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In-vitro Interaction of Artemisinin with Intact Human Erythrocytes, Erythrocyte Ghosts, Haemoglobin and Carbonic Anhydrase

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Abstract

The kinetics of the interaction of the antimalarial compound artemisinin with human erythrocytes, erythrocyte ghosts, haemoglobin and carbonic anhydrase were evaluated in-vitro. Artemisinin plasma concentrations, measured by HPLC (high pressure liquid chromatography), decreased with time during incubations with whole blood and erythrocyte suspensions of varying haematocrit. Artemisinin concentrations declined more rapidly during incubations under oxygen-poor as compared to oxygen-rich conditions. Artemisinin concentrations did not decrease during incubation with erythrocyte ghosts suspended in plasma suggesting that the drug does not bind avidly to red blood cell membranes. There was no decline in concentrations of artemisinin in the presence of carbonic anhydrase. The disappearance of the drug in solutions containing haemoglobin was very rapid and was even more so when the incubation was performed under an argon- instead of oxygen-rich atmosphere. The results suggest that drug blood clearance may be considered for inclusion in a pharmacokinetic model, but does not invalidate in-vivo plasma concentration-time data and their relevance for clinical effects. Furthermore, caution is advised when relating measurements of in-vitro potency to drug levels in patients. Finally, the enhanced artemisinin disappearance when oxygen tension is low may contribute towards the explanation of the selective toxicity of the endoperoxide drugs to *Plasmodium falciparum* parasite.

With the increasing resistance of malaria parasites to conventional drugs such as chloroquine, Fansidar, mefloquine and quinine, the development of new antimalarial drugs is urgently needed. Artemisinin and its derivatives are a timely contribution in this regard.

Artemisinin, a sesquiterpene lactone peroxide isolated from *Artemisia annua* L., and its derivatives, have in Southeast Asia become widely used in the treatment of resistant *Plasmodium falciparum* malaria. An endoperoxide bridge appears necessary for antimalarial activity, since deoxyartemisinin, which lacks such a bridge, has virtually no antimalarial effect (Brossi et al 1988).

Artemisinin concentrates in malaria haemozoin where it is covalently bound to haemin (Hong et al 1994). Intraparasitic haem iron is known to catalyse the reductive decomposition of the drug with consequent formation of free radicals, the formation of which has been associated with the parasiticidal effects (Meshnick et al 1993). The inhibitory effect of artemisinin on *P. falciparum* growth in-vitro was antagonised by antioxidants and that of artesunate, a hemisuccinate derivative, potentiated by oxidant drugs such as miconazole and doxorubicin (Krungkrai & Yuthavong 1987).

Concentrations of arteether, an ethyl ether derivative, decreased with time during incubation with whole blood (Edwards et al 1992). By using radiolabelled arteether and assessing the recovery of radioactivity from incubations with plasma, haemoglobin and erythrocyte ghosts, it was suggested that the interaction between the compound and blood components involved the red cell membrane. Asawamahasakda et al (1994) described that radiolabelled artemisinin and dihydroartemisinin are taken up and concentrated in isolated red cell membranes but not by intact erythrocytes and demonstrated that dihydroartemisinin binds to peripheral membrane proteins located on the cytoplasmic face of the red cell. Muhia et al (1994) found that after storage of artemisinin or artemether, a methylether derivative, in whole blood at room temperature there were significant losses of the two compounds. Using radiolabelled artemether, they demonstrated that haemolysis products and ferriprotoporphyrin-containing substances catalyse the decomposition of the compound.

The objectives of the present study were to investigate the kinetics of the in-vitro interaction of artemisinin with intact human erythrocytes, erythrocyte ghosts, haemoglobin and carbonic anhydrase, using a specific method for drug quantitation and to evaluate the influence of oxygen tension. Such information was considered valuable when specifying handling procedures for blood samples collected for pharmacokinetic analyses.

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Materials and Methods

Disappearance in whole blood and erythrocyte suspensions in plasma

Fresh heparinized human blood, erythrocytes and plasma of type A^+ were obtained from the University Hospital, Uppsala, Sweden. Artemisinin was supplied by Guilin Pharmaceutical Company, People's Republic of China. Erythrocytes, washed three times with isotonic phosphate buffer (100 mM, pH 7·4), were suspended in plasma to give three different erythrocyte suspensions of varying haematocrit (5, 18 and 33% v/v). Erlenmeyer flasks were predosed with artemisinin by adding the drug dissolved in acetonitrile and allowing the solvent to evaporate prior to the experiment. One hundred mL of whole

blood, plasma or one of the three erythrocyte suspensions were added to the flasks to produce initial artemisinin concentrations of 400, 800 and 1600 ng mL^{-1}. Two incubations were run in parallel: one exposed to carbogen gas (95% O_2–5% CO_2) and the other to a mixture of argon-carbon dioxide (95% Ar–5% CO_2) both from AGA, Sweden. Experiments were performed under gentle agitation at 37°C. Ten samples of 1 mL were taken from each incubation flask at various time points between 0 and 8 h. The samples were kept at 4°C for 10 min and then centrifuged at 3500 *g* for 5 min. The plasma was removed and stored at − 20°C until analysis within one month. The haematocrit, pH and pO_2 in each incubation were determined at 0, 4 and 8 h during the experiments using a Adams Readacrit CT-3400, a Metrohm 632 pH-meter and a AVL-Compact 2 Blood gas analyser, respectively.

Interaction with erythrocyte ghosts suspended in plasma

Erythrocyte ghosts were prepared according to the method of Dodge et al (1962). Briefly, to fresh human erythrocytes of type A^+, washed three times with isotonic phosphate buffer (100 mM, pH 7·4), was added ice-cold hypotonic phosphate buffer (5 mM, pH 7·4). After 10 min the lysed erythrocytes were centrifuged at 30 000 *g* for 20 min (Sorvall RC-5B, GSM rotor) and the membranes harvested after washing 5 times with hypotonic buffer.

Suspensions of ghosts in plasma of 1, 5 or 9% v/v were prepared and incubated with artemisinin as described above at an initial drug concentration of 1600 ng mL^{-1}.

Haemoglobin and carbonic anhydrase interactions

Human haemoglobin (MW ~64 500) and carbonic anhydrase II (MW ~30 000), both from Sigma, USA, were dissolved in phosphate buffer (100 mM, pH 7·4) to give concentrations of 90 and 0·45 mg mL^{-1} respectively, and added to different E-flasks containing artemisinin to provide initial drug concentration of 1500 ng mL^{-1}. To a third flask containing artemisinin was added phosphate buffer only. Carbonic anhydrase and phosphate buffer experiments were performed under air. The incubation with haemoglobin was performed under either carbogen gas or an argon-carbon dioxide mixture with the pH and pO_2 determined at 0 and 8 h. From each incubation flask, gently agitated at 37°C, six samples of 300 μL were taken at various time points (0–8 h). The samples were immediately ultrafiltered (MPS-1, Amicon, USA) at 37°C on a Sorvall RC-5B centrifuge with a SM 24 rotor at 1500 *g* for 6 or 8 min for carbonic anhydrase and haemoglobin solutions, respectively. YM3 (3 000 d cut-off) and YMT (30 000 d) filters were used for carbonic anhydrase and haemoglobin solutions, respectively. Protein leakage through the membrane was checked by addition of 35% perchloric acid and checking for absence of precipitate in the filtrate.

Extraction procedure

Plasma samples (0·5 mL) were mixed with 1·0 mL potassium phosphate buffer (0·3 M, pH 3·5) and 0·1 mL 30% H_2O_2, placed at 4°C for 15 min and then extracted with 4 mL hexane/*n*-butanol (93·75 : 6·25). Three mL of the organic phase was transferred to a new test tube and evaporated under N_2 at 40°C. The residue was dissolved in 0·3 mL mobile phase (acetonitrile : acetate buffer; 60 : 40, pH 4·0), vortexed and centrifuged at 1000 *g* (Hettich EBA 30) for 5 min. All the chemicals were from Merck (Germany).

Analysis of the samples

Artemisinin was quantitated using a reversed-phase HPLC-system with on-line post-column alkali derivatization and UV-detection according to a slightly modified method of Edlund et al (1984). Buffer samples were injected directly into the HPLC. The HPLC equipment consisted of two LC-9A pumps, a SPD-6A detector and an C-R5A integrator, all from Shimadzu (Japan) and an CMA/2000 autoinjector cooled to 7°C. A Chromsphere 3, C18 (100 mm × 4·6 mm, i.d.) column with a R2-guard, both from Chrompack (Netherlands) was used for separation. Prior to UV-detection (289 nm) the column effluent was allowed to mix with 1 M KOH-solution in 95% ethanol (Kemetyl AB, Sweden) at 70°C in a 5-m knitted teflon capillary coil. The limit of quantitation in plasma was 10 ng mL^{-1}, at which level the intraassay coefficient of variation for replicate samples was 12%, and with an interassay coefficient of variation less than 6%, based on 6 quality control samples in each run at concentrations between 40 and 1000 ng mL^{-1}.

Data analysis

The half-life for artemisinin disappearance and the area under the plasma concentration-time curve (AUC), evaluated until the last sampling time point, were calculated by log-linear regression and by the trapezoidal rule, respectively.

Results

In all incubations containing red blood cells or haemoglobin, artemisinin medium concentrations first increased due to dissolution of pre-dosed drug, then declined with a rate markedly increased under oxygen-poor, compared with oxygen-rich, atmospheric conditions (Table 1). No dose-dependency in the disappearance of artemisinin was found whether in the presence of carbogen gas or argon-carbon dioxide mixture.

The decline of artemisinin plasma concentrations in erythrocyte suspensions became more pronounced with increasing haematocrit under an argon-carbon dioxide atmosphere (Fig. 1) but was not influenced by haematocrit in the presence of carbogen gas. Artemisinin was stable in plasma irrespective of atmosphere during the incubations.

Artemisinin concentrations did not change during incubation with 1, 5 and 9% erythrocyte ghost suspensions in plasma regardless of experimental condition (Fig. 2).

Artemisinin was stable in phosphate buffer and in the carbonic anhydrase solution. The drug exhibited a rapid disappearance with a half-life of approximately 14 min in the 90 mg mL^{-1} haemoglobin solution under an oxygen-rich atmosphere. Under the argon-carbon dioxide atmosphere, all artemisinin concentrations except one single determination at zero time were below the limit of quantitation.

Oxygen tension was expectedly higher in mediums exposed to carbogen gas compared with those exposed to the argon-carbon dioxide mixture (Table 2). Medium pH values were stable throughout the experiments.

Discussion

Earlier observations regarding the degradation of artemisinin derivatives by human blood components led us to investigate

Table 1. Artemisinin AUCs and disappearance half-lives as measured in plasma following incubations under either carbogen gas or an argon-carbon dioxide atmosphere in whole blood or erythrocyte suspensions of varying haematocrit.

Atmosphere	95% O_2–5% CO_2			95% Ar–5% CO_2		
Initial Artemisinin concn (ng mL^{-1})	400	800	1600	400	800	1600
Whole blood						
$t_{\frac{1}{2}}$ (h)	9·5	10·5	10·7	1·5	1·7	2·8
AUC (min μg mL^{-1})	169	332	691	70	184	391
AUC/D (min mL^{-1})	4·2	4·2	4·3	1·8	2·3	2·4
RBC 5%						
$t_{\frac{1}{2}}$ (h)	20·3	40·6	17·8	7·1	8·5	9·1
AUC (min μg mL^{-1})	161	330	645	130	283	569
AUC/D (min mL^{-1})	4·0	4·1	4·0	3·3	3·5	3·5
RBC 18%						
$t_{\frac{1}{2}}$ (h)	4·2	16	21·3	1·9	2·0	3·0
AUC (min μg mL^{-1})	155	337	664	94	212	455
AUC/D (min mL^{-1})	3·9	4·2	4·2	2·4	2·7	2·8
RBC 33%						
$t_{\frac{1}{2}}$ (h)	7·7	8·3	7·7	1·5	1·4	0·9
AUC (min μg mL^{-1})	141	295	602	69	146	312
AUC/D (min mL^{-1})	3·5	3·7	3·8	1·7	1·8	2·0

$t_{\frac{1}{2}}$ = Half-life. AUC/D = Area under the plasma concentration–time curve divided by the dose. Each value represents one measurment.

the kinetics of this interaction using a specific, non-radiolabel method for drug quantitation. Furthermore, the influence of oxygen tension and also the influence of dose- or haematocrit-dependencies, or both, were investigated. In agreement with previous studies (Edwards et al 1992; Muhia et al 1994) we observed a disappearance of artemisinin when it was incubated in whole blood or erythrocyte suspensions. In addition, we found a marked difference in artemisinin disappearance rates depending on whether the incubations were performed under an atmosphere rich or poor in oxygen. This finding was consistent in all incubations with either whole blood, erythrocyte suspensions, and, as far as could be ascertained, also with haemoglobin-containing solutions. We hold it unlikely that the difference in plasma concentration decline rates reflect a change in the capacity for uptake or non-specific binding in the blood cells. With the evidence to support artemisinin degradation being catalysed by haem iron (Meshnick et al 1991, 1993), our results indicate the rate of this decomposition to be oxygen-dependent. A direct competition between oxygen and the peroxide bridge for binding to haemoglobin may exist. The enhanced disappearance of artemisinin in the absence of oxygen leads to the deduction that an increased formation rate of free radicals under conditions of low oxygen tension, such as those expected within the *Plasmodium falciparum* parasite (Trager & Jensen 1976; Allison & Eugui 1983), may contribute to the selective toxicity of the artemisinin class of antimalarials.

The rate of artemisinin disappearance increased with higher haematocrit of the erythrocyte plasma suspensions under the argon-carbon dioxide atmosphere. However, the disappearance was more rapid in the 33%-erythrocyte suspension compared

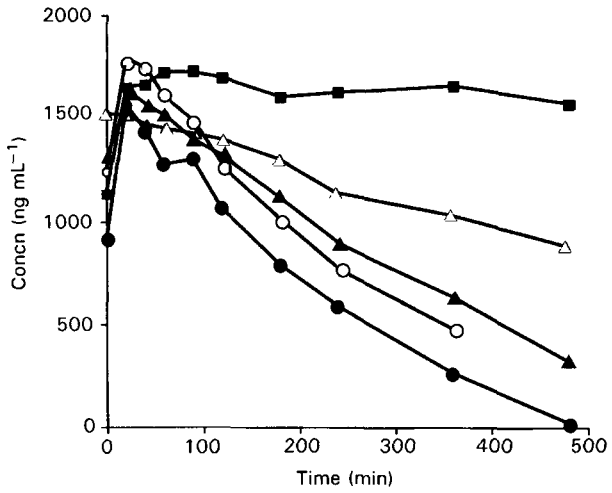

FIG. 1. Artemisinin plasma concentrations in-vitro during incubation with human whole blood (○), plasma (■) and erythrocyte-plasma suspensions of 5% (△), 18% (▲), 33% (●) haematocrits under a 95% : 5% argon-carbon dioxide atmosphere in flasks pre-dosed to yield initial drug concentrations of 1600 ng mL^{-1}.

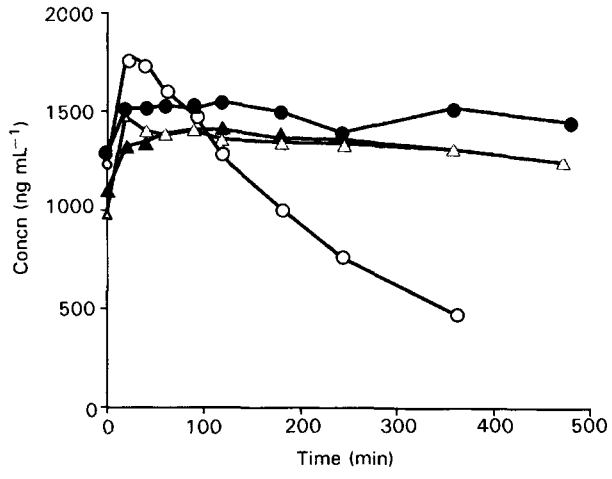

FIG. 2. Artemisinin plasma concentrations in-vitro during incubation with human whole blood (○) and with erythrocyte ghosts 1% (●), 5% (▲), 9% (△) v/v suspended in plasma under a 95% : 5% argon-carbon dioxide atmosphere in flasks pre-dosed to yield initial drug concentrations of 1600 ng mL^{-1}.

Table 2. pH and pO_2 during incubations with whole blood, plasma, erythrocyte and erythrocyte ghost suspensions under either an oxygen-rich or oxygen-poor atmosphere.

Atmosphere	95% O_2–5% CO_2			95% Ar–5% CO_2		
Time (h)	0	4	8	0	4	8
pO_2 (kPa)	23 ± 6·9	83·3 ± 2·6	82·8 ± 4·2	23 ± 6·9	2·4 ± 2·0	2·2 ± 2·6
pH	7·4 ± 0·2	7·2 ± 0·1	7·2 ± 0·2	7·4 ± 0·2	7·3 ± 0·1	7·2 ± 0·1

Values are mean + s.e.m. (n = 18 per time-point).

with whole blood, whereas the opposite was expected. This could not be attributed to different degrees of haemolysis in the incubations. A difference in oxygen levels may have existed although this was not evident when comparing oxygen tensions for these incubations. An interaction of the compound with other blood cells can not be ruled out.

In contrast to previous observations of dihydroartemisinin binding to erythrocyte membranes but not to intact red blood cells (Asawamahasakda et al 1994), our findings clearly demonstrate an interaction between artemisinin and intact red blood cells and do not confirm any quantitative binding to erythrocyte membranes. This apparent discrepancy may be explained by our quantitation method being selective for unchanged artemisinin unlike in previous studies which were based on radiolabelled compound.

It has been proposed that the putative binding of artemisinin compounds to erythrocyte membranes invalidates pharmacokinetic investigations based on plasma concentrations and their relevance to pharmacological response (Edwards et al 1992). However, should there be significant binding to blood cell membranes (for other derivatives aside from artemisinin), one may expect such binding to be non-specific and of no relevance to the therapeutic effects of the drug. Such interactions would be of little concern since they would not influence intraparasitic concentration of unbound drug, for which the determining factor is the plasma level of the unbound drug. We therefore conclude that pharmacokinetic studies based on plasma drug levels are appropriate and that their interpretation carry clinical relevance. Further investigations may demonstrate whether it is necessary to include drug disappearance in blood as an irreversible clearance in a pharmacokinetic model. However, with an in-vivo plasma half-life of approximately 2 h in patients (Hassan Alin et al 1996), the contribution of drug irreversibly lost due to interaction with blood cells to the overall elimination is likely to be marginal. It is, on the other hand, evident that when collecting and handling blood samples one should ensure uniform treatment and plasma separated without undue delay.

Our results also illustrate the precariousness of relating clinically observed drug concentrations with potency parameters such as IC50 determined by in-vitro susceptibility tests. The Candle Jar method (Jensen & Trager 1977) employs conditions quite similar to our experiment with 5% haematocrit under an argon-carbon dioxide atmosphere in which the artemisinin half-life was about 8 h. Thus actual drug concentrations during and especially towards the termination of an in-vitro susceptibility incubation may be expected to have declined considerably from the spiked, initial concentrations used in the data analysis.

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