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The role of glutathione in the neurotoxicity of artemisinin derivatives *in vitro*

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Abstract

The role of antioxidants in the neurotoxicity of the antimalarial endoperoxides artemether and dihydroartemisinin was studied *in vitro* by quantitative image analysis of neurite outgrowth in the neuroblastoma cell line NB2a. Intracellular glutathione concentrations were measured by high performance liquid chromatography with fluorescence detection. Both dihydroartemisinin (1 μM) and a combination of artemether (0.3 μM) plus haemin (2 μM) significantly inhibited neurite outgrowth from differentiating NB2a cells to 11.5 ± 11.0% (SD) and 19.6 ± 15.2% of controls, respectively. The inhibition by artemether/haemin was prevented by the antioxidants superoxide dismutase (109.7 ± 47.8% of control), catalase (107.0 ± 29.3%) glutathione (123.8 ± 12.4%), L-cysteine (88.0 ± 6.3%), *N*-acetyl-L-cysteine (107.8 ± 14.9%), and ascorbic acid (104.3 ± 12.7%). Dihydroartemisinin-induced neurotoxicity was completely or partially prevented by L-cysteine (99.5 ± 17.7% of control), glutathione (57.9 ± 23.4% of control), and *N*-acetyl-L-cysteine (57.3 ± 9.5%), but was not prevented by superoxide dismutase, catalase, or ascorbic acid. Buthionine sulphoximine, an inhibitor of γ-glutamylcysteine synthetase, significantly increased the neurotoxic effect of non-toxic concentrations of artemether/haemin (0.1 μM/2 μM) and dihydroartemisinin (0.2 μM), suggesting that endogenous glutathione participates in the prevention of the neurotoxicity of artemether/haemin and dihydroartemisinin. Artemether/haemin completely depleted intracellular glutathione levels, whereas dihydroartemisinin had no effect. We conclude that although glutathione status is an important determinant in the neurotoxicity of endoperoxides, depletion of glutathione is not a prerequisite for their toxicity. This is consistent with their mechanisms of toxicity being free radical-mediated damage to redox-sensitive proteins essential for neurite outgrowth, or alteration of a redox-sensitive signalling system which regulates neurite outgrowth.

Keywords: Neurotoxicity; Artemisinin; Glutathione; Free radical; Antioxidant; Malaria

1. Introduction

Artemisinin is a sesquiterpene lactone endoperoxide isolated from the Chinese medicinal plant *Artemisia annua L* (qinghao) that has been used for over a thousand years as a herbal remedy for fever and malaria. Artemisinin and its derivatives have impressive activity against multidrug-resistant forms of *Plasmodium falciparum* both *in vivo* and *in vitro* [1–6]. When injected intramuscularly, certain artemisinin derivatives, including artemether and dihydroartemisinin, cause dose-related neuropathology in the caudal brain stems of dogs, rats, monkeys, and mice [7–15]. The neuropathology is apparent in the absence of neurological signs or deficits in behavioural performance [8,9,12]. More recently, a human case of ataxia and slurred speech was described after treatment with the artemisinin derivative artesunate [16]. We and others have previously shown that artemisinin and its derivatives are also toxic to neuronal and glial cells in culture [17,18].

The antimalarial action of the artemisinin derivatives *in vitro* appears to involve the intraparasitic iron- or haem-catalysed cleavage of the endoperoxide bridge to generate toxic free radicals or intermediates [19–22]. This is supported by the fact that antioxidants (e.g. α-tocopherol, cata-

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Abbreviations: AEM, artemether; DHA, dihydroartemisinin; and SOD, superoxide dismutase.

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lase, dithiothreitol, ascorbic acid, and glutathione) antagonise the antimalarial activity of these drugs [23,24]. Conversely, free radical-generating compounds (e.g. doxorubicin, miconozole, castecin, and armetin) [23,25], as well as inhibitors of endogenous antioxidants (e.g. buthionine sulphoximine, which inhibits glutathione synthesis), enhance their antimalarial activity [26].

The mechanism of neurotoxicity may also involve the iron-mediated cleavage of the endoperoxide bridge to produce toxic free radicals. We have previously demonstrated that derivatives which lack the endoperoxide bridge are not significantly neurotoxic *in vitro* [17] and that iron, in the form of haemin, enhances the *in vitro* neurotoxicity of derivatives that contain an endoperoxide bridge [27]. This is consistent with the fact that neuronal cells are very vulnerable to oxidative damage [28].

To confirm these original observations and to determine the possible protective role of free radical scavengers, we show here the effects of enzymatic and non-enzymatic antioxidants on the neurotoxicity of artemether/haemin (AEM/haemin) and dihydroartemisinin (DHA) *in vitro*. Toxicity is measured as the outgrowth of neurites from the neuronally derived tumour cell line, mouse neuroblastoma NB2a. The effects on neurotoxicity of glutathione depletion by an inhibitor of glutathione synthesis (buthionine sulphoximine) are also examined.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. Tissue culture flasks and culture plates were obtained from Falcon/Fred Baker. Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1, horse serum, foetal bovine serum, penicillin/streptomycin solution, and gentamicin were purchased from GIBCO BRL. Cell-permeable SOD-mimetic (MnTMPyP; manganese (III)-tetrakis(1-methyl-4-pyridyl)porphyrin) was purchased from Alexis Corporation. HPLC grade acetic acid and acetonitrile were purchased from Fisher Scientific. Dr. P. Buchs (SAPEC) supplied AEM and DHA.

2.2. Cell culture

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

2.3. Measurement of neurite outgrowth

NB2a cells were plated on to 48-well culture plates at a cell density of 16,000 cells/mL. After allowing 24 hr for the cells to adhere to the plates, they were preincubated for 2 hr in the presence of the following antioxidants at concentrations that had previously been determined to have no significant effect on their own: the enzymatic antioxidants SOD (300 U/mL), catalase (100 U/mL), SOD/catalase (200 U/mL + 75 U/mL), and SOD-mimetic (1 μM); and the non-enzymatic antioxidants glutathione (100 μM), L-cysteine (1 mM), *N*-acetyl-L-cysteine (2 mM), ascorbic acid (100 μM), and α-tocopherol (1 μM). As neither α-tocopherol nor the SOD-mimetic was soluble in medium, they were initially dissolved in dimethyl sulphoxide; an equal concentration of dimethyl sulphoxide was included in the controls for these agents. Differentiation was then initiated by removal of the culture medium from the wells and replacement with serum-free medium plus 1 mM dibutyryl cyclic AMP. Cells then continued to differentiate and generate neurites for a further 24 hr in the presence of the above antioxidants on their own or in the presence of AEM/haemin (0.3 μM/2 μM) or DHA (1 μM). In one set of experiments, differentiating cells were preincubated for 6 hr with 50 μM buthionine sulphoximine in the presence or absence of AEM/haemin (0.1 μM/2 μM) or DHA (0.2 μM).

In all cases, the cells were then 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 finally washed with PBS. Fixed and stained cells were viewed on 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 sample 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 [29].

2.4. Measurement of endogenous glutathione

NB2a cells were plated on to 24-well plates at 20,000 cells/mL and after 24 hr were induced to differentiate (as described above) in the presence or absence of AEM/haemin (0.3 μM/2 μM), DHA (1 μM), buthionine sulphoximine alone (50 μM), or buthionine sulphoximine (50 μM) plus AEM/haemin (0.1 μM/2 μM) or DHA (0.2 μM). After a further 24 hr, the incubation medium was removed from the wells and replaced with 200 μL fresh serum-free medium containing 20 μL bromobimane (3 mM, in *N*-ethylmorpholine; pH 8.0) for measurement of glutathione by the method of Cotgreave and Moldeus [30]. The glutathione–bimane conjugates formed were previously characterised by LC–MS as described by Pirmohamed *et al.* [31]. The plate

was incubated in the dark for 5 min and the protein then precipitated with 100% (v/v) trichloroacetic acid (2 μL). Subsequently, the bottom of each well was scraped and the contents transferred to microcentrifuge tubes. Protein was sedimented by centrifugation at 850 $\times$ *g* for 5 min. Fifty microlitres of the supernatant was injected on to the HPLC column (5 μ Prodigy ODS (2), 150 $\times$ 4.60 mm, Phenomenex) and glutathione adducts were eluted with mobile phase (0.25% (v/v) acetic acid/9% (v/v) acetonitrile, pH 3.7) for 6 min, followed by 75% (v/v) acetonitrile for 4 min, with a re-equilibrium period of 4 min with mobile phase. The flow rate was 1 mL/min throughout. The eluent was monitored with a fluorescence detector (Hitachi 1080) set for excitation at 394 nm and emission at 480 nm. The limit of detection for glutathione was 0.05 μM; at 10 μM, the coefficient of variation was $<$ 10%.

2.5. Statistical analyses

Unless otherwise stated, data were normally distributed and were subjected to two-way ANOVA and Bonferroni-modified *t*-test for multiple comparisons. In the case of non-parametric data, a two-way ANOVA and Neuman–Keuls test for multiple comparisons were carried out. Differences were considered statistically significant at the $P < 0.05$ level. Values of *N* represent separate experiments.

3. Results

3.1. Effects of antioxidants on artemether/haemin-induced neurotoxicity

AEM/haemin (0.3 μM/2 μM) significantly inhibited NB2a neurite outgrowth to 19.6 $\pm$ 15.2% of controls (mean $\pm$ SD; $P < 0.001$, N = 4) and this inhibition was prevented by the enzymatic antioxidants SOD (109.7 $\pm$ 14.1% of control) and catalase (107.0 $\pm$ 29.3%; Fig. 1, both $P < 0.001$, N = 4), but was not prevented by SOD plus catalase or SOD-mimetic (Fig. 1). Significant protection against AEM/haemin-induced neurotoxicity was achieved by the non-enzymatic antioxidants glutathione (123.8 $\pm$ 12.4% of control), L-cysteine (88.0 $\pm$ 6.3%), *N*-acetyl-L-cysteine (107.8 $\pm$ 14.9%), and ascorbic acid (104.3 $\pm$ 127%; Fig. 2, all $P < 0.001$, N = 4), but was not prevented by α-tocopherol (Fig. 2).

Not only did SOD plus catalase, SOD-mimetic, and α-tocopherol fail to protect against AEM/haemin-induced toxicity, but even at concentrations which were not toxic on their own, these antioxidants apparently had pro-oxidant actions [32–35] and enhanced the neurotoxicity of AEM/haemin so that cells were non-adherent and neurite outgrowth could not be measured (Figs. 1 and 2).

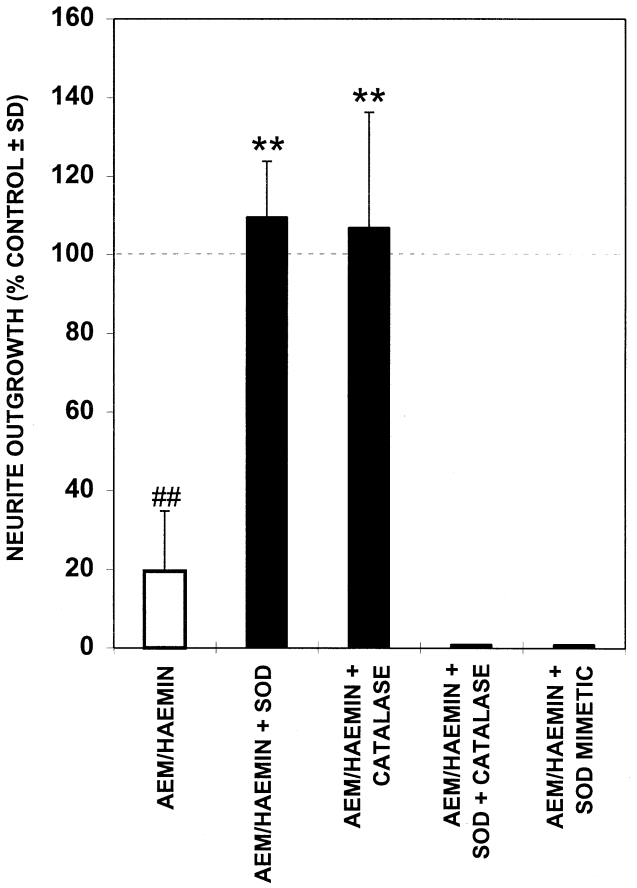

Fig. 1. Effects of AEM/haemin (0.3 μM/2 μM) in the presence or absence of 300 units/mL of SOD, 100 units/mL of catalase, 200 units/mL of SOD + 75 units/mL of catalase, or 1 μM SOD-mimetic on NB2a neurite outgrowth measured by image analysis. Results are the means $\pm$ SD of 4 separate experiments. ## represents a significant difference between AEM/haemin-treated cells in the absence of antioxidant and untreated control cells at the $P < 0.001$ level. ** represents a significant difference between AEM/haemin-treated cells in the presence or absence of antioxidant at the $P < 0.001$ level.

3.2. Effects of antioxidants on dihydroartemisinin-induced neurotoxicity

DHA (1 μM) significantly inhibited neurite outgrowth to 11.5 $\pm$ 11.0% of controls ($P < 0.001$, N = 4). None of the enzymatic antioxidants tested (SOD, catalase, SOD plus catalase, or SOD-mimetic) reduced this neurotoxicity (Fig. 3). DHA-induced neurotoxicity was completely or partially prevented by the non-enzymatic antioxidants glutathione (57.9 $\pm$ 23.4% of control), L-cysteine (99.5 $\pm$ 17.7%), and *N*-acetyl-L-cysteine (57.3 $\pm$ 9.5%; Fig. 4, $P < 0.001$, $P < 0.001$, and $P < 0.05$ respectively, N = 4). Ascorbic acid did not protect against the inhibition of neurite outgrowth produced by DHA (Fig. 4).

As with AEM/haemin, not only did SOD plus catalase, SOD-mimetic, and α-tocopherol not protect against DHA toxicity, but these antioxidants had pro-oxidant actions [32–35] and enhanced the neurotoxicity of DHA so that cells

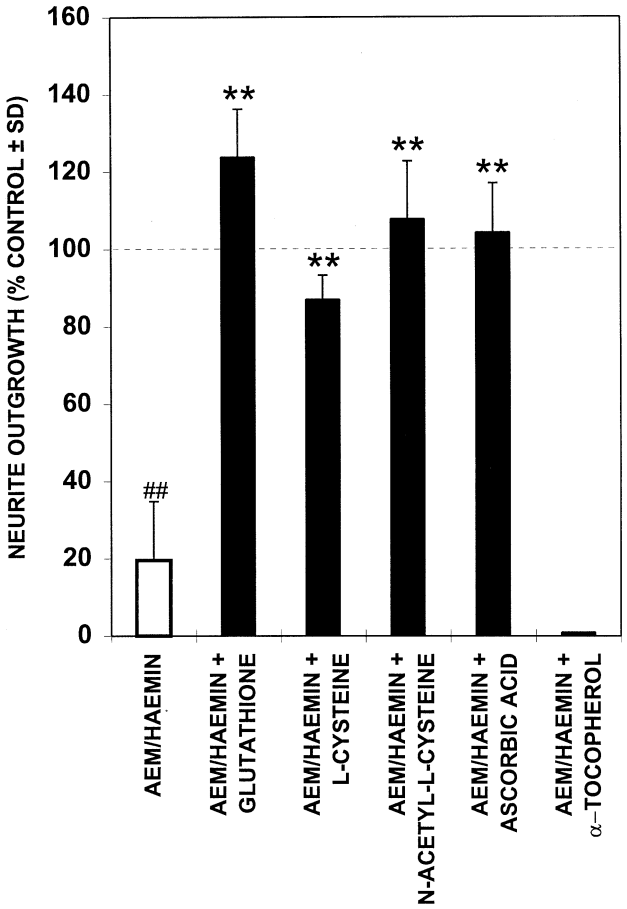

Fig. 2. Effects of AEM/haemin (0.3 μM/2 μM) in the presence or absence of 0.1 mM glutathione, 1 mM L-cysteine, 2 mM *N*-acetyl-L-cysteine, 0.1 mM ascorbic acid, and 1 μM α-tocopherol on NB2a neurite outgrowth measured by image analysis. Results are the means ± SD of 4 separate experiments. ## represents a significant difference between AEM/haemin-treated cells in the absence of antioxidant and untreated control cells at the $P < 0.001$ level. ** represents a significant difference between AEM/haemin-treated cells in the presence or absence of antioxidant at the $P < 0.001$ level.

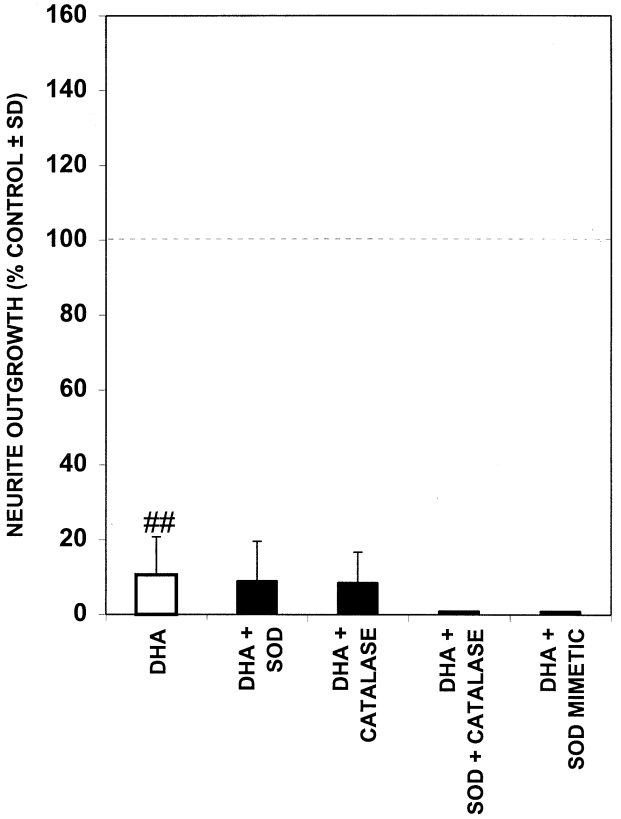

Fig. 3. Effects of DHA (1 μM) in the presence or absence of 300 units/mL of SOD, 100 units/mL of catalase, 200 units/mL of SOD + 75 units/mL of catalase, or 1 μM SOD-mimetic on NB2a neurite outgrowth measured by image analysis. Results are the means ± SD of 4 separate experiments. ## represents a significant difference between DHA-treated cells in the absence of antioxidant and untreated control cells at the $P < 0.001$ level. ** represents a significant difference between DHA-treated cells in the presence or absence of antioxidant at the $P < 0.001$ level.

were non-adherent and neurite outgrowth could not be measured (Figs. 3 and 4).

3.3. Effects of glutathione depletion on AEM/haemin- and DHA-induced neurotoxicity

Although buthionine sulphoximine (50 μM), AEM/haemin (0.1 μM/ 2 μM), and DHA (0.2 μM) had no significant effect on NB2a neurite outgrowth in their own right (Fig. 5), buthionine sulphoximine, when added in combination, significantly increased the neurotoxic effect of AEM/haemin and DHA by 97% and 96%, respectively (i.e. to 3.0 ± 5.2% and 2.8 ± 4.9% of control values respectively; Fig. 5, $P < 0.001$, N = 3, two-way ANOVA and Neuman–Keuls test).

3.4. Measurement of intracellular glutathione

Buthionine sulphoximine, at a concentration that had no effect on neurite outgrowth, lowered intracellular glutathione to 9.5 ± 4.7% of control (Fig. 6, $P < 0.001$, N = 4). AEM/haemin lowered intracellular glutathione to 27.6 ± 18.9% of control value (Fig. 6, $P < 0.001$, N = 4), whereas haemin alone had no significant effect (96.2 ± 5.9%; N = 4). Unlike AEM/haemin, DHA did not significantly affect endogenous glutathione levels (92.5 ± 6.6%; N = 4).

4. Discussion

In healthy volunteers, peak plasma concentrations of AEM, arteether, or DHA can reach 0.5 to 2.5 μM, with the highest concentration attained with DHA, the main metabolite of AEM, after AEM administration [36–40]. The artemisinin derivatives readily cross the blood–brain barrier in rats [41]. Thus, although evidence for serious clinical neurotoxicity of artemisinin derivatives has not emerged, the concentrations of artemisinin derivatives used in this study are relevant to those likely to be achieved after administration to patients.

The enzymatic antioxidants SOD and catalase and the non-enzymatic antioxidants glutathione, L-cysteine, *N*-acetyl-L-cysteine, and ascorbic acid completely protected

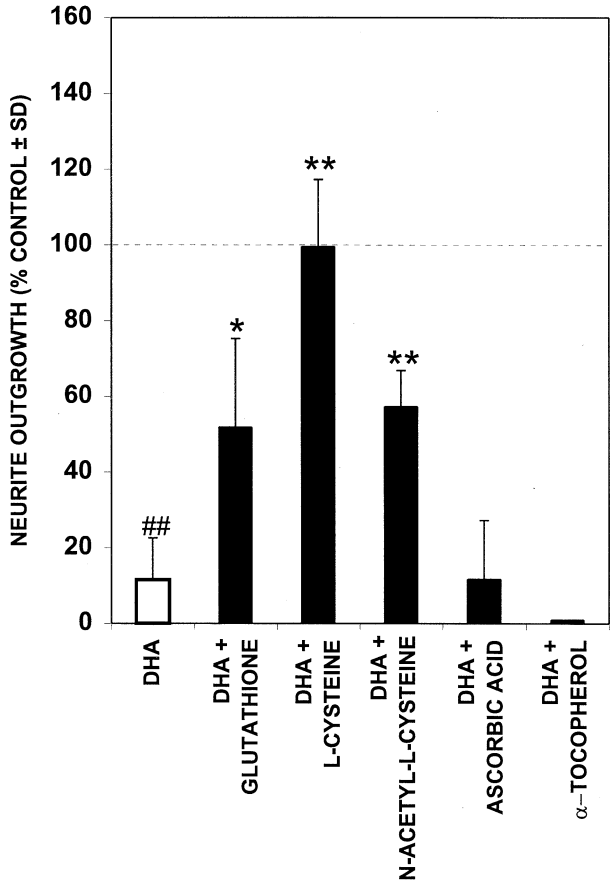

Fig. 4. Effects of DHA (1 μM) in the presence or absence of 0. 1 mM glutathione, 1 mM L-cysteine, 2 mM *N*-acetyl-L-cysteine, 0.1 mM ascorbic acid, and 1 μM α-tocopherol on NB2a neurite outgrowth measured by image analysis. Results are the means SD of 4 separate experiments. ## represents a significant difference between DHA-treated cells in the absence of antioxidant and untreated control cells at the $P < 0.001$ level. * and ** represent a significant difference between DHA-treated cells in the presence or absence of antioxidant at the $P < 0.05$ and $P < 0.001$ level, respectively (two-way ANOVA and Neuman–Keuls test)

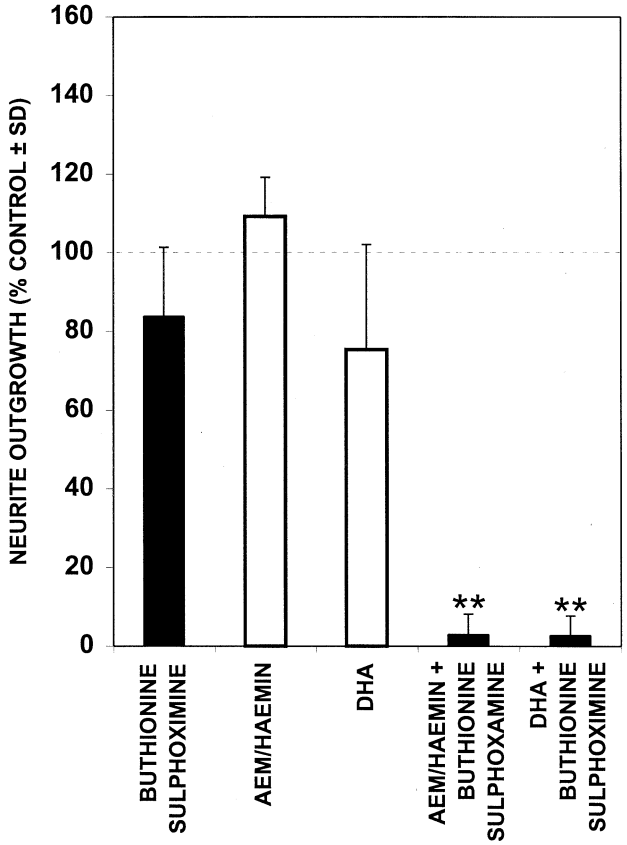

Fig. 5. Effects of buthionine sulphoximine (50 μM) alone or AEM/haemin (0.1 μM/2 μM) or DHA (0.2 μM) in the presence or absence of buthionine sulphoximine on NB2a neurite outgrowth measured by image analysis. Results are the means $\pm$ SD of 3 separate experiments. ** represents a significant difference between AEM/haemin-treated or DHA-treated cells in the presence or absence of buthionine sulphoximine at the $P < 0.005$ level (two-way ANOVA and Neuman–Keuls test).

against AEM/haemin-induced neurotoxicity, implying that superoxide radicals, hydrogen peroxide, and hydroxyl radicals were formed during the reaction of AEM and haemin. The fact that both non-cell-permeant antioxidants (SOD and catalase) and cell-permeant antioxidants (glutathione, L-cysteine, *N*-acetyl-L-cysteine, and ascorbic acid) prevented toxicity suggests that the ultimate neurotoxin was present both inside and outside the cells. Buthionine sulphoximine significantly enhanced the toxicity of AEM/haemin, indicating that endogenous glutathione plays a role in protecting NB2a cells from AEM/haemin, possibly by interacting with the free radicals produced from the reaction of this artemisinin derivative with haem-bound iron. An interaction between drug-derived radicals and glutathione is also supported by the fact that AEM/haemin depleted the intracellular levels of glutathione. However, glutathione depletion per se was not toxic to the cells, since buthionine sulphoximine alone had no effect on neurite outgrowth, despite significantly reducing intracellular glutathione.

These results suggest that the mechanism of neurotoxicity of AEM/haemin in neuroblastoma cells *in vitro* involves the formation of superoxide radicals, hydrogen peroxide, and hydroxyl radicals, which may damage extracellular or intracellular membranes by lipid peroxidation or protein oxidation or may damage DNA. Previous studies have demonstrated that artemisinin induces lipid peroxidation [24] and causes a dose-dependent decrease in the glutathione concentration and the amount of unsaturated fatty acids in normal and malaria-infected red blood cells [42]. Furthermore, artemisinin/haemin leads to oxidation of protein thiols in isolated red cell membranes [20,42] and AEM, in the presence of iron, cleaves supercoiled DNA, calf thymus DNA, or salmon DNA [43].

The neurotoxicity of DHA *in vitro* clearly differs from the neurotoxicity of AEM/haemin: only the thiol antioxidants (glutathione, L-cysteine, and *N*-acetyl-L-cysteine) protected against DHA-induced neurotoxicity. These results suggest that the production of activated oxygen free radicals (such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals) is not the principal mechanism of DHA neurotoxicity *in vitro*. DHA produces carbon-centred radi-

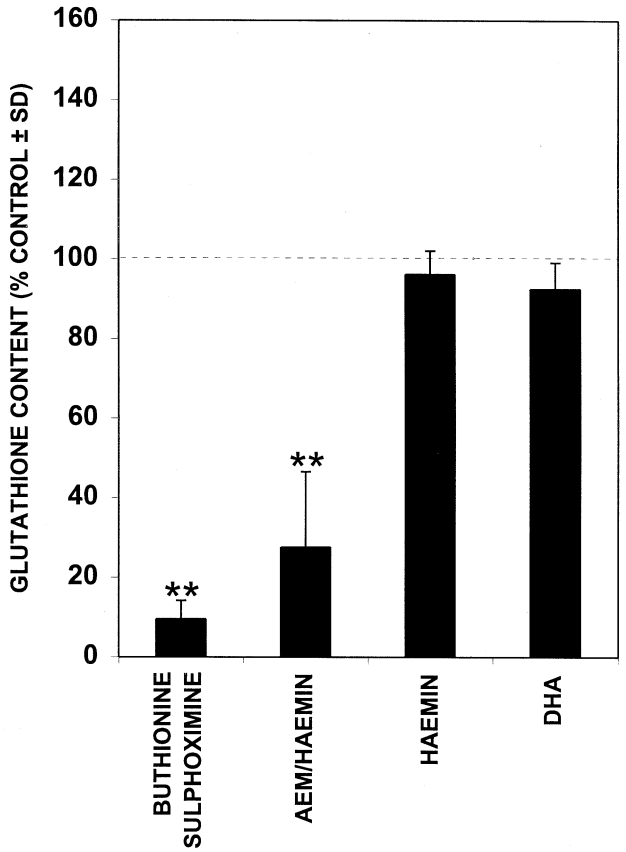

Fig. 6. Effects of buthionine sulphoximine (50 μM), AEM/haemin (0.3 μM/2 μM), haemin (2 μM), or DHA (1 μM) on the glutathione content of NB2a cells. Results are the means ± SD of 4 separate experiments. ** represents a significant difference between buthionine sulphoximine-treated cells or AEM/haemin-treated cells and untreated control cells at the $P < 0.001$ level.

cals that alkylate and damage thiol groups on cellular proteins. Only cell-permeant antioxidants protected against the toxicity of DHA, indicating that intracellular proteins are the targets for modification by DHA.

This idea that carbon-centred radicals may damage thiol-bearing protein is supported by the fact that thiol-containing compounds (glutathione, L-cysteine, and *N*-acetyl-L-cysteine) can bind and protect thiol groups on proteins from damage by alkylation or oxidation [44,45]. Artemisinin can react with low-molecular-weight thiols [46]. Furthermore, carbon-centred radicals produced from the iron-mediated cleavage of the endoperoxide bridge of DHA [47–49] are known to bind extensively to the thiol groups of cysteine residues [50]; DHA also binds specifically to thiol groups of proteins in NB2a cells [11,51] and rat brain homogenate [51]. This could lead to the damage to mitochondrial membranes and rough endoplasmic reticulum that we have previously demonstrated by electron microscopy [52].

Surprisingly, not only did SOD plus catalase, SOD-mimetic, and α-tocopherol not protect against AEM/haemin and DHA-induced toxicity, but these antioxidants actually enhanced the neurotoxicity of the artemisinin derivatives. This phenomenon is probably due to the fact that these antioxidants are capable of themselves producing free radicals. For example, SOD-mimetic and α-tocopherol are able to produce superoxide radical, and SOD plus catalase can form both superoxide radicals and hydrogen peroxide (i.e. catalase is a haemoprotein which, if reduced, can auto-oxidise to form superoxide radicals, which would in turn be converted by SOD to hydrogen peroxide) [32–35].

Glutathione depletion by buthionine sulphoximine significantly enhanced the toxicity of DHA, suggesting that endogenous glutathione plays a role in protecting NB2a cells from DHA-induced toxicity. Since DHA itself did not itself deplete endogenous glutathione, depletion of glutathione is clearly not obligatory for DHA-induced inhibition of neurite outgrowth. One must therefore also conclude that the glutathione depletion induced by AEM/haemin is a consequence of, rather than a prerequisite for, the inhibition of neurite outgrowth.

These results with buthionine sulphoximine are supported by a previous study which demonstrated that glutathione depletion (by buthionine sulphoximine) significantly increased the toxicity of hydrogen peroxide and *t*-butyl hydroperoxide in rat hepatoma-derived cells [53]. However, the toxicity of these agents could only be demonstrated at extremely high concentrations (compared with the toxicity produced by DHA in glutathione-depleted NB2a cells), probably because of their inability to produce carbon-centred radicals.

In conclusion, glutathione status may affect susceptibility to the neurotoxic effects of artemisinin derivatives, but depletion of glutathione is not a prerequisite for their toxicity. This is consistent with free radical-mediated damage to redox-sensitive proteins that may form part of the complex signalling system that regulates neurite outgrowth. Further work should be aimed at identifying these specific targets and the precise chemical nature of the ultimate neurotoxin.

Acknowledgments

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