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Stability of Artemisinin in Aqueous Environments: Impact on its Cytotoxic Action to Ehrlich Ascites Tumour Cells

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Abstract

We have recently shown artemisinin to be cytotoxic against Ehrlich ascites tumour cells. The aim of this study was to investigate the stability of this compound in the aqueous environment of the in-vitro Ehrlich ascites tumour cell system (RPMI 1640 cell culture medium supplemented with 10% foetal bovine serum (RPMI/FBS) with reference to its cytotoxic action.

Literature data show that artemisinin can react with Fe^{2+} yielding reactive intermediates leaving artemisinin G as a major end-product. The current study showed that only excess addition of Fe^{2+} to artemisinin in distilled water, phosphate-buffered saline (PBS) and RPMI/FBS and incubation for 24 h led to degradation of artemisinin and yielded artemisinin G. If Fe^{2+} was not added results from HPLC analysis were indicative of complete recovery of artemisinin from distilled water and RPMI/FBS, with or without cells, at 37°C for at least 24 h. In addition, incubation of artemisinin in RPMI/FBS with or without cells at 37°C for 24 h before cytotoxicity assay did not change its cytotoxic action.

On the basis of these results, we suggest that cytotoxicity to tumour cells was caused by unchanged artemisinin. This is not so for the antimalarial activity of artemisinin and derivatives, for which the presence of a pool of (haem) Fe^{2+} is a prerequisite resulting in free radicals or electrophilic intermediates or both.

Artemisinin (Fig. 1, **1**), a sesquiterpene lactone endoperoxide isolated from the plant *Artemisia annua* L. (Asteraceae), is the parent compound of a novel class of antimalarials which is effective against multi-drug-resistant *Plasmodium falciparum* strains (Klayman 1985; Woerdenbag et al 1994). Previously, we have reported that artemisinin and a number of derivatives were cytotoxic to Ehrlich ascites tumour cells (Woerdenbag et al 1993; Beekman et al 1997). The cytotoxic action of artemisinin to tumour cells might be caused by a chemical reaction of the compound or its degradation products with cellular targets, or as a result of interaction of the intact compound without chemical reaction.

It is known that artemisinin can react with free Fe^{2+} ions or haem. Several authors have investigated this reaction and the end-products were characterized. However, these reactions were performed in non-physiological environments under circumstances optimum for the reaction (Posner et al 1995; Haynes & Vonwiller 1996a, b). In another study, the reaction of artemisinin with (haem) Fe^{2+} was found to yield free-radical or electrophilic intermediates toxic to malaria parasites inside red blood cells (Meshnick et al 1993, 1996).

It is possible that reactions needed for the antimalarial activity of artemisinin also account for its cytotoxic activity. The possible reaction of artemisinin and Fe^{2+} was studied in aqueous environments, including the medium of the in-vitro tumour-cell test-system consisting of RPMI 1640 culture medium with 10% foetal bovine serum (RPMI/FBS). This reaction was compared with the behaviour of artemisinin in aqueous environments without the addition of Fe^{2+}. Similarities in the extent of degradation of artemisinin and the appearance of a characteristic end-product might reveal whether reaction of artemisinin with Fe^{2+} or other reactants, present in the medium in small amounts, is the basis of the cytotoxicity of artemisinin to tumour cells.

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Materials and Methods

Test compound

The sesquiterpene lactone artemisinin (Fig. 1, **1**) isolated from the plant *Artemisia annua* L. (Asteraceae) was kindly provided by Artecef BV (Maarssen, The Netherlands). The identity and purity of the compound were checked by standard spectroscopic and chromatographic techniques. Before each experiment a fresh stock solution of the compound was prepared in 100% dimethylsulphoxide.

Artemisinin and Fe^{2+} in aqueous solution

A stock solution of artemisinin (MW 282, 100 mM in 100% dimethylsulphoxide) was diluted with either 5 mL distilled water, or in phosphate-buffered saline (PBS, 1·6 mM KH_2PO_4, 6·5 mM $Na_2HPO_4.12H_2O$, 0·137 mM NaCl, 2·7 mM KCl, pH 7·4), or with the tumour cell system RPMI 1640 cell culture medium (Gibco, Paisley, Scotland) supplemented with 10% foetal bovine serum (FBS, Gibco). The end-concentration of artemisinin was 0·1 mM; this was incubated with 0, 0·1, 1, or 10 mM $Fe(NH_4)_2(SO_4)_2.6H_2O$. Incubations were performed in polystyrene Petri dishes with ventilation ridges (Greiner, Frickenhausen, Germany) at 37°C in a humidified incubator with 5% CO_2. At 0 and 24 h, 1·0-mL samples were taken and extracted twice with toluene (Merck, Darmstadt, Germany,

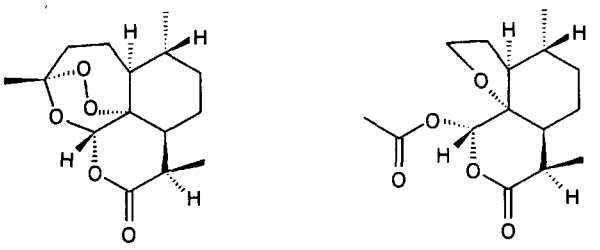

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FIG. 1. Structural formulae of artemisinin (**1**) and artemisinin G (**2**).

p.a.; 1·0 mL) in 2-mL Eppendorf cups. The toluene–water mixtures were vortex-mixed and subsequently centrifuged to achieve good separation. The toluene phase was evaporated to dryness, and stored at −20°C until further analysis. The reaction of artemisinin and Fe^{2+} was monitored by TLC (see below for technical details).

Artemisinin in aqueous solutions

Artemisinin was diluted (100 mM stock in 100% dimethylsulphoxide) either in 5 mL distilled water, or in PBS pH 7·4 or 8, or in RPMI 1640 cell culture medium with 10% FBS with or without 10^6 tumour cells mL^{-1}. A cloned Ehrlich ascites tumour cell line was used (EN2). The final concentration of artemisinin was 0·1 mM. Incubation and extraction of the compound were performed as described above. Samples were taken at 0, 24 and 72 h. The EN2 cells, as determined with the trypan blue exclusion method, were still viable after 72 h incubation in the presence of 0·1 mM artemisinin.

Analysis of artemisinin and degradation products

The dried residues of the toluene extracts were dissolved in toluene (20 μL) and 1 μL was used for TLC analysis on 20 × 10 cm silica gel GF254 TLC plates (Merck) in an unsaturated chamber with 40–60 petroleum ether–diethyl ether (1:1) as mobile phase. The development distance was 6 cm. Detection was accomplished by dipping in a reagent solution consisting of sulphuric acid (96%, 1 mL), glacial acetic acid (50 mL) and anisaldehyde (0·5 mL) for a few seconds followed by heating at 105°C for 6 min. Artemisinin was revealed as a pink-red spot of R_F 0·51. The reaction of artemisinin and $Fe(NH_4)_2(SO_4)_2.6H_2O$ yielded the orange spot of artemisinin G (Fig. 1, **2**) of R_F 0·31. This compound is the most important end-product of the reaction and has been described by several authors (Wei et al 1992; Posner et al 1995; Haynes & Vonwiller 1996b). Under 366 nm UV light artemisinin was fluorescent orange whereas artemisinin G was green. GC-MS and ^1H NMR data for artemisinin G were in agreement with those obtained by Wei et al (1992). Artemisinin was quantified by comparing the intensity of the spots with those obtained from a series of standard solutions. Scanning of the intensities was performed with the HP Scanjet IICx and the software Aldus Photostyler 2·0 and SigmaScan Image.

For HPLC analysis the residues of the toluene extracts were dissolved in 200 μL methanol and artemisinin was hydrolysed in alkaline solution as described elsewhere (Pras et al 1991). The derivative of artemisinin (Q260) was separated on a 100 × 4·6 mm i.d. Chromsep Microspher C_{18} column equipped with a guard column (Chrompack, Middelburg, The Netherlands). The mobile phase was 11:9 phosphate buffer (10 mM $NaH_2PO_4.H_2O$, 5 mM $Na_2HPO_4.2H_2O$)–methanol, pH 7·0, at a flow rate of 1 mL min^{-1}. The artemisinin derivative was detected at 260 nm. The retention time was approximately 4 min. Standard solutions were derivatized and analysed with the other samples. Q260 is not stable for longer (<1 week).

Cytotoxicity assay for artemisinin

The cloned Ehrlich ascites tumour cells (EN2) were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS, 0·2 mg mL^{-1} streptomycin and 200 int. units mL^{-1} penicillin G, at 37°C in a shaking incubator. The doubling time of the cells was ca 12 h. Exponentially growing cells were used for all experiments. In all experiments over 95% of the cells excluded trypan blue. The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed as described previously (Beekman et al 1996), but in the current study the cells were continuously (72 h) exposed to the test compound. Incubations were performed as described above. Samples were taken after 24 h and diluted to appropriate concentrations in fresh RPMI 1640 medium with 10% FBS. When the pre-incubation was performed with cells, the cells were precipitated by centrifugation and the supernatant was used for the experiment. The concentration of a compound resulting in 50% growth inhibition (IC50) was determined and used as a parameter for cytotoxicity.

Statistics

Student's *t*-test was used for statistical evaluation of the data.

Results and Discussion

Artemisinin and $Fe(NH_4)_2(SO_4)_2.6H_2O$ were incubated in aqueous solution. The occurrence of a reaction was demonstrated by TLC, results from which were indicative of a significant decrease in the amount of artemisinin and the concomitant appearance of the main product artemisinin G (Fig. 1, **2**) as an orange spot (Table 1). GC-MS and ^1H NMR data for artemisinin G were in agreement with those found by Wei et al (1992). Artemisinin could be quantitated by this TLC method; TLC was used for analysis of artemisinin because artemisinin G could not be detected by the HPLC method. The TLC spot obtained for artemisinin G was used for visual monitoring of the reaction.

Table 1 shows the recoveries of 0·1 mM artemisinin after incubation with concentrations of Fe^{2+} ranging from 0–10 mM for 0 and 24 h. Reaction of artemisinin and 1 mM Fe^{2+} occurred in water after incubation for 24 h, but not in PBS or RPMI/FBS medium. The reason for this might be that ions present in both PBS and the RPMI/FBS interacted with Fe^{2+} ions. Precipitation was visible with 1 and 10 mM Fe^{2+} in PBS, but not in RPMI/FBS. Because reaction with artemisinin was obtained on addition of 10 mM Fe^{2+}, this high concentration apparently exceeded the Fe^{2+}-trapping capacity of PBS and RPMI/FBS. In general, at 0 h the recovery of artemisinin from the solutions was approximately 100% with 0–10 mM Fe^{2+} added. In PBS, the recoveries obtained at 0 h were somewhat lower than those at 24 h. This might be because of the low rate of dissolution of artemisinin in PBS. Surprisingly, artemisinin in RPMI/FBS and 10 mM Fe^{2+} reacted at 0 h resulting in reduced artemisinin recovery and the appearance

Table 1. Percentage recovery of artemisinin from 0·1 mM solutions mixed with 0, 0·1, 1 and 10 mM $Fe(NH_4)_2(SO_4)_2.6H_2O$ ($=Fe^{2+}$) in distilled water, phosphate-buffered saline (PBS), and RPMI 1640 cell culture medium +10% foetal bovine serum (RPMI/FBS).

Time (h)	Amount of Fe^{2+} (mM)	Distilled water	Phosphate-buffered saline	RPMI 1640 cell culture medium + 10% foetal bovine serum
0	0	98·8+5·7	85·2 ± 8·7	94·1 ± 19·4
	0·1	93·1 ± 6·1	79·8 ± 6·0	96·9 ± 15·9
	1	97·1 ± 14·8	79·4 ± 7·6	97·8 ± 17·3
	10	73·5 ± 26·2	77·8 ± 9·2	4·9 ± 7·5*
24	0	124 ± 12·0	108 ± 22·4	112 ± 14·7
	0·1	102 ± 11·4	103 ± 24·1	104 ± 9·6
	1	26·6 ± 10·4*	110 ± 18·0	91·8 ± 4·4
	10	0*	2·4 ± 3·2*	5·8 ± 5·8*

Values (mean ± s.d., n = 4) were determined by quantitative TLC. Incubation was performed at 37°C for 0 and 24 h. Significantly reduced recovery of artemisinin was always accompanied by visual detection of the orange-coloured spot of artemisinin G. *$P < 0·005$, significantly different from result for 0 mM Fe^{2+}.

of artemisinin G. In view of the putative Fe^{2+}-trapping capacity of RPMI/FBS, the reason for this remains unknown. In conclusion, it was demonstrated that the reaction of artemisinin and Fe^{2+} did occur in PBS and RPMI/FBS, but only when Fe^{2+} was added and artemisinin was present at a molar ratio of at least 100:1 after 24 h incubation.

To investigate the cytotoxicity of artemisinin after reaction with Fe^{2+}, both reactants (1 mM) were incubated in 85:15 dimethylsulphoxide–water or in RPMI/FBS for 30 min. The reaction was monitored by TLC. EN2 cells treated with a 100-fold-diluted sample of these reaction mixtures did not have any effect whereas artemisinin alone at this concentration resulted in almost complete inhibition of growth (72 h incubation in the MTT assay, data not shown). Thus, the end-products of the reaction, which includes artemisinin G as a major product, were not cytotoxic. No enhancement of cytotoxicity to EN2 cells (cultured in RPMI/10% FBS) was found when artemisinin (1 nM–1 μM) was incubated with $Fe(NH_4)_2(SO_4)_2.6H_2O$ (0·1 nM–10 μM) in the presence of cells after 72 h exposure in the MTT assay. Obviously, the reaction of artemisinin and Fe^{2+} did not occur in these concentration ranges. This might be because of the Fe^{2+}-trapping capacity of the culture medium as suggested above. In principle, free-radical intermediates formed by the reaction of artemisinin with haem or Fe^{2+} are likely to be cytotoxic; Fe^{3+} does not react with artemisinin (Zhang et al 1992; Meshnick et al 1993). The in-vitro cytotoxicity of artemisinin derivatives to neuroblastoma cells was not changed by Fe^{2+} (Parker et al 1994), but haem enhanced the cytotoxic effect (Smith et al 1997). Yet it has been demonstrated that the toxicity of artemisinin to red-blood-cell membrane proteins can be enhanced by incubation with both Fe^{2+} and haem (Meshnick et al 1993; Wei & Sadrzadeh 1994). Artemether, a methyl ether of dihydroartemisinin, seemed to be degraded by haem, which is thought to be responsible for the instability of artemether in whole blood (Muhia et al 1994). Apparently, the haem-iron entity was somehow kept in the reduced state, because it is known that free haem in an aqueous environment is immediately oxidized by oxygen (i.e. formation of haem-Fe^{3+}).

HPLC analysis was used to determine the recovery of 0·1 mM artemisinin from water, or RPMI/FBS with or without cells. Table 2 shows that the recovery of artemisinin was complete after incubation at 37°C in each solution for 24 h. In contrast, the reaction of artemisinin and Fe^{2+} was followed by a sharp decrease in the recovery of artemisinin (Table 1). Artemisinin and its derivative arteether were found to be stable when stored at 37°C in water, plasma or serum for 24 h (Edwards et al 1992; Bakhshi et al 1997). Despite complete recovery of artemisinin, TLC analysis revealed the presence of an additional faint pink-red spot after 0 h incubation in any solution; this increased in intensity after 24 h incubation in RPMI/FBS. It had the same R_F value (0·34) as artemisinin G, but its colour was identical to that of artemisinin in daylight and under UV at 366 nm. GC-MS of this compound recovered from the TLC plate showed its mass to be 298, 16 higher than that of artemisinin. In contrast, artemisinin G has a molecular mass of 282. Further elucidation of the structure of the unknown compound was greatly inhibited by the small yield. The ^1H NMR spectrum of artemisinin incubated in RPMI/FBS at 37°C for 48 h was not different from that of artemisinin

Table 2. Percentage recovery of artemisinin from 0·1 mM solutions in distilled water, RPMI 1640 cell culture medium + 10% foetal bovine serum (RPMI/FBS), and RPMI/FBS with 10^6 cloned Ehrlich ascites tumour cells (EN2) mL^{-1}.

Time (h)	Distilled water	RPMI 1640 cell culture medium +10% foetal bovine serum	RPMI 1640 cell culture medium +10% foetal bovine serum +10^6 cloned Ehrlich ascites tumour cells (EN2) mL^{-1}
0	103 ± 7·1	101 ± 2·8	102 ± 2·9
24	106 ± 22·3	95·5 ± 3·8	94·7 ± 4·2
72	98·9 ± 7·6	67·0 ± 3·4*	68·5 ± 1·6*

Values (mean ± s.d., n = 3) were determined by HPLC. Incubation was performed at 37°C for 0, 24 and 72 h. *$P < 0·01$, significantly different from result for water.

standard. Because NMR is a very sensitive method, which needs a pure compound for a good spectrum, we inferred that the unknown compound must be present at trace levels only. Although the unknown could be detected by TLC, we consider this compound to be a minor impurity.

After 72 h recovery of artemisinin declines significantly to approximately 70% in RPMI/FBS with or without cells, but not in water. Artemisinin might be degraded by chemical hydrolysis of the lactone, but if so it is unclear why artemisinin was not degraded in water. In addition, lactone hydrolysis is catalysed by acid, which is not present in the RPMI/FBS cell culture medium buffered at pH 7·2. The identical decrease of the recovery of artemisinin in RPMI/FBS with or without cells suggested that cellular metabolic reactions (i.e. enzymatic degradation) did not play a role. TLC analysis showed artemisinin alone and the unknown compound described in the previous paragraph. Because the TLC method used is rather non-specific, sesquiterpene-related degradation compounds would also have been detected. We believe that binding of artemisinin or possible degradation products to components present in the medium might account for reduced recovery from RPMI/FBS, and RPMI/FBS containing EN2 cells after 72 h incubation. Apparently, the binding was resistant to the toluene extraction method. Artemisinin has been found to bind strongly to albumin (Yang et al 1993), which is present in the RPMI/FBS cell culture medium.

The effect of pre-incubation of artemisinin on its cytotoxic action was determined by measuring its effect on EN2 tumour cells in the MTT assay after 72 h incubation. The IC50 value without pre-incubation was $0{\cdot}86 \pm 0{\cdot}31$ μM. Pre-incubation of artemisinin for 24 h in RPMI/FBS with or without cells yielded IC50 values of $0{\cdot}91 \pm 0{\cdot}30$ and $1{\cdot}2 \pm 0{\cdot}29$ μM respectively (mean $\pm$ s.d., $n \geq 3$). There are no significant differences between these values. Thus, pre-incubation of artemisinin for 24 h did not affect its cytotoxic activity as detected with the MTT assay.

We were able to recover artemisinin completely from the aqueous solutions. Basically, it cannot be excluded that cytotoxicity to tumour cells was caused by a very small undetectable fraction of artemisinin yielding free-radical intermediates or toxic degradation products, or both. However, end-products from the reaction of artemisinin and Fe^{2+} were not cytotoxic (see above). In addition, the cytotoxic activity of artemisinin was not influenced by pre-incubation of the compound in aqueous environments. Free-radical intermediates might have been formed during the pre-incubation experiment. Because of the instability of such intermediates they cannot result in cytotoxicity after the long period of 24 h pre-incubation. Significant artemisinin cytotoxicity to EN2 cells cultured in RPMI/FBS was already apparent after 2 h incubation in the MTT assay (Beekman et al 1996). Reactive intermediates might still have destroyed vital nutrients of the RPMI/FBS medium, but before the MTT assay the artemisinin was diluted with fresh medium. The tripeptide glutathione might inhibit the reactive intermediates arising from the possible reaction of artemisinin and Fe^{2+} present in the medium. However, we found that glutathione depletion by buthionine sulphoximine (Griffith & Meister 1979) did not influence the cytotoxicity of artemisinin (data not shown).

Artemisinin and its derivatives contain an endoperoxide which is responsible for the reaction with (haem) Fe^{2+} and the formation of free radical or electrophilic intermediates, or both, resulting in toxicity against malaria parasites (Meshnick et al 1993, 1996). Deoxyartemisinin, which has an ether bridge instead of the endoperoxide bridge, was inactive against malaria parasites (Klayman 1985). We found that deoxyartemisinin was 100 times less toxic than artemisinin against EN2 cells (Beekman et al 1997). Thus, the endoperoxide group also seemed to play an important role in cytotoxicity against tumour cells. The same conclusion was drawn by others investigating the effect of artemisinin and derivatives on neuronal cancer cells (Wesche et al 1994; Fishwick et al 1995). These investigations on neuronal cancer cells were performed because of the in-vivo neurotoxicity of artemsinin and derivatives (Brewer et al 1994). Although Smith et al (1997) suggested that reaction of the endoperoxide with haem was responsible for neurotoxicity, the mechanism of action remains unclear. The current study showed that artemisinin was able to react with Fe^{2+} in RPMI/FBS, but only when Fe^{2+} was added exogenously in large amounts. HPLC analysis provided evidence for the stability of artemisinin in different aqueous environments without added Fe^{2+}, including culture medium with EN2 tumour cells. Apparently, traces of Fe^{2+} or other reactants present in the medium were not sufficient to react with artemisinin. Although the endoperoxide has a weak covalent bond, it was found to be remarkably stable in neutral environments without Fe^{2+} added (Zeng et al 1983; Lin et al 1985). In contrast with the antimalarial action of artemisinin, reaction of the endoperoxide group might not be responsible for the cytotoxic activity against EN2 tumour cells. Instead, the intact endoperoxide might play a role in cytotoxicity. Because artemisinin inhibited growth (Beekman et al 1996), we suggest that the activity of this compound might be based on reversible binding to cellular targets involved in the cell cycle.

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