven days before the sessions (from Genovese et al., 2001)



Since the histopathological expression of the artemisinin neurotoxicity was recognized as a very specific and special effect, efforts have been undertaken to investigate the mechanism for the induction of these effects, and a number of *in vitro* studies have been performed to this end.

Figure 14 Toxicokinetic profile for daily intramuscular injections of arteether in either sesame oil or aqueous cremophor formulation (from Li et al., 2002).

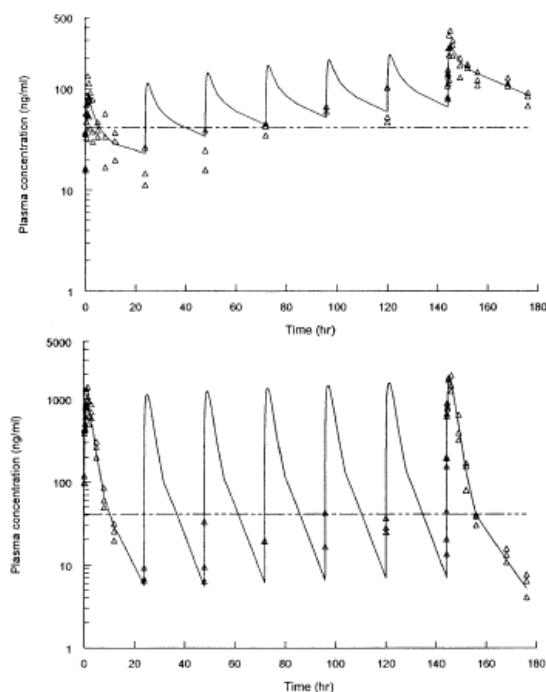
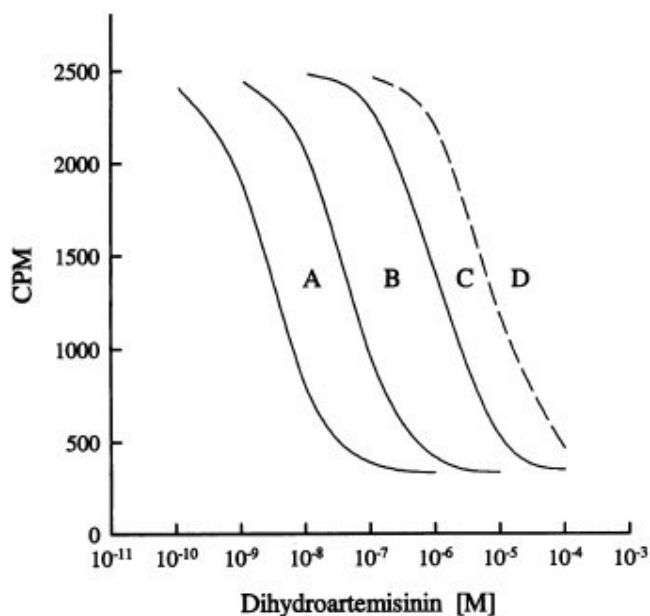


FIGURE 4. Plasma concentration-time profiles measured by high-performance liquid chromatography-with reductive electrochemical detector (triangles) and computer fitted curves by pharmacokinetic parameters (solid line) of arteether in sesame oil (top) and 1:2 cremophore/0.9% saline (bottom) following a 25 mg/kg, multiple, intramuscular injection once a day for seven days in male rats ($n = 3$). The minimal detected adverse effect level was estimated to be 41.32 ng/ml (dashed line).

In a panel of several neuronal and glial cells (foetal rat primary neuronal cells, foetal rat secondary astrocytes, and transformed neuroblastoma cell lines derived from rats and mice), artemisinin derivatives were shown to damage only neuronal cells in a concentration- and time-dependent manner (see figure 15); extending the time of exposure from 45 minutes to 16 hours resulted in a decrease of the EC_{50} from about 10 μ M to $5 \cdot 10^{-3}$ μ M. Neuronotoxic activity, estimated as release of lactate dehydrogenase into the culture medium or as inhibition of L-[14 C]- or L[4,5- 3 H]-leucine uptake, was observed to be highest for artemimol and artesunate (Wesche et al., 1994). In another assay system, investigating the proliferation of undifferentiated neuroblastoma and glioma cells and the inhibition of neurite outgrowth from neuroblastoma cells, similar results were obtained, and it was confirmed that the endoperoxide bridge of artemisinin derivatives was a necessary component for this toxicity; again, artemimol was demonstrated to be of higher toxicity than artemether or arteether. It was suggested that reactions with cytoskeletal proteins, e.g., cross-linking reactions, might form the mechanistic basis of this specific neurotoxicity (Fishwick et al., 1995). In the presence of an exogenous metabolic activation system from liver able to convert artemether and arteether into the primary metabolite artemimol, neuronal toxicity has been shown also to increase for these compounds (McLean and Ward, 1998).

Figure 15 Concentration- and time-dependence of neuronal toxicity due to *in vitro* treatment with artemimol (DQHS, from Wesche et al., 1994)

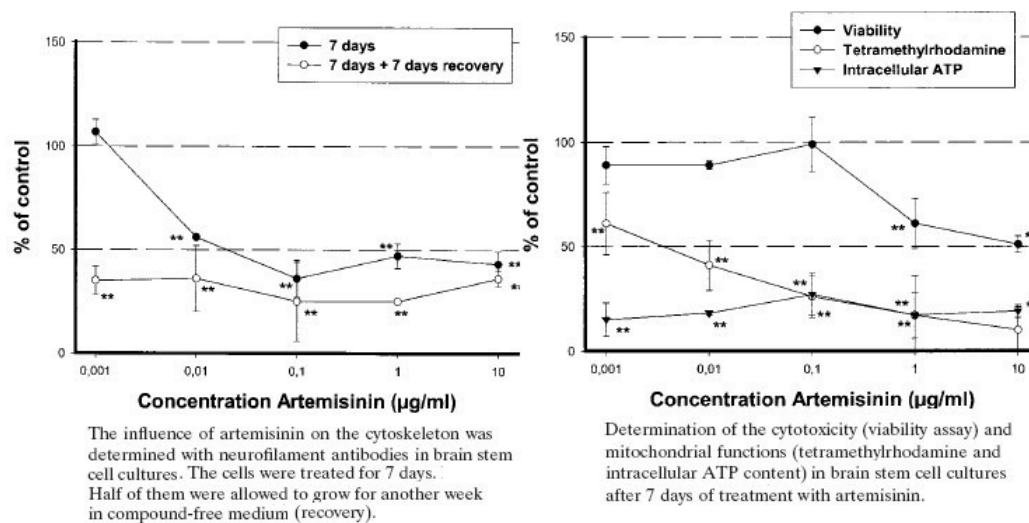


Representative plot of inhibition of L-[¹⁴C]leucine uptake in NG108-15 neuroblastoma cells by DQHS at various time points. Cells were incubated with DQHS for 16 h (A), 24 h (B), 8 h (C), and 0.75 h (D) followed by drug removal with subsequent assay at 24 h.

The involvement of oxidative stress in this neuronal toxicity has been made plausible not only by the necessity for an intact endoperoxide structure, but also by the findings of enhanced toxicity of artemisinins in the presence of haemin or iron, and of the respective protective effects of ascorbic acid and glutathione (Smith et al., 1997; Smith et al., 1998; Smith et al., 2001). Furthermore, not only a reduction in the cytoskeleton, but also an inhibition of the respiratory chain in mitochondria has been demonstrated, expressed as significantly reduced intracellular concentrations of ATP and reduced inner mitochondrial membrane potential (measured by the extent of tetramethylrhodamine fluorescence), which consequently was considered to be a second mode of action (see figure 16). The specific sensitivity of brain stem neuronal cells was explained by their less active antioxidant system (Schmuck et al., 2002).

The question of whether the nonclinical observations of this neurotoxicity would have any influence on the safety assessment of artemisinins in humans has been raised and tried to be answered in a number of ways. In the first instance, the pharmacokinetic properties and the distribution of these compounds into the CNS have to be considered for an estimate of the potential magnitude of any problem in humans. From nonclinical studies it has been estimated that brain levels of artemether and arteether should amount to only about 10 % of the respective plasma concentrations; the lesser lipophilicity of artesunate and artemimol - considered to be more neurotoxic than artemether and arteether - should lead to even lower brain concentrations. Whether this holds for situations in which the blood-brain barrier could be compromised, such as in cerebral malaria, has been a point for debate. A small clinical study in 6 patients with cerebral or severe *P. falciparum* malaria treated with an i.v. injection of 120 mg artesunate (2.0 - 2.86 mg/kg) showed indeed that, although no artesunate could be detected in cerebrospinal fluid, concentrations of artemimol were measurable and increasing with time; the levels were, however, small and amounted to less than 10 % of concentrations needed to produce neurotoxicity *in vitro* (Davis et al., 2003). In accordance with this, clinical studies have generally failed to detect CNS disturbances related to artemisinin administration (van Vught et al., 2000; Kissinger et al., 2000), although in a more recent case-control study a small hearing loss has been observed

Figure 16 Influence on the number of neurofilaments, and inhibition of mitochondrial function in primary brain stem neuronal cells, by artemisinin (from Schmuck et al., 2002)



in patients with uncomplicated malaria treated with artemether-lumefantrine compared to untreated controls; the study has been criticized in several points, and the differences in hearing acuity between patients and controls may be considered small and possibly more related to the occupation (construction workers) than to an influence of the medication (Toovey and Jamieson, 2004).

4.7 Other toxicity

Nonclinical studies with other toxicity endpoints (immunotoxicity, phototoxicity, local tolerance) have not been performed. Especially local tolerance can be considered to be sufficiently known from human experience, for intramuscular as well as for intravenous application. Also other toxicities such as immunotoxicity and phototoxicity have not been observed to be serious problems in clinical experience, and the lack of non-clinical studies in these areas should therefore not be considered as a deficiency of the documentation on non-clinical safety of artemisinins in general.

4.8 Toxicology Assessment

As target organs of artemisinin toxicity the haematopoietic, cardiovascular, gastrointestinal, hepatic and central nervous systems have been identified, most of which can be considered as reversible. They can be graded into different categories according to their potential impact on human patients. Haematopoietic effects are expected anyway to be an attendant syndrome of malaria, and the hepatic effects are minor, reversible and of a very low histopathological grade. Gastrointestinal disturbances with loss of appetite and body weight loss may also not be seen as potentially causing serious side effects when considering the clinical seriousness of the disease-to-be-treated. Cardiovascular effects with the potential to prolong the QT interval and thus to have the potential for inducing possibly lethal arrhythmias may be regarded as more serious, although the relevant effects have been recorded in nonclinical studies at doses and exposures in excess of what might be "normal" exposures in patients.

In the various submissions, a similar general toxicology profile for all artemisinin derivatives tested, and for all modes of application, is apparent. There are no major target organs of toxicity other than the CNS (neurotoxicity, see 4.6). The most prominent and constant findings are haematotoxic effects with decreases in haemoglobin and haematocrit levels, and decreased reticulocyte counts in all species investigated. Liver effects have also been observed with changes in serum enzyme activities concomitant with mild to moderate

histopathological alterations. Slight to moderate prolongation of the QT interval has been recorded in some studies and seems to be a class effect of the artemisinins

The two nonclinical toxicology areas where the most serious concerns arise, are the reproductive toxicity and the neurotoxicity of artemisinins.

Reproductive toxicity is characterized by a very pronounced embryotoxicity with a steep dose-response relationship, and teratogenic effects have been recorded only at doses bordering to the induction of complete embryotoxicity. The teratogenic effects consist mainly of malformations of the cardiac system and the large vessels, as well as of a characteristic syndrome of bone changes. Several aspects need to be considered in the interpretation of these findings and their extrapolation to the human situation. The underlying damage has been found to be a specific toxicity to the erythroid precursor cells in the blood islands of the yolk sac, with a consequent anaemia and hypoxia of the growing embryo, at a time in development, when simple diffusion of oxygen and nutrients is no longer sufficient to supply the developing tissues. Since this is a general step in the development of the mammalian embryo, it can be assumed that the same, or similar, effects should occur in species other than rodents, too, and that humans would be no exception to this basic mechanism. The doses needed in rodents and rabbits to induce embryotoxicity correspond closely to the human therapeutic doses on a mg/kg basis, but good toxicokinetic data on the exposure of yolk sac, placenta and growing embryos at the most sensitive stage (development of erythroid precursor cells as well as of the vascular and cardiac systems) are not yet available. Since, on the one hand, the concentrations needed to induce relevant damage to rat embryos *in vitro* do correspond closely to the exposure levels (in terms of plasma concentrations) that are obtained with embryotoxic and teratogenic doses in rats *in vivo*, and since, on the other hand, there are no straightforward indications for a major embryotoxicity or teratogenicity problem in first trimester pregnancies, the apparent differences in the expression of such effects needs a different explanation. Pharmacokinetics do not seem, at the moment, to provide such an explanation. The differences observed between animals and humans with respect to kinetic parameters of parent artemisinins and their common major metabolite, artemimol, do indicate a much higher exposure of humans in terms of C_{max} and AUC. Further research needs to be done in this area to provide better insight into the potential for human risk. Nevertheless, certain theoretical considerations may point to possibilities for explaining the apparent differences.

In terms of risk to the human conceptus, it has to be considered that the development of the circulatory system in the growing human embryo takes place between days 21 and 35 post conception, with some processes finished around day 50. This would mean that the most sensitive period for the generation of any cardiovascular defects in humans should be in the early part of the first trimester, possibly at a time when pregnancy is not even recognized. Additionally, the relative time span of exposure to artemisinins, during a course of malaria treatment, in relation to the extent of time needed for the development of the haematopoietic and cardiovascular systems is very much reduced in humans as compared to rodents, and the *in vitro* experiments with rat embryos have indeed provided evidence for the need of higher concentrations to produce a similar extent of damage when using a shorter duration of exposure. Observational reports on treatment with artemisinins in pregnancy have not produced evidence for increased rates of teratogenic events in humans, although the power of these observations is restricted due to the relatively low number of treatments in confirmed first trimester pregnancies. It may also be speculated that this could be additionally due to the much higher probability of embryotoxicity compared with the respective teratogenicity, followed by a lack of recognition of embryotoxic events in very early pregnancy (i.e., judged to be a late menstrual bleeding); furthermore, the longer gestation time in humans could be responsible for a certain propensity to catch up on early developmental retardations such as those caused by delayed development of the circulatory system.

This interpretation is supported by the views of the experts in the WHO informal consultation meetings (WHO, 2003), in which for the clinical implications it is stated that:

- 2.1 *It is not possible to predict from the animal findings the exact nature of the harmful effects that might be produced in women. However, the predominant experimental findings do suggest that if there were similar clinical effects, they would be likely to become apparent during the first trimester of pregnancy, e.g. **as early loss of pregnancy or difficulty in becoming pregnant.***
- 2.2 *The clinical information is encouraging in not showing any evidence of harm to mothers or fetuses at any stage of pregnancy.*

The number of women and their babies formally studied after dosing during the first trimester of pregnancy being small (44 in SE Asia, 80 in Africa), any conclusion about safety can only be of limited power. No study has been specifically designed to examine early pregnancy loss.

A much larger number of women has been followed after treatment during the second and third trimesters (417 in SE Asia, 207 in Africa) and there is greater confidence in the safety of treatment during that period, based on clinical findings and the results of animal experimentation.

Neurotoxicity, on the other hand, can be considered as better characterized in the nonclinical view. Although regarded as a class effect of artemisinins, it is recognized that differences exist between the various derivatives, with the more lipophilic compounds (artemether, arteether, arteminol) having a greater potential for neurotoxic activity than the more hydrophilic ones (artesunate). The consensus opinion with regard to the potential of artemisinins for eliciting neurotoxic effects, primarily in the brain stem but potentially also in other areas of the brain, is that these effects are neither dependent on the C_{max} reached in plasma, nor on the plasma AUC, but rather on the continuous exposure of (brain stem) neurons to a minimal concentration of the toxic agent for a minimal length of time, in rats in the order of some days (Gordi and Lepist, 2004). Although the hazard for neurotoxic effects is thus clearly recognized, the risk to humans is less clear. In the first instance, it has to be recognized that the exposure profile that has been made responsible for the induction of neurotoxicity is less probable to be obtained with oral application, while especially intramuscular injection of artemisinins in an oil-based formulation would be expected to lead to protracted absorption and exposure. The observation in a small number of patients treated with intravenous doses of artesunate that arteminol could be determined in cerebrospinal fluid at a more or less constant level over a certain period of time, but reaching concentrations of only about 10% of those needed to induce neurotoxicity *in vitro* seems to corroborate this interpretation. There have also been no unequivocal and undisputed reports of observations of neurotoxicity in patients treated with artemisinins, and the effects tested for, such as auditory changes, may be difficult to assess anyway, since the disease itself may produce smaller or larger damage to the CNS. Again, continued research under closely controlled conditions will be needed to provide better answers. However, since the recommendations of the WHO favour the use of combinations of artemisinins with longer acting antimalarials, for which a much shorter duration of treatment can be envisaged than for artemisinin monotherapy, the risk to patients may be judged to be low, because the exposure requirements, defined in animals for initiating the chain of events leading to outright neurotoxicity, may not be fulfilled in patients.

5. INTEGRATED SUMMARY

Artemisinin, its ether derivatives artemether and arteether, its primary metabolite arteminol and the ester derivative artesunate are exhibiting superior activity against *Plasmodium falciparum* and also against *Plasmodium vivax*. The compounds can be administered in various ways, and oral, intramuscular, intravenous and rectal formulations are known. The substances, with the exception of artesunate, are very lipophilic and not readily soluble in water, which led to the development of oil-based suspensions as intramuscular formulations.

Nonclinical pharmacological studies have extensively investigated the antiplasmodial properties of artemisinins in *in vitro* as well as *in vivo* models, and have proven their excellent efficacy. Their activity is primarily directed against the asexual blood stages of the parasite's life-cycle, with additional activity against gametocytes. They provide for a rapid onset of action and a rapid clearance of the parasite from the blood which, in consequence, leads to a rapid reduction of clinical symptoms of the disease such as fever. Their activity is based on the intact endoperoxide bridge of the molecules which may react with ferrous ions or haem to generate radicals and to increase intracellular oxidative stress. Their mode of action is not definitely clear, but may involve the inhibition of the SERCA-type PfATPase6 enzyme of the parasite. One of the assets of artemisinin derivatives is the lack of resistance despite extensive use in some regions with otherwise high levels of resistance against other drugs, notably chloroquine. Although isolates have been found that exhibit reduced sensitivity towards these compounds, there are no reports yet of treatment failures confirmed to be due to artemisinin resistance. Studies on the activity of artemisinins in combination with other antimalarials have provided evidence for additive or synergistic activities.

The pharmacokinetics of artemisinins have been investigated in nonclinical systems. Artemisinins are rapidly absorbed from the gastrointestinal tract upon oral administration, and they exhibit a short half-life in plasma. When applied as intramuscular injection, absorption is more protracted and leads to plasma levels that are sustained for longer periods of time; repeated administration by this route also leads to accumulation and higher plasma levels. This property may be important in terms of the risk assessment regarding the potential neurotoxicity of artemisinins.

The metabolism of artemisinins involves the formation of dihydroartemisinin (artemimol) as the first step. Artemisinin and its ether derivatives are converted by cytochrome P450 enzymes, while artesunate - as an ester of artemimol - is hydrolyzed by esterases; thus, artesunate tends to disappear very fast from circulation, while the metabolism of the other compounds is less rapid, especially when administered parenterally. Since, however, artemimol is as active as, or even more so than, the different parent compounds, there is no loss of antimalarial activity upon the metabolic conversion to artemimol. Artemisinins do induce their own metabolism, and therefore, plasma levels and exposures tend to decrease upon repeated dosing. Further biotransformation yields a number of additional metabolites, mainly excreted as glucuronides. Combination with other antimalarials did in most cases not influence the pharmacokinetics of artemisinins; interactions with cytochrome P450 inhibitors are known (e.g., for artemether and grapefruit juice - Riamet SmPC, 2005), but these will neither affect the efficacy nor the toxicity of the respective derivative.

Artemisinins are generally regarded to be of low toxicity. The main target organs of toxicity are the haematopoietic system with reversible decreases in the number of reticulocytes as the main effect, the cardiovascular system with prolongation of the QT interval, the gastrointestinal system with loss of appetite and weight loss, the liver with some slight changes in hepatic serum enzyme activities and concomitant minor histopathology, and especially the central nervous system with a very specific pattern of neuronal damage seen in the brain stem. This latter neurotoxicity has been regarded as a serious problem, and much investigational efforts have been devoted to its experimental clarification. The pattern that emerged from these studies is that the extent of this neurotoxicity is dependent on the nature of the compound, on the route of administration, and on the nature of the formulation. These factors determine the pharmacokinetic profile of the administered drug, and the neurotoxic effects have been found to depend neither on the maximal concentration in plasma (C_{max} , as a surrogate marker for the concentration reached in the brain) nor on the total exposure (plasma AUC), but on the continuous exposure to a certain minimal concentration; if this minimal concentration was not sustained over a prolonged period of time, no damage to the CNS has been observed. There are still a number of open questions to be resolved, among which the problem of the actual exposure of the CNS in these nonclinical studies is evident, and - although no unequivocal evidence for the induction of neurotoxic effects in malaria patients has been observed and reported - the possibility of the induction of very slight and nearly imperceptible neurological damage (especially in the auditory area) will have to be carefully assessed, in order to exclude all possibilities of such side effects. In addition, artemisinins are very toxic to the developing embryo, exhibiting a very steep dose-response relationship.

Teratogenic effects have not been found in most investigations, but as has been pointed out when such effects were found in rats and rabbits, this may have been due to two factors, namely that teratogenicity seems to be restricted to a narrow dose range in which embryotoxicity is already high, and that the frequency of observable effects is rather low; in consequence, differences in such observations to untreated controls might just not have reached statistical significance. The possible mode of action for these reproductive toxicities primarily involves a cytotoxic action of artemisinins on embryonic blood cells and in second line an inhibitory action on the embryonic vascular system, with consequent damage to the developing heart and growth retarding effects on other tissues. Again, while there have been no reports on negative influences of treatment with artemisinins on pregnancy outcomes, the potential embryotoxicity and teratogenicity needs to be assessed further.

In summary, there is sufficient evidence from nonclinical publications, from confidential nonclinical information contained in submission dossiers to the WHO Prequalification Scheme, and especially from clinical experience of more than two decades of use for the efficacy and safety of artemisinins in the treatment of malaria. Although two major areas of potential serious concern have emerged out of the results from nonclinical studies, namely neurotoxicity and reproductive toxicity, these toxicities have been judged as not to change the risk-benefit ratio to an extent that use of artemisinins in patients should be restricted to any major extent. On the one hand, clinical experience and clinical study data have not shown these two toxicities to be of importance to humans, since in no instance significant effects could be observed. For the neurotoxicity, the requirement that plasma levels of artemisinins (parent compound plus artemimol) should remain above a certain threshold level (estimated to be in the order of about 40 ng/ml) for the whole time between sequential doses may be assumed not to apply, since the

short half-lives of the respective molecules leads to practically undetectable levels at around 8 hours after each dose. On the other hand, doses inducing embryotoxicity and teratogenicity in rats and rabbits are very near, or even below, human therapeutic doses, and therefore, such effects might be expected to occur. However, the hypothesis that embryotoxicity as well as the associated teratogenic effects are induced by an action of the compound on the embryonal processes involved in the formation of erythrocytic precursor cells and on embryonal angiogenesis leads to the assumption that embryotoxicity, if it occurs in humans, would not be noticed, or only noticed as “late menses”. As a matter of precaution, the use of artemisinins in the first trimester of pregnancy has been contraindicated. Since malaria in pregnancy, however, is a very great risk for the lives of both, mother and developing embryo/foetus, treatment options have to be available in these cases, too.

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