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Enhanced *In Vitro* Neurotoxicity of Artemisinin Derivatives in the Presence of Haemin

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ABSTRACT. The role of haem in the neurotoxicity of artemisinin derivatives has been studied *in vitro* by examining neurite outgrowth measured by image analysis and cellular metabolism of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) measured spectrophotometrically in the neuroblastoma cell line NB2a, and by examining binding of radiolabelled dihydroartemisinin to NB2a cell and rat brain proteins. In the cases of artemether, dihydroartemisinin, and arteether, haemin (ferriprotoporphyrin IX) significantly increased the dose-related inhibition of neurite outgrowth from differentiating NB2a cells and significantly increased the dose-dependent inhibition of MTT metabolism. Inhibition of neurite outgrowth and metabolism of MTT in the presence or absence of haemin ranged from 72% to 93% and from 27% to 49% at a drug concentration of 300 nM. Haemin also significantly increased the dose-related binding of radiolabelled dihydroartemisinin to proteins from NB2a cells approximately twofold and to rat brain between three- and sixfold. Haemin did not enhance the neurotoxicity of desoxyarteether, a structural analogue of arteether with an ether linkage in the place of the endoperoxide bridge. It is suggested that haemin may catalyse the transformation of these derivatives via an interaction with the endoperoxide bridge of the artemisinin derivative to produce free radicals or electrophilic intermediates that are toxic to neuronal cells. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:5–10, 1997.

KEY WORDS. artemisinin; neurotoxicity; haem; iron; neuroblastoma; free radical

Artemisinin and its derivatives have become increasingly important as antimalarial drugs with impressive activity against multi-drug resistant forms of *Plasmodium falciparum* both *in vivo* and *in vitro* [1, 2]. Artemisinin is the active principle of qinghaosu, an extract of a Chinese medicinal plant (qinghao or *Artemisia anmua* L.) that has been used for over a thousand years as an herbal remedy for malaria. It is a sesquiterpene lactone with an endoperoxide bridge (Fig. 1). Despite wide clinical experience, there is concern that these drugs are being used against a background of limited pharmacological information. Few detailed pharmacological studies in humans have been performed, and investigations of metabolism have been confined to animal and microbial models [3–5].

As if to emphasize this deficiency, dose-related and anatomically specific neuropathology was demonstrated in two independent studies in dogs and rats injected IM with moderately high doses of artemether or arteether [6].§ The neuropathies were specific to the caudal brain stem in both animal models and were characterized by swelling of axonal processes and spheroid formation with associated axonal degeneration and necrosis in myelinated axons. Moreover, studies carried out in *Macaca mulatta* monkeys showed dose-dependent neuropathology in the same regions as in the rodent and canine brains [7]. These histological changes have been found in the absence of neurological signs or deficits in behavioral performance [7, 8]. In addition to these *in vivo* findings, studies *in vitro* have shown that artemisinin and its derivatives are toxic to neuronal and glial cells in culture [9, 10].

The antimalarial action of the artemisinin derivatives appears to involve the intraparasitic iron- or haem-catalysed cleavage of the endoperoxide bridge to generate unstable organic free radical intermediates. These free radicals, or an electrophile, may alkylate specific parasitic proteins, causing cell death [11–14]. Because brain contains a very high concentration of iron [15], either haem or free iron may also be involved in the neurotoxic effects of these derivatives *in vivo*. That is, the mechanism of antimalarial action of the artemisinin derivatives and the mechanism of neurotoxicity may be identical. In this study, we have examined the effects of haemin on the neurotoxicity of artemisinin derivatives *in vitro* by measuring cellular metabolism and axonal or neurite maintenance in the neuronally derived tumour cell line, mouse neuroblastoma NB2a, and the binding of radiolabelled dihydroartemisinin to NB2a cell and rat brain homogenates.

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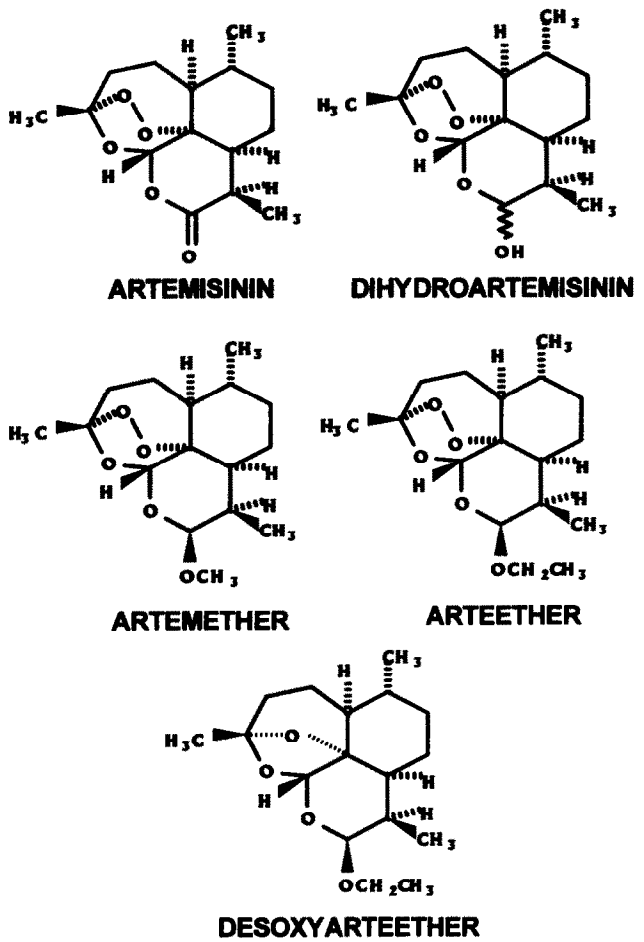

FIG. 1. Chemical structures of artemisinin and its derivatives: dihydroartemisinin, artemether, arteether, and desoxyarteether.

MATERIALS AND METHODS

Materials

Tissue culture flasks and culture plates were obtained from Falcon/Fred Baker Scientific (Runcorn, Cheshire, U.K.). DMEM[‖] with Glutamax-1, horse serum, foetal calf serum, penicillin/streptomycin solution, and gentamicin were purchased from Gibco BRL (Life Technologies Limited, Uxbridge, Middlesex, U.K.). Dibutyryl cyclic AMP, paraformaldehyde, Coomassie Brilliant Blue G, PBS tablets, PIPES, MTT, Triton-X-100, and haemin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Artemether, arteether, dihydroartemisinin, and desoxyarteether were supplied by Dr. P. Buchs (SAPEC, Lugano, Switzerland). ^{14}C-radiolabelled dihydroartemisinin was supplied by the Research Triangle Institute, Research Triangle Park (North Carolina).

[‖] *Abbreviations:* DMEM, Dulbecco's Modified Eagle's Medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulphonic acid).

Cell Culture

NB2a cells were grown in culture flasks with 5 ml of medium in a humidified 37°C incubator with 5% CO_2. Culture medium used for growing NB2a cells consisted of high glucose DMEM with Glutamax-1, supplemented with 5% (v/v) horse serum, 5% (v/v) foetal calf serum, and 100 units/ml penicillin plus 100 μg/ml streptomycin, with optional 25 μg/ml gentamicin.

Measurement of Neurite Outgrowth and MTT Metabolism

NB2a cells were plated onto 48- or 96-well culture plates at a cell density of 16,000 cells/ml to measure neurite outgrowth and cellular metabolism respectively. After 24 hr, the cells were induced to differentiate and generate neurites in the presence of the artemisinin derivatives and/or haemin by the following method: the culture medium was removed from the wells and replaced with serum-free medium plus 0.5 mM dibutyryl cyclic AMP containing artemether, arteether, dihydroartemisinin, or desoxyarteether at concentrations of 100, 200, and 300 nM for measurement of neurite outgrowth, or 100 and 300 nM for measurement of cellular metabolism, in the presence or absence of 2 μM haemin. Preliminary experiments demonstrated that 2 μM haemin was the highest concentration that itself had no effect on neurite outgrowth or MTT metabolism; neurite outgrowth was potentiated by 1 μM haemin and inhibited by haemin at concentrations greater than 2 μM. The cells were incubated for a further 24 hr, and then the following measurements were carried out.

MEASUREMENT OF NEURITE OUTGROWTH. Cells were fixed with 4% (w/v) formaldehyde in PBS for 10 min at room temperature, stained for 3 min with Coomassie Blue cell stain (0.6% [w/v] Coomassie Brilliant Blue G in 10% [v/v] acetic acid, 10% [v/v] methanol, and 80% [v/v] PBS), and then washed with PBS and viewed with a light microscope (Zeiss Axiovert 35M) linked by a video camera to a Kontron Vidas 2.0 image analyser. Subsequently, 10 different fields of approximately 20 cells were chosen for each antimalarial drug and control. A program was written using the available functions of the image analyser to permit the automatic measurement of the total length of neurites (in pixels) for the cells in a given field and to express the results as the average length of neurites per cell.

MEASUREMENT OF MTT METABOLISM. MTT (20 μl, 1 mg/ml) was added to each well and the plate incubated for a further 4 hr. Dehydrogenase enzymes present in metabolically active NB2a cells bioreduced the MTT to a coloured formazan product that was solubilized with 20% (v/v) Triton-X-100 in 0.5 M HCl (50 μl/well), and the plate was shaken for 10–15 min before the absorbance was measured at 570 nm on a microplate reader. The absorbance of the formazan product measured at 570 nm is thus related to the number of viable cells in culture.

Binding of ^{14}C-labelled Dihydroartemisinin

NB2a CELLS. NB2a cells were added to a flask at a density of approximately 1.5 × 10^6 cells/flask and then were induced to differentiate as described previously. ^{14}C-dihydroartemisinin (3.75–75 μM) with or without haemin (2 μM) was also added to the flasks. After 24 hr, the cells were harvested and washed in 100 mM PIPES (pH 6.8); the cell proteins were precipitated in 90% acetone at −20°C. The protein was then resuspended in PIPES, and aliquots were used to determine the protein-bound dihydroartemisinin by liquid scintillation counting.

RAT BRAIN HOMOGENATE. Cortex was removed from six rats (weighing approximately 250 g) and frozen immediately. Rat cortex homogenate (10 mg/ml in PIPES) was incubated for 24 hr at 37°C with 3.75–75 μM ^{14}C-dihydroartemisinin with or without 2 μM haemin. The cortex protein was then precipitated in 90% acetone at −20°C, and ^{14}C-dihydroartemisinin binding was assessed by liquid scintillation counting.

RESULTS

Neurite Outgrowth

Haemin alone (2 μM) and artemether alone at concentrations up to 300 nM had no significant inhibitory effect on NB2a neurite outgrowth (Fig. 2). However, 2 μM haemin when in combination with artemether at 200 and 300 nM significantly increased the inhibition of neurite outgrowth in a dose-related manner, by 51% and 82%, respectively, when compared with artemether alone (Fig. 2; both $P < 0.001$, N = 4, two-way ANOVA and Bonferroni modified *t*-test). Dihydroartemisinin was the only derivative tested that significantly inhibited neurite outgrowth in its own right, by 24% and 35% at concentrations of 200 and 300 nM (Fig. 2; $P < 0.05$ and $P < 0.001$, N = 4). Haemin significantly increased this inhibition of neurite outgrowth at these concentrations in a dose-related manner, by 45% and 72% (both $P < 0.001$, N = 4). Arteether and desoxyarteether up to a concentration of 300 nM had no inhibitory effect on neurite outgrowth in their own right (Fig. 3). However, haemin in combination with 100, 200, and 300 nM arteether significantly inhibited neurite outgrowth in a dose-dependent manner, by 22%, 68%, and 93%, respectively, when compared with arteether alone (Fig. 3; $P < 0.01$, $P < 0.001$, and $P < 0.001$, N = 3). Unlike the other derivatives, desoxyarteether in combination with haemin produced no increase in the inhibition of neurite outgrowth (Fig. 3).

MTT Metabolism

Haemin alone (2 μM) had no inhibitory effect on metabolism of MTT by NB2a cells (Fig. 4) Artemether at a concentration of 300 nM inhibited MTT metabolism by 15% ($P < 0.05$, N = 7). However, haemin in combination with artemether at 100 and 300 nM significantly increased the inhibition of MTT metabolism in a dose-related manner, by 20% and 49% when compared with artemether alone (both $P < 0.001$, N = 7). As with the neurite outgrowth study, dihydroartemisinin significantly inhibited MTT metabolism in its own right, by 18% and 29% at concentrations of 100 and 300 nM (Fig. 4, $P < 0.01$ and $P < 0.001$, N = 7), and this inhibition was significantly potentiated in the presence of haemin, by 13% and 39% ($P < 0.05$ and $P < 0.001$, N = 7). Arteether at concentrations up to 300 nM had no inhibitory effect on metabolism of MTT (Fig. 5). However, haemin plus arteether at concentrations of 100

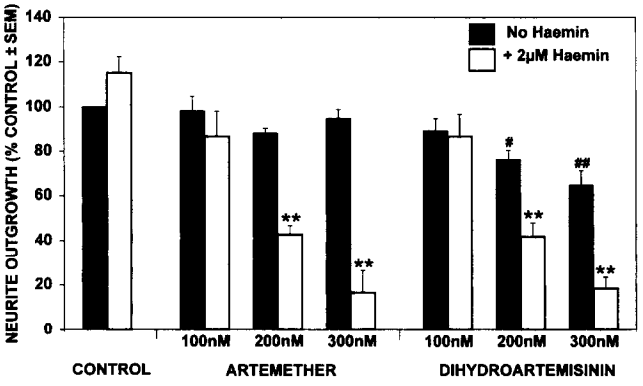

FIG. 2. Effects of artemether and dihydroartemisinin in the presence or absence of 2 μM haemin on neurite outgrowth measured by image analysis. Results are the means ± SEM of four separate experiments. ** represents a significant difference between drug-treated cells in the presence or absence of haemin at the $P < 0.001$ level (two-way ANOVA and Bonferroni modified *t*-test). # and ## represent a significant difference between drug-treated cells in the absence of haemin and untreated control cells at the $P < 0.05$ and $P < 0.001$ levels, respectively (two-way ANOVA and Bonferroni modified *t*-test).

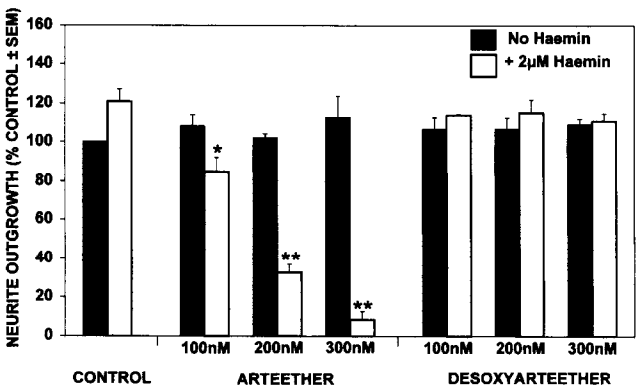

FIG. 3. Effects of arteether and desoxyarteether in the presence or absence of 2 μM haemin on neurite outgrowth measured by image analysis. Results are the means ± SEM of three separate experiments. * and ** represent a significant difference between drug-treated cells in the presence or absence of haemin at the $P < 0.01$ and $P < 0.001$ levels, respectively (two-way ANOVA and Bonferroni modified *t*-test).

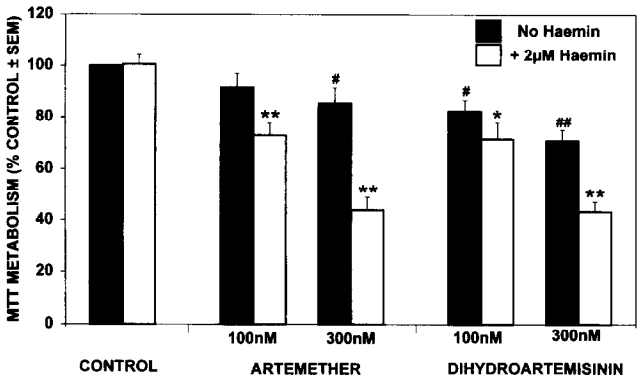

FIG. 4. Effects of artemether and dihydroartemisinin in the presence or absence of 2 μM haemin on metabolism of MTT measured spectrophotometrically. Results are the means ± SEM of seven separate experiments. * and ** represent a significant difference between drug-treated cells in the presence or absence of haemin at the $P < 0.05$ and $P < 0.001$ levels, respectively (two-way ANOVA and Bonferroni modified *t*-test). # and ## represent a significant difference between drug-treated cells in the absence of haemin and untreated control cells at the $P < 0.05$ and $P < 0.001$ levels, respectively (two-way ANOVA and Bonferroni modified *t*-test).

and 300 nM significantly inhibited MTT metabolism by 8% and 27%, respectively, compared with arteether alone ($P < 0.05$ and $P < 0.001$, N = 7). Desoxyarteether at concentrations up to 300 nM had no inhibitory effect on MTT metabolism, and, as with neurite outgrowth, haemin did not increase the inhibition of MTT metabolism by desoxyarteether (Fig. 5).

^{14}C-Dihydroartemisinin Binding

Haemin (2 μM) significantly increased the binding of ^{14}C-dihydroartemisinin to both rat brain homogenate and NB2a cells at all concentrations of dihydroartemisinin used. In differentiated NB2a cells, the haemin-induced increase in ^{14}C-dihydroartemisinin binding was 2-fold at 75 μM and 2.3-fold at 3.75 μM (Fig. 6, all $P < 0.005$, N = 3). In rat cortex homogenate, the haemin-induced increase in ^{14}C-dihydroartemisinin binding ranged from 3.3-fold at 75 mM to 6.1-fold at 3.75 μM (Fig. 7, all $P < 0.001$, N = 6). The results were not due simply to formation of a haemin–drug adduct being precipitated with acetone, because no such precipitation occurred in the absence of cells or brain homogenate.

DISCUSSION

Results reported here substantiate our previous findings and those of Wesche and colleagues [10] that artemisinin and its derivatives are toxic to neuronal cells *in vitro*. They confirm that dihydroartemisinin is the most neurotoxic derivative and the desoxy-analogues lacking the endoperoxide bridge show no significant neurotoxicity [9, 10].

In the present study, haemin significantly potentiated the inhibition of neurite outgrowth and the inhibition of cellular metabolism of MTT produced by nanomolar concentrations of the artemisinin derivatives artemether, dihydroartemisinin, and arteether, and it significantly increased the binding of ^{14}C-labelled dihydroartemisinin to NB2a cell and rat brain protein. However, haemin did not increase the inhibition of neurite outgrowth or MTT metabolism produced by desoxyarteether, a compound without the endoperoxide bridge.

The amount of haemin used was based on the maximum concentrations that had no effect on neurite outgrowth or MTT metabolism in its own right. In mice infected with *Plasmodium berghei*, levels in brain of haemozoin, the haem polymer, can reach 0.8 ng/mg tissue [19]. Assuming that the catalytic capacity of 1 g haemozoin is the same as the cata-

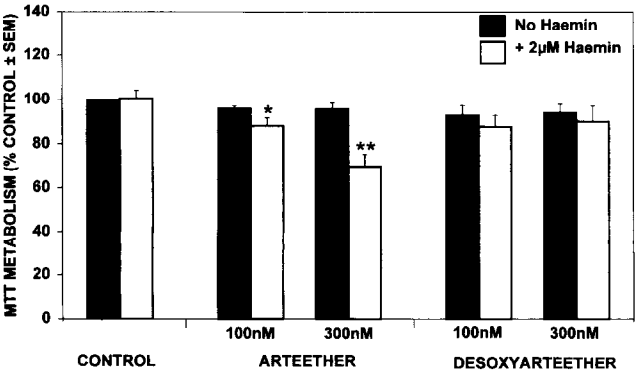

FIG. 5. Effects of arteether and desoxyarteether in the presence or absence of 2 μM haemin on metabolism of MTT measured spectorphotometrically. Results are the means ± SEM of seven separate experiments. * and ** represent a significant difference between drug-treated cells in the presence or absence of haemin at the $P < 0.05$ and $P < 0.001$ levels, respectively (two-way ANOVA and Bonferroni modified *t*-test).

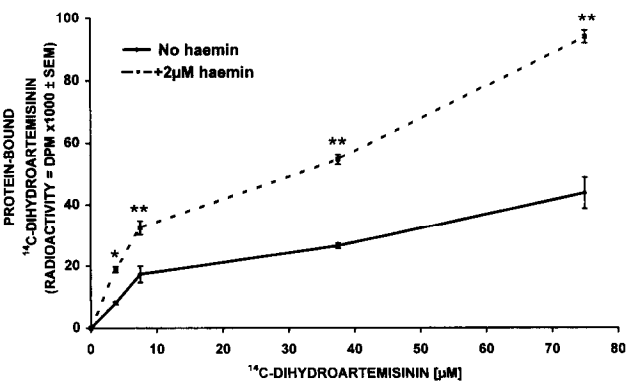

FIG. 6. The binding of ^{14}C-dihydroartemisinin to NB2a cell proteins in the presence or absence of 2 μM haemin. Results are the means ± SEM of three separate experiments. * and ** represent a significant difference between cells incubated with ^{14}C-dihydroartemisinin in the presence or absence of haemin at the $P < 0.01$ and $P < 0.001$ levels, respectively (two-way ANOVA and Bonferroni modified *t*-test).

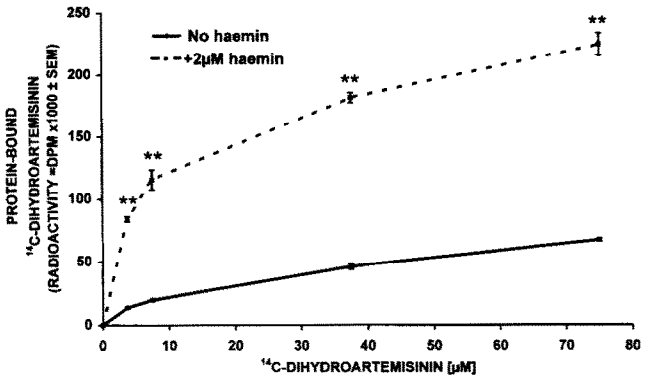

FIG. 7. The *in vitro* binding of ^{14}C-dihydorartemisinin to rat brain protein in the presence or absence of 2 μM haemin. Results are the means ± SEM of six separate experiments. ** represents a significant difference between rat brain homogenate incubated with ^{14}C-dihydroartemisinin in the presence or absence of haemin at the $P < 0.001$ level (two-way ANOVA and Bonferroni modified *t*-test).

lytic capacity of 1 g haemin, this would be equivalent to approximately 1 μM haemin. Potentiation of the effects of the drugs on neurite outgrowth also occurred in the presence of 1 μM haemin (results not shown), but the data were complicated by the fact that 1 μM haemin potentiated neurite outgrowth in its own right. The amount of haemin used may therefore have a clinical correlate in cerebral malaria, a condition for which the artemisinin derivatives are indicated.

The fact that artemether, arteether, and dihydroartemisinin in combination with haemin exhibit a greater effect on neurite outgrowth than on MTT metabolism (up to 93% decrease for neurite outgrowth compared with up to 49% for MTT metabolism) supports the view that neurite outgrowth, which measures toxic effects on axonal/neurite maintenance, is a more specific measure of potential neurotoxicity than MTT metabolism, which measures a general effect on neuronal survival. This is also suggested by our earlier findings, which show that the artemisinin derivatives have a greater effect on NB2a neurite outgrowth than on NB2a cell proliferation measured by the uptake of [^{3}H]thymidine [9]. There may, of course, be other properties of neurones that are affected by artemisinin derivatives at different concentrations.

The mechanism of neurotoxicity may be the same as the mechanism of antimalarial action of the artemisinin derivatives. Free radical intermediates may cause oxidative damage to neuronal cells by lipid peroxidation or protein oxidation in neuronal membranes or the cytoskeleton, since artesunate (a hemi-succinyl derivative of artemisinin) induces lipid peroxidation in both infected and uninfected erythrocytes and protein thiol oxidation in isolated red cell membranes [11, 20].

Both haem and free iron (FeII) accelerate the decomposition of hydrogen peroxide and organic peroxides into free radicals and also readily catalyse the cleavage of the endoperoxide bridge of artemisinin, possibly via an iron-oxo intermediate, to produce a carbon-centered free radical [12, 13, 16–18]. Because haemin significantly potentiates the *in vitro* neurotoxicity produced by artemether, dihydroartemisinin, and arteether, but not desoxyarteether, it is possible that haemin is catalysing the conversion of these artemisinin derivatives via an interaction with the endoperoxide bridge to produce free radicals.

The results of the ^{14}C-binding studies support this idea. Even in the absence of haemin, considerable drug–protein binding occurs, and in the presence of haemin this binding is increased by between two- and sixfold; haemin, and protein oxidation/alkylation therefore appear to be in some way involved in the toxicity of the artemisinin derivatives.

An alternative interpretation of the data might be that free iron is released from haemin during the reaction with the artemisinin derivatives to cause neuronal damage, since other alkyl peroxides have been shown to degrade haem [21]. Moreover, free iron is thought to be released from haem during haemoglobin digestion by the parasite in infected red blood cells [22]. However, neither free iron (ferrous sulphate [FeII]) nor the iron chelator desferrioxamine influences the degree of toxicity induced by dihydroartemisinin *in vitro* in the rat neuroblastoma × glioma hybrid cells NG108-15 [23]. Furthermore, the iron chelator desferrioxamine-hydroxyethyl starch does not prevent arteether-induced neuropathology in rats.§ Also, the brain-iron carrier protein transferrin significantly increases the toxicity of dihydroartemisinin in molt-4 cancer cells, which, like neurons, have a high cell surface concentration of transferrin receptors [24]. Therefore, iron in a complex or bound to protein, but not free iron, may be involved in potentiating the toxicity of the artemisinin derivatives.

Free radicals are very reactive, short-lived species that structurally rearrange to produce other reactive intermediates. Two such products of the iron-catalysed cleavage of the endoperoxide bridge of artemisinin are a highly electrophilic epoxide and a 1,5-diketone [17, 18, 25], both of which are potent alkylating agents and react with and damage specific neuronal proteins.

The effect on neurite growth and/or maintenance is consistent with the view that artemisinin derivatives may bind to and damage components of the neuronal cytoskeleton necessary for axonal maintenance. This hypothesis is supported by our study, which suggests that the toxic effects of artemisinin derivatives may involve protein alkylation. It is possible that the 1,5-diketone metabolite may cross-link cytoskeletal proteins in the same way that the hexacarbon diketones such as 2,5-hexanedione exert their neurotoxic effects [26], because artemisinin is known to bind in infected erythrocytes to actin and also to the protein spectrin, which is closely related to the neuronal cytoskeletal maintenance protein fodrin [24, 27].

In summary, we have demonstrated that haemin accelerates neurotoxicity induced by artemisinin derivatives *in vitro* and increases their binding to NB2a cell and rat brain proteins. Further studies are required to confirm the molecular mechanism of neurotoxicity of the artemisinin de-

rivatives *in vivo* and to determine whether it will be possible to design novel derivatives that retain antimalarial activity with minimal neurotoxicity.

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