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The acute neurotoxicity of mefloquine may be mediated through a disruption of calcium homeostasis and ER function *in vitro*

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Abstract

Background: There is no established biochemical basis for the neurotoxicity of mefloquine. We investigated the possibility that the acute *in vitro* neurotoxicity of mefloquine might be mediated through a disruptive effect of the drug on endoplasmic reticulum (ER) calcium homeostasis.

Methods: Laser scanning confocal microscopy was employed to monitor real-time changes in basal intracellular calcium concentrations in embryonic rat neurons in response to mefloquine and thapsigargin (a known inhibitor of the ER calcium pump) in the presence and absence of external calcium. Changes in the transcriptional regulation of known ER stress response genes in neurons by mefloquine were investigated using Affymetrix arrays. The MTT assay was employed to measure the acute neurotoxicity of mefloquine and its antagonisation by thapsigargin.

Results: At physiologically relevant concentrations mefloquine was found to mobilize neuronal ER calcium stores and antagonize the pharmacological action of thapsigargin, a specific inhibitor of the ER calcium pump. Mefloquine also induced a sustained influx of extra-neuronal calcium via an unknown mechanism. The transcription of key ER proteins including GADD153, PERK, GRP78, PDI, GRP94 and calreticulin were up-regulated by mefloquine, suggesting that the drug induced an ER stress response. These effects appear to be related, in terms of dose effect and kinetics of action, to the acute neurotoxicity of the drug *in vitro*.

Conclusions: Mefloquine was found to disrupt neuronal calcium homeostasis and induce an ER stress response at physiologically relevant concentrations, effects that may contribute, at least in part, to the neurotoxicity of the drug *in vitro*.

Background

Mefloquine is a prophylactic antimalarial drug that is also used for malaria chemotherapy. Adverse central nervous system (CNS) events have been associated with its use [1,2]. Severe CNS events requiring hospitalization occur

in 1:10,000 and 1:200–1200 patients taking mefloquine for chemoprophylaxis or treatment, respectively [1,2]. Milder CNS events (e.g. dizziness, headache and insomnia) are a more frequent occurrence, being experienced by up to 25% of patients receiving chemoprophylactic doses

and 90% of those receiving therapeutic doses [1,2]. Higher blood levels of mefloquine are reached under therapeutic regimens (2.1–23 μM) compared with those for prophylaxis (3.8 μM) [3,4]. The relative incidence of adverse effects is, therefore, probably dose-related, although the concomitant effect of malaria during treatment cannot be dismissed. It is likely, then, that the adverse neurological events associated with mefloquine have a biochemical basis.

To date, no satisfactory biochemical explanation of the neurotoxicity of mefloquine has been proposed. However, two lines of evidence suggest that the endoplasmic reticulum might be a neuronal target of mefloquine. Go and others [5,6] showed that mefloquine affects calcium flux into and out of isolated mammalian microsomes obtained from skeletal muscle and neuronal tissue. These observations suggest that mefloquine might disrupt ER calcium homeostasis, but are difficult to interpret since the experiments were not conducted in an intact cell system. In an unrelated study, we investigated the effects of mefloquine on global transcription in rat neuroblastoma (NG108) cells using microarrays [7]. Mefloquine caused a striking upregulation of growth arrest and development protein 153 (GADD153), a transcription factor selectively modulated under conditions of ER stress [8]. Taken together, these observations suggest that mefloquine might perturb the function of the ER via a disruption of calcium homeostasis. Most ER-active agents (e.g. thapsigargin and ryanodine) deplete the ER calcium pool either by (i) inhibiting the ER calcium pump and thereby preventing the replenishment of the ER calcium pool or (ii) triggering ER (ryanodine or inositol 1,4,5-trisphosphate (IP_3) activated) calcium release channels [9]. These phenomena can be visualized by fluorescence microscopy as a transient increase in cytoplasmic calcium concentrations (for example see [10]). Nominally calcium-free media are used so that the effect can be distinguished from calcium influx mediated via plasma membrane channels. If the test agent can be shown to antagonize the release of calcium by a known ER agonist, it can then be concluded that the ER calcium pool is a target of the compound.

ER function is critical if protein synthesis and folding is to successfully occur in eukaryotic cells [11,12]. ER stress, induced by cellular events as diverse as disrupted calcium homeostasis, elevated levels of reactive oxygen species and viral infection, leads to accumulation of dysfunctional ER chaperones and misfolded proteins [11]. Cells respond by one or both of two conserved stress response pathways. The first, the ER overload response, triggers the activation of the transcription factor NF κB and the subsequent upregulation of inflammatory proteins [13]. The second, the unfolded protein response (UPR) induces three divergent cellular events: (i) the activation of tran-

scription factor ATF6 leading to the upregulation of ER chaperones such as protein disulfide isomerase, (ii) upregulation of the pro-apoptotic transcription factor GADD153 and (iii) suppression of protein synthesis via phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) by pancreatic eIF2 α kinase (PEK/PERK, [11,12]). Upregulation of the ER chaperones, GADD153 and PERK in response to a toxic insult would constitute direct evidence of an ER stress response.

In the present study, using laser scanning confocal microscopy, we show that the ER calcium pool is a target of mefloquine in rat neurons, as, under calcium free conditions, the drug induces elevations in intracellular calcium concentrations and antagonizes the pharmacological effect of thapsigargin, a known inhibitor of the ER calcium pump. Further, we show that, in the presence of extra-neuronal calcium, mefloquine induces a sustained elevation of cytoplasmic calcium levels via an unknown mechanism. The disruption of calcium homeostasis by mefloquine appears to be related, in terms of dose-effect and kinetics of action, to the acute neurotoxicity of the drug. In a microarray study we also show that mefloquine induces the transcription of key ER stress response genes. Collectively, the data suggest that mefloquine may inhibit neuronal ER function and disrupt neuronal calcium homeostasis at physiologically relevant concentrations *in vitro*.

Methods

Materials

Racemic (*erythro*) mefloquine was obtained from the WRAIR chemical inventory system. The calcium-sensitive dye Fluo3 was purchased from Molecular Probes, Eugene, OR. Reagents used in cell culture and neurotoxicity/neuroprotection assays were obtained from Sigma, St Louis, MO. Biosource International, Rockville, MD, and GIBCO/Life Technologies, Grand Island, NY.

Preparation of primary neuronal cell cultures

Primary neuronal cultures of fetal rat forebrains were prepared as described previously [14]. Briefly, the forebrains of fetal rat pups (embryonic day 15) were isolated, and the cells were dispersed by repeated mechanical trituration in neuronal culture medium (NCM = Ham's F-12: Basal Medium Eagle, 1:1; supplemented with dextrose, 0.6 g/l; glutamine, 0.35%; and Pen-Strep, 1%). Following centrifugation ($900 \times g$; 5 min) and re-suspension in fresh NCM, the cells were counted and plated onto poly-L-lysine-coated 48 well plates at a density of 0.5×10^6 cells/well. For imaging experiments the neurons were transferred to 4-chamber cover glass slides that had been previously coated with poly-L-lysine followed by collagen. Each chamber contained 1×10^6 cells. For the array studies, the neurons (20×10^6 neurons per flask) were

transferred to 75 cm² flasks. To suppress glial growth, cultures were treated with cytosine β -D-arabinofuranoside (10⁻⁵ M) after 3 days *in vitro*. All cultures were maintained in an incubator (5% CO₂; 37°C) for at least 6 days and no more than 10 days prior to use.

Confocal microscopy

The approach utilized for confocal microscopy experiments is similar to that employed in an earlier study [14]. Neurons (on chamber slides) were treated with 5 μ M Fluo-3-AM for 1 h. After 1 h in an incubator, the cells were rinsed with fresh Mg²⁺-free Locke's solution (NaCl, 154 mM; KCl, 5.6 mM; NaHCO₃, 3.6 mM; CaCl₂, 2.3 mM; glucose, 5.6 mM; HEPES, 5 mM; pH 7.4), and returned to the incubator for an additional 15 minutes prior to initiating the imaging experiments. Changes in neuronal calcium homeostasis were monitored using a Bio-Rad Radiance 2000 confocal imaging system. Changes in cytoplasmic calcium were recorded as fluctuations in the emitted fluorescence of Fluo-3-complexed calcium at 530 nm (excitation was 488 nm). Sequential image scans of fields containing 5–25 neurons were used to construct temporal profiles of the effects of the different agonists. Typically, scans were made at 10 or 15 s intervals. To compare the fluorescence levels in different neurons (which were often in slightly different focal planes) on different days, the fluorescence readings at each time point were normalized to the first one taken for each neuron.

Effects of mefloquine on ER calcium homeostasis

In the first series of experiments, mefloquine's effects on mobilization of ER calcium stores were investigated. The neurons (chamber slides) were rinsed twice with fresh low calcium Locke's buffer (NaCl, 154 mM; KCl, 5.6 mM; NaHCO₃, 3.6 mM; CaCl₂, 0.92 mM; glucose, 5.6 mM; HEPES, 5 mM; EGTA, 1 mM; pH 7.4) and immersed in the same solution (240 μ l per chamber). Neurons were scanned at 10 s intervals. Mefloquine (final concentration of 80 μ M) or DMSO (0.2%), thapsigargin (1 μ M), glutamate (1 μ M) and CaCl₂ (1.6 mM), were added after scans 3, 33, 63 and 67 respectively. All drugs were prepared in calcium-free Locke's solution containing no EGTA. Data analysis was conducted only for neurons in which an increase in cytoplasmic calcium was observed after the addition of calcium chloride.

Effects of lower mefloquine doses on ER calcium homeostasis

In a second series of experiments, the effects of lower mefloquine doses on ER calcium homeostasis were investigated. The neurons (chamber slides) were rinsed twice with fresh low calcium Locke's buffer containing DMSO (NaCl, 154 mM; KCl, 5.6 mM; NaHCO₃, 3.6 mM; CaCl₂, 0.92 mM; glucose, 5.6 mM; HEPES, 5 mM; EGTA, 1 mM; DMSO, 0.625%; pH 7.4) and immersed in the same solu-

tion (240 μ l per chamber). Neurons were scanned at 10 s intervals. Mefloquine (2, 10, 30, 60, 80 or 200 μ M), thapsigargin (1 μ M), glutamate (1 μ M) and CaCl₂ (1.6 mM final concentration) were added sequentially as outlined earlier, except that for the lowest mefloquine dose experiment, 2 and 10 μ M mefloquine were added to the same well after scans 3 and 18 respectively. Selection of neurons for data analysis was conducted as described above. For each mefloquine concentration in each experiment, the maximum increase in the cytoplasmic calcium concentration relative to the baseline was determined (at least four replicate experiments were conducted for each mefloquine concentration). For example, the maximum relative increase in cytoplasmic calcium concentration induced by mefloquine at 80 μ M in one of the experiments was 2.95, as illustrated in Figure 1. Concentration-response effects were then expressed as a percentage change in terms of the mean relative increase in cytoplasmic calcium observed at the highest mefloquine concentration. The highest mefloquine concentration used was 200 μ M, since this was the lowest concentration tested that induced almost 100% loss of viability after five minutes exposure (Figure 3).

Effect of mefloquine on cytoplasmic calcium homeostasis and its antagonisation by thapsigargin

The effect of mefloquine on whole cell calcium homeostasis was also investigated. The neurons (chamber slides) were rinsed twice with fresh Locke's buffer containing physiological levels of calcium (NaCl, 154 mM; KCl, 5.6 mM; NaHCO₃, 3.6 mM; CaCl₂, 2.3 mM; glucose, 5.6 mM; HEPES, 5 mM; pH 7.4) and immersed in the same solution (240 μ l per chamber). Neurons were scanned at 15 s intervals. Mefloquine (80 μ M), thapsigargin (4 μ M) or DMSO (0.4%) were added after scan 13 and DMSO (0.2%) or mefloquine (80 μ M) after scan 28.

Detection of transcriptional changes in key ER stress response genes using microarrays

Neurons (75 cm² culture flasks) were treated with 0.2% DMSO (controls), 2, 10 or 80 μ M mefloquine in Locke's buffer (containing calcium) for five minutes. This treatment time was chosen because the depletion of the ER calcium store by mefloquine occurs within this time period. After 5 min, the media were replaced with MEM containing 0.02% DMSO (for the control, 10 and 80 μ M mefloquine treatment groups) or 2 μ M mefloquine for a further 24 h. Therefore, neurons were exposed to the lowest mefloquine concentration for 24 h. A recovery period of 24 h is required following an insult with an ER-agonist to induce a maximal ER stress response [8]. After 24 h, total RNA was extracted from the flasks using RNA-STAT 60 as previously described [7]. The experiment was repeated five times on independent occasions.

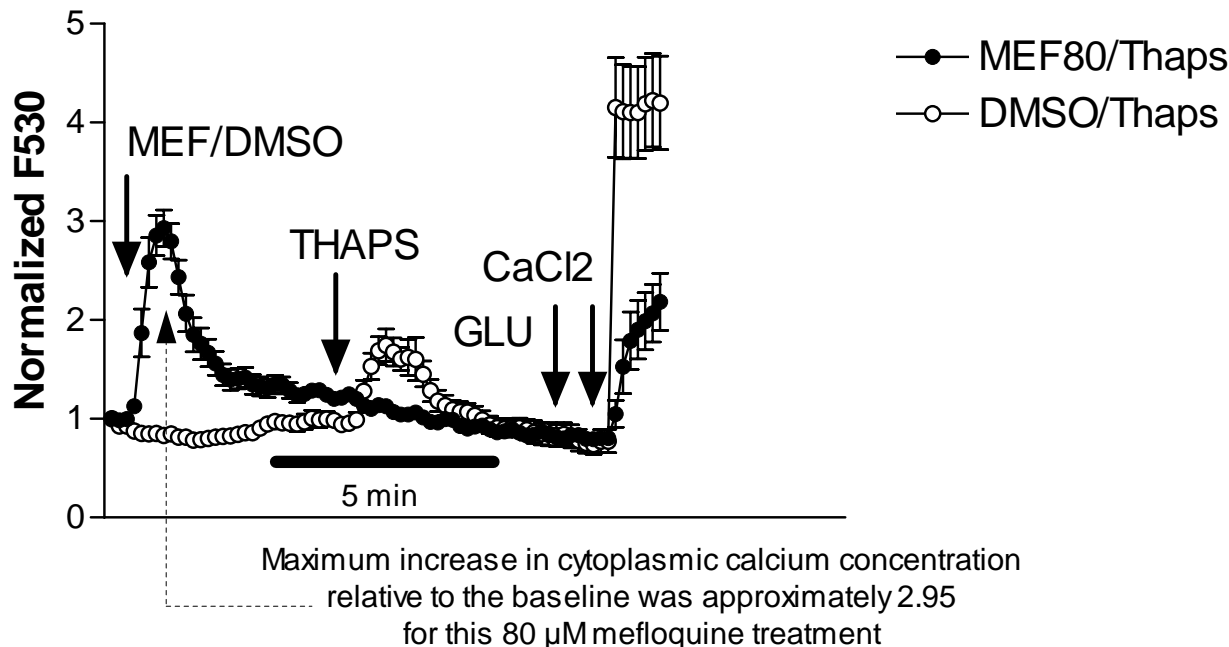


Figure 1

Effects of mefloquine on ER calcium homeostasis. The effects of mefloquine on cytoplasmic calcium levels in rat neurons were investigated using confocal microscopy. Neurons were loaded with the calcium-sensitive dye Fluo-3 that was replaced with low-calcium Locke's buffer after 1 h. The neurons were scanned at 10 s intervals to measure baseline fluorescence prior to the addition of DMSO (0.2%) or mefloquine (Mef, 80 μM) followed by thapsigargin (Thaps, 1 μM). Arrows indicate additions after scans 2 and 37, respectively. Mefloquine increased cytoplasmic calcium and antagonized the pharmacological action of thapsigargin, suggesting that the drug mobilizes the ER calcium store. The lack of a subsequent glutamate response (Glu, 1 μM after scan 63) demonstrates the external medium was substantially devoid of free calcium, whilst the presence of a subsequent CaCl₂ (1.6 mM after scan 67) response indicates that the neurons remain viable at the termination of the experiment.

Detailed procedures for preparation of cDNA and fluorescently labeled cRNA, hybridization, staining, and scanning of gene chips and assay monitoring are outlined in earlier literature [15]. The platform chosen for global expression profile was the Rat Genome U34 Array (Affymetrix, Santa Clara, California), which contains probes for a total of 8799 expressed sequence tag (EST) clusters and genes (including controls). RNA (10 μg) extracted from each individual flask was hybridized to a single gene chip (i.e. a total of 20 chips were used). Affymetrix analysis software (version 5) was used to gen-

erate average difference (AD) values for each gene for each treatment (Affymetrix, Santa Clara, California). AD values represent the difference in mean fluorescence between positive and mismatch probe cells for each gene, and are hereafter referred to as expression values.

The Affymetrix website <http://www.affymetrix.com> was searched using the terms 'PEK/PERK', 'GADD153' and the gene names for ER chaperones previously identified as being upregulated during an ER stress response [12], and where they were represented by probe-sets on the U34

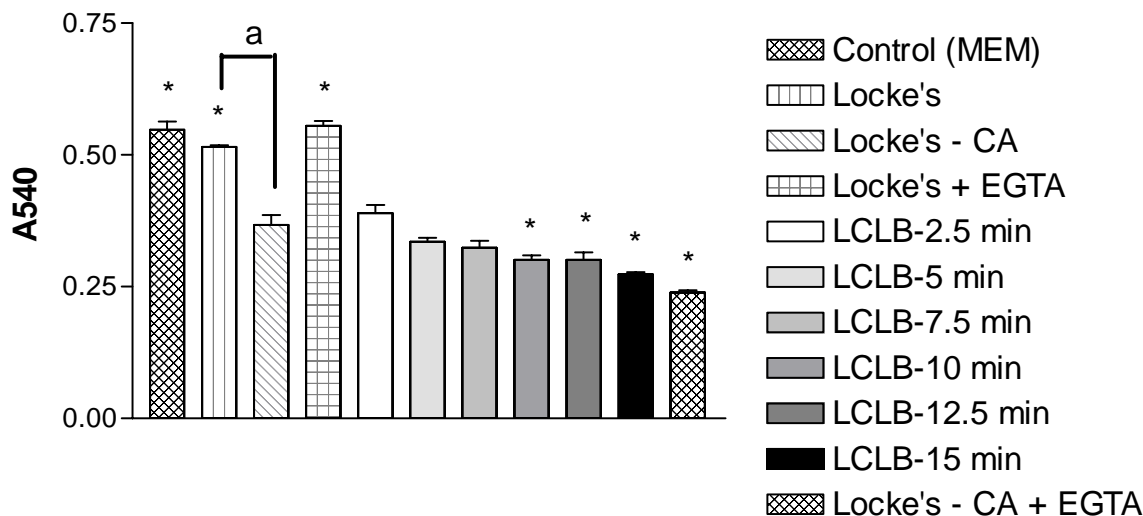


Figure 2

Effects of low calcium Locke's buffer on neuronal viability. Neurons were exposed to MEM (controls), Locke's buffer with and without Ca²⁺ and/or 1 mM EGTA for 15 min or to a low calcium Locke's buffer (LCLB) containing 920 μM Ca²⁺ and 1 mM EGTA for 2.5, 5, 7.5, 10, 12.5 and 15 min. Viability was assessed using the colorimetric MTT assay, where a loss of viability is reflected in a decrease in absorbance at λ = 540 (A₅₄₀). The substitution of normal Locke's buffer for one containing no added calcium results in a loss of neuronal viability (27%, Welch's test, P < 0.005, n = 8, indicated by a superscript a). This solution was not used for the mefloquine studies because control experiments showed that glutamate elicited a calcium response in neurons exposed to this buffer, presumably because the rinsing procedure did not remove all residual free calcium. A 10 min exposure to LCLB was required to reduce viability below that of the calcium-free Locke's buffer (one way ANOVA and Dunnett's test, P < 0.05, n = 44, * designates a significant change). Use of this buffer does not elicit a glutamate response, and therefore should not contain substantial amounts of free calcium. Experiments that utilized this buffer to investigate the effect of mefloquine on neuronal calcium homeostasis were performed within this ten-minute window.

arrays, corresponding accession numbers were identified. The expression values for these accession numbers were imported directly into Partek Pro 2000. No additional data normalization or scaling methods were employed (as these procedures were performed previously by the Affymetrix software). One-way ANOVAs were performed to compare the mean expression levels of each of the genes amongst the different treatment groups. A significant change in mean expression level was assumed to have

occurred where ANOVA P-values were < 0.05. A less conservative significance threshold was considered appropriate since the total number of hypotheses (i.e. tests of significance) conducted was small. For a particular treatment group, a gene was considered to have differentially modulated expression where the fold-change in expression exceeded 1.3. Fold-changes (FC) in expression were defined as: = mean mefloquine expression level/mean DMSO expression level. Where a gene was represented by

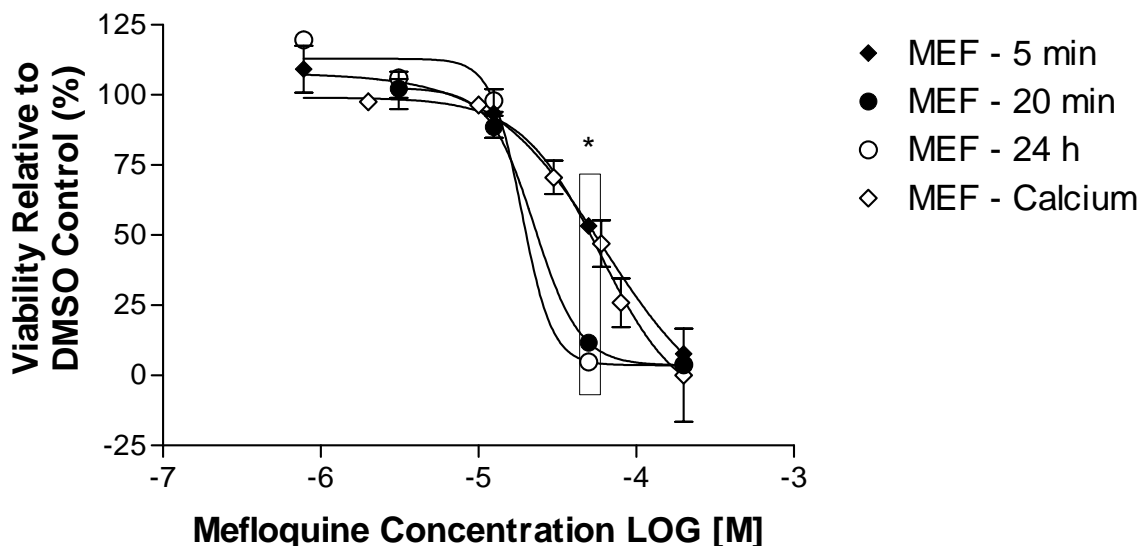


Figure 3

Dose-response curves for mefloquine's effects on neuronal cell viability and mobilization of ER calcium stores. Neurons were exposed to mefloquine (0.75–200 μM) for 5 min, 20 min or 24 h. Viability was assessed using the colorimetric MTT assay. Dose-response data is presented as mean viability ($\% \pm \text{SEM}$ compared to appropriate DMSO controls) and represents data from at least three pooled experiments. For the ER-calcium experiments, changes in neuronal intracellular calcium concentrations induced in response to different mefloquine treatments were monitored in real time using confocal microscopy. Dose-response effects are expressed as a percentage of the maximal elevation of cytoplasmic calcium occurring at 200 μM mefloquine. The dose-response curves for mefloquine's effects on neuronal viability (5 min exposure) and ER calcium store mobilization are superimposable, as are the 20 min and 24 h exposure curves. At a concentration of 50 μM , mefloquine exhibited greater toxicity after 20 min and 24 h exposure as compared to 5 min exposure (one way ANOVA and Dunnett's t-test, $P < 0.0001$, $n = 12$, see asterixed points on graph). Therefore, it appears that the neurotoxic effects of mefloquine occur within 20 min of exposure, but cannot be accounted for solely by the disruptive effect of mefloquine on ER calcium homeostasis.

more than one probe set, data is presented only for those probe-sets for which the ANOVA P -value was < 0.05 .

Viability measurements

Neuronal viability was assessed using the colorimetric MTT assay as described previously [14]. All experiments were conducted in Mg^{2+} -free Locke's solution unless otherwise indicated. Neurons were exposed to graded concentrations of mefloquine for 5 or 20 min and 24 h. After

treatment, mefloquine dilutions were removed and replaced with MEM (Gibco). The neurons were incubated for a further 24 h. The next day MTT was added to each well such that the final concentration of the dye was 150 $\mu\text{g/ml}$. Plates were then returned to the incubator for 1 h at which time unincorporated MTT was removed, and the plates were allowed to air dry. The purple formazan product indicative of viable cells was then dissolved by adding 250 μl acidified isopropanol (95% isopropanol; 5% 2N

HCl), and 200 μ l aliquots were collected. The absorbance of these aliquots was measured on an ELISA plate reader at 540 nm. The effects of mefloquine were compared to appropriate DMSO controls.

The acute toxicity of the low calcium Locke's solution was also investigated. Neurons were exposed to the buffer at 2.5 minute intervals over a 15 min time period. Effects of the various treatments were compared to appropriate controls (Locke's solution with and without calcium and/or 1 mM EGTA for 15 minutes). Viability was assessed as above except that the neurons were not incubated overnight in MEM prior to performing the MTT assay.

Neuroprotective effect of thapsigargin

The effect of mefloquine (10 or 80 μ M for 5 min), before and after pretreatment with thapsigargin (4 μ M for 5 min), on the viability of rat neurons was assessed as described above. The degree of neuroprotection afforded by thapsigargin was basically the extent to which the drug reversed the decline in absorbance (viability) induced by mefloquine and was calculated as previously described [14].

Statistical analyses, data analysis and presentation

For the confocal experiments the data are presented as mean normalized Fluo-3 fluorescence \pm the standard error of the mean (SEM) and are representative of at least three replicate experiments. Toxicity and neuroprotection data are presented as mean (%) viability or raw A_{540} values (\pm SEM) and represent the results of at least three experiments in every case. Samples sizes are outlined in the table or figure legends. For the toxicity experiments, the significance of multiple treatments was assessed using one-way ANOVA and Dunnett's t-test. For the neuroprotection experiment, the data were analyzed using a two-way ANOVA, with a P -value < 0.05 for the interaction between mefloquine and thapsigargin taken to represent an antagonistic effect of the two compounds. Statistical procedures for the analysis of microarray data are discussed above. Fifty percent inhibitory or effective concentrations and 95% confidence intervals (IC_{50} or ED_{50}) for the effects of mefloquine on neuronal viability and calcium homeostasis were calculated using Prism software.

Results

Effects of 80 μ M mefloquine on ER calcium homeostasis

As can be seen from examination of the first peak in Figure 1, mefloquine caused a transient increase in intracellular calcium levels whereas DMSO did not. Mefloquine also antagonized the subsequent transient elevation of cytoplasmic calcium induced by the addition of thapsigargin as can be seen from examination of the second series of peaks in Figure 1. A subsequent addition of glutamate failed to elicit a calcium response, whereas addition of cal-

cium chloride to the medium did. The antagonisation by mefloquine of the ER calcium release induced by thapsigargin, a specific inhibitor of the ER calcium pump, suggests that mefloquine mobilizes ER calcium stores. Glutamate induces the activation of plasma membrane influx channels, therefore its failure to illicit a calcium response shows that the external media contained low levels of free calcium. Therefore the elevation of intracellular calcium cannot be attributed to influx from an external source. The subsequent ability of the neurons to elicit a response after restoration of normal levels of extracellular calcium shows that the neurons selected for the confocal microscopy experiments remained viable for the duration of these experiments.

Low-calcium Locke's buffer does not substantially reduce neuronal viability

The neurotoxicity of the low calcium Locke's buffer was investigated over time. As can be seen from Figure 2, substitution of normal Locke's buffer for one containing no added calcium results in a loss of neuronal viability (27% reduction, Welch's test, $P < 0.005$, $n = 8$). This solution was not used for the mefloquine studies because control experiments showed that glutamate elicited a calcium response in neurons exposed to this buffer (data not shown), presumably because the rinsing procedure did not remove all residual free calcium. We utilized a low calcium (920 μ M) Locke's buffer supplemented with EGTA (1 mM) for the confocal microscopy experiments. As can be seen from Figure 2, exposure to this buffer for 10 minutes was required to reduce the viability of the neurons below that induced by treatment of neurons with Locke's containing no added calcium (one way ANOVA and Dunnett's test, $P < 0.05$, $n = 44$). All mefloquine calcium response experiments were conducted within this time frame, and care was taken to analyze data only from neurons that were shown to be viable at the termination of the experiment (as indicated by the $CaCl_2$ -induced calcium response).

Effect of lower mefloquine concentrations on ER calcium homeostasis and relationship to time-dependent neurotoxicity

We next investigated the effects of lower mefloquine doses on neuronal ER calcium homeostasis (i.e. in low-calcium Locke's buffer). At concentrations of at least 30 μ M, mefloquine induced a transient increase in cytosolic calcium levels (Table 1, one-way ANOVA, Dunnett's t-test, $P < 0.05$, $n = 29$). The effect was dose-related, with an ED_{50} of 66 μ M (95% confidence interval, 33 – 130 μ M). The IC_{50} of mefloquine's effect on neuronal viability after 5 min exposure was 62 (19–204) μ M, and the dose response curves of the two effects are super-imposable (Figure 3). These data suggest that the two effects may be related. The IC_{50} s of mefloquine's effect on neuronal via-

Table 1: Dose-response effects of mefloquine on elevation of cytosolic calcium levels induced via liberation of the ER store.

Concentration in μM (# of experiments)	Relative increase in cytosolic CA^{2+} due to ER calcium release (range)	Magnitude of effect relative to 200 μM mefloquine (%)
DMSO (4)	1.0 (1.0–1.0)	0
2 (4)	1.0 (1.0–1.2)	2.4
10 (4)	1.1 (1.0–1.2)	3.6
30 (5)	1.5 (1.2–1.7)	30
60 (4)	1.9 (1.8–2.6)	53
80 (4)	2.5 (2.1–2.9)	74
200 (4)	3.2 (2.6–4.1)	100

bility were 23 (18–28) and 19 (11–32) μM for exposure times 20 min and 24 h respectively. The concentration-effect curves are super-imposable. Also, at a concentration of 50 μM , mefloquine exhibited a greater neurotoxic effect after 20 min or 24 h exposure as compared to a 5 minute exposure (one way ANOVA and Dunnett's t-test, $P < 0.0001$, $n = 12$). Therefore, it appears that most of mefloquine's neurotoxic effects occur within 20 min of exposure, but cannot be accounted for solely by the disruptive effect of mefloquine on ER calcium homeostasis. Consequently, we investigated the effect of mefloquine on neuronal calcium homeostasis in calcium-containing media.

Effect of mefloquine on whole cell calcium homeostasis

The effects of mefloquine on neuronal calcium homeostasis in calcium-containing medium were investigated. As shown in Figure 4, mefloquine (80 μM) caused an immediate and sustained rise in cytoplasmic calcium levels. In some experiments, this sustained elevation of cytoplasmic calcium was preceded by a transient rise as observed in low calcium Locke's (as in Figure 1). The sustained elevation of cytoplasmic calcium can only be due to entry from an external source, as it was not observed in the low-calcium medium.

Thapsigargin antagonizes the disruptive effect of mefloquine on calcium homeostasis and protects neurons from cellular injury

Pre-treatment with thapsigargin (4 μM) antagonised both the initial rise in intracellular calcium attributable to the effect of mefloquine on the ER calcium store, and the subsequent sustained influx of external calcium (Figure 4). We next investigated the possibility that thapsigargin pre-treatment might have a protective effect on neurons exposed to mefloquine. Rat neurons were pretreated with either thapsigargin (4 μM) or DMSO (0.4%) for 5 min prior to the addition of DMSO (0.2%) or mefloquine (10 or 80 μM) for 5 min. The drugs were then removed and incubated in calcium-containing MEM medium for 24 h prior to viability assessment using the MTT assay. As can be seen in Figure 5, pretreatment with thapsigargin

reduced the decline in viability (absorbance) induced by mefloquine by 60%. There was a significant interaction between thapsigargin and mefloquine, indicating that the drugs behave antagonistically (two way ANOVA, $P < 0.0001$, $n = 48$).

Mefloquine upregulates the transcription of important ER stress response genes

The Rat Genome U34 array was found to contain probes representing the ER chaperones glucose-regulated protein (GRP) 78, protein disulfide isomerase, GRP94, calreticulin and endoplasmic reticulum protein 29 (EPR29), GADD153 and PERK. Mefloquine (80 μM) upregulated the transcription of four of five ER resident chaperones, GRP78, PDI, GRP94 and calreticulin, GADD153 and PERK (one way ANOVA $P < 0.05$, $n = 20$, $FC > 1.3$, Table 2). Mefloquine induced no transcriptional changes at 2 μM (24 h) or 10 μM (5 min). Relative transcriptional changes at each concentration for GRP78, GADD153 and PERK are shown in Figure 6. Mefloquine induced no significant change in the levels of λ -tubulin mRNA (Table 2, Figure 6). λ -tubulin was called present by Affymetrix software in all 20 samples (mean $P = 0.00106$), and the mean control (DMSO) AD (\pm SEM) value was 444 (± 88).

Discussion

There are relatively few studies that have attempted to address the possibility that a biochemical explanation may lie behind the neurotoxicity of mefloquine. However, previous literature suggested that mefloquine might disrupt ER calcium homeostasis. Firstly, Go and others [5,6] showed that mefloquine alters calcium flux into and out of isolated mammalian microsomes obtained from skeletal muscle and neuronal tissue. Secondly, mefloquine has been shown to cause a striking upregulation of GADD153, a transcription factor selectively modulated under conditions of ER stress [8]. However, the depletion of the ER calcium pool on the one hand, and a consequent induction of an ER stress response have not been shown to occur simultaneously in intact primary neurons. In the

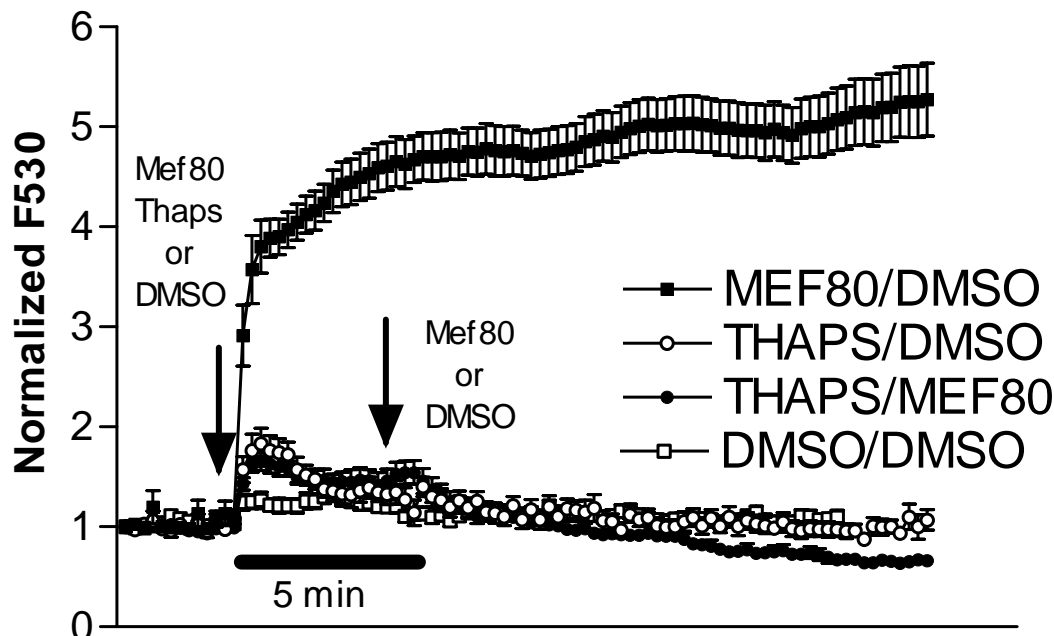


Figure 4
Effects of mefloquine on whole cell calcium homeostasis and their antagonisation by thapsigargin. The effects of mefloquine on neuronal whole cell calcium homeostasis were investigated using confocal microscopy and Locke's buffer containing physiological calcium levels (2.3 mM). Neurons were loaded with the calcium-sensitive dye Fluo-3 that was replaced with normal Locke's buffer after 1 h. Neurons were scanned at 15 s intervals. Mefloquine (MEF80, 80 μ M), thapsigargin (THAPS, 4 μ M) or DMSO (0.4%) were added at scan 13 (as indicated by the first arrow). Subsequently (second arrow at scan 28), DMSO (0.2%) or mefloquine (80 μ M) were added. Mefloquine (but not DMSO) caused an immediate and sustained rise in the cytosolic calcium concentration. This elevation is due to an influx across the plasma membrane as it was not observed in neurons bathed in low calcium Locke's buffer. The effect was antagonized by prior treatment with thapsigargin. As this agent is a specific inhibitor of the ER calcium pump, the data suggest that the effects of mefloquine on calcium homeostasis are mediated at the level of the ER in rat neurons.

present study, utilizing confocal microscopy and Affymetrix arrays, we have shown that at least one component of the neurotoxicity of mefloquine can be attributed to an effect on the ER calcium store, and that the drug activates a UPR similar to that induced by agents known to disrupt ER function.

We employed confocal microscopy as a tool to investigate the effects of mefloquine on calcium homeostasis. This approach has an advantage over that employed by Go et

al [6], in that calcium homeostasis can be assessed in real-time in live, intact neurons. Our data show that, in nominally calcium free medium, mefloquine induces a transitory elevation of cytoplasmic calcium levels and antagonizes a similar effect induced by thapsigargin. The most reasonable interpretation of these data is that mefloquine mobilizes a thapsigargin-sensitive calcium pool, and, since thapsigargin is known to mobilize the ER calcium pool, that this is also the case for mefloquine. The ER calcium pool can be mobilized in two ways. Firstly, an

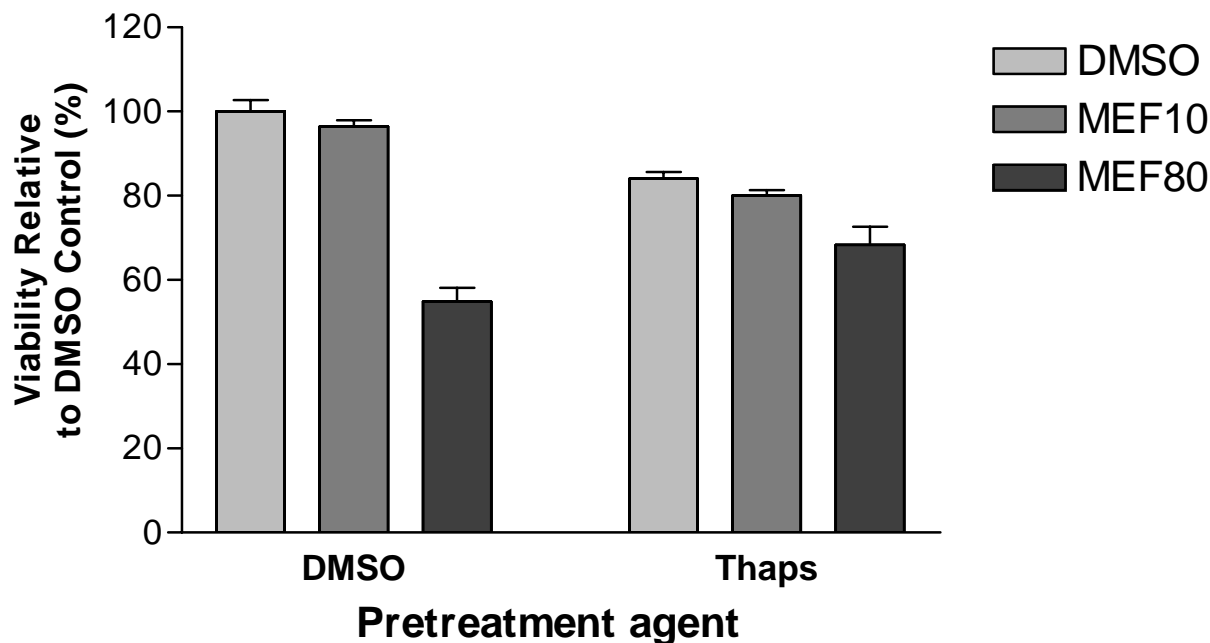


Figure 5

Effects of mefloquine and thapsigargin treatment on viability of rat neurons *in vitro*. A: The effect of mefloquine (Mef, 10 or 80 μ M for 5 min), before and after pretreatment with thapsigargin (Thaps, 1 μ M or DMSO, 0.4% for 5 min), on the viability of rat neurons was assessed using the MTT assay. Data shown are from eight replicate experiments. There was a significant interaction between mefloquine and thapsigargin (two way ANOVA, $P < 0.01$, $n = 48$). Thapsigargin had a neuroprotective effect against the toxic action of mefloquine, as indicated by the partial prevention (by 60%) of the reduction in viability (A_{540} units) caused by mefloquine.

inhibitory effect of an ER-active agent (e.g. thapsigargin) on the ER Ca^{2+} -ATPase results in a decrease in the rate of calcium influx across the ER membrane. As a consequence of a passive leak of calcium ions across the ER membrane into the cytoplasm, the store becomes depleted and a transient elevation in the cytoplasmic calcium concentration occurs. Alternatively, some ER-active agents (e.g. ryanodine) deplete the calcium store by activating calcium release channels. Either of these effects could account for our observations.

Compromised ER function (induced by numerous stressors including depletion of ER calcium stores) may lead to the accumulation of unfolded proteins in the ER [11].

These events may trigger the UPR, in which the transcription of genes encoding ER-resident chaperones is upregulated. The ability of mefloquine to upregulate the transcription of PDI, GRP78, GRP94 and calreticulin is consistent with this hypothesis. Triggering of the UPR also results in the suppression of global protein synthesis, as PERK is activated and phosphorylates EIK2 α , which can only interact with the appropriate ribosomal binding sites in an unphosphorylated state [11,12]. While the upregulated transcription of PERK is not required for its activation, its over expression can lead to the enhanced phosphorylation (and therefore inactivity) of EIK2 α [16]. Therefore, its up-regulation by mefloquine supports the hypothesis that the drug triggers an UPR. The third com-

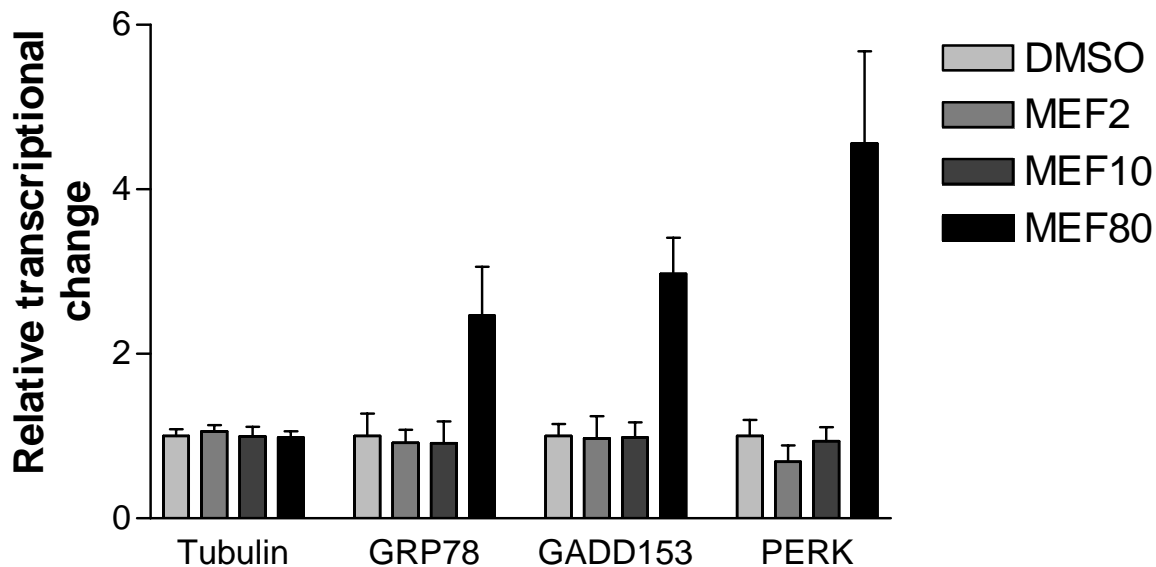


Figure 6

Effects of mefloquine on the transcription of ER stress response genes. Changes in the mRNA levels of tubulin, GADD153, GRP78 and PERK induced in rat neurons after exposure to mefloquine (5 min treatment followed by 24 h wash-out) were quantitated using Affymetrix U34 Rat Genome arrays. Mefloquine (80 μM) upregulated the transcription of GADD153, GRP78 and PERK but not tubulin (one way ANOVA, $P < 0.05$, $n = 20$), suggesting that the drug induces an ER stress response at this concentration.

ponent to the UPR cascade is an induction of the ER transcription factor GADD153 [11,12], an effect also observed after mefloquine treatment. Thus it appears that mefloquine induces an UPR similar to that induced by agents (e.g. thapsigargin) known to cause ER stress [8].

The effects of mefloquine on ER calcium homeostasis have been examined previously [5,6]. In experiments with isolated rabbit skeletal muscle microsomes, mefloquine was found to inhibit the uptake of calcium, with an IC_{50} of approximately 43 μM [6]. This effect was exacerbated by treatment with thapsigargin, a specific inhibitor of the ER calcium pump [6]. Further, the rate of ATP hydrolysis by the ER Ca^{2+} -ATPase was reduced in the presence of

mefloquine (K_i of = 53 μM). These authors concluded that the ER calcium pump might be a target of mefloquine. As discussed above, our own data are also consistent with such a mechanism. The observation by Go et al. [6], that the effects of mefloquine and thapsigargin on microsomal calcium uptake are synergistic, might seem at first to contradict ours. However, it should be pointed out that in our study, we treated neurons with these two agents sequentially, so that, in effect, one of the agents was acting on a depleted ER calcium pool. Under the conditions employed in our study, it is not surprising that mefloquine antagonized the effect of thapsigargin.

Table 2: Changes in the transcriptional modulation of ER stress response genes induced by mefloquine in rat neurons.

Protein	Genbank accession	ANOVA P	Fold-change at:			ANOVA P < 0.05 and FC > 1.3 at 80 μ M?
			2 μ M	10 μ M	80 μ M	
ER chaperones						
GRP78	S63521	0.0186	1.0	1.0	2.6	YES
PDI	X02918	0.000219	1.0	1.0	1.7	YES
GRP94	S69315	0.004307	1.1	1.0	0.8	NO
GRP94	S69316	0.000165	1.0	1.0	2.2	YES
ERP29	U63482	0.23012	0.8	0.9	1.3	NO
Calreticulin	D78308	0.0062	1.0	1.0	1.5	YES
ER-relevant transcription factors						
GADD153	U30186	0.00207	1.0	1.0	3.0	YES
EIF2 α kinases						
PEK/PERK	AF096835	0.000587	0.7	0.9	4.6	YES
Controls						
Tubulin	AB015946	0.926	1.2	1.0	1.0	NO

Li et al [5] also found that mefloquine inhibited calcium uptake into isolated microsomes from dog brain neurons – presumably via the same mechanism – although the drug was much less potent (IC_{50} of 272 μ M) than we are reporting it to be. We observed an approximately four-fold more potent effect of mefloquine on ER calcium homeostasis in rat neurons (IC_{50} of 66 μ M). It would be tempting to speculate that, because mefloquine's effects on rat neuronal ER calcium homeostasis were more potent than that for dog-brain microsomes, there might exist a species difference in sensitivity to the drug. However, this would be premature, since the two studies have employed markedly different experimental systems to observe similar phenomena. Li et al. also observed that mefloquine inhibited IP_3 -induced calcium release from isolated dog brain microsomes [5]. Our results do not preclude such a role for mefloquine in neuronal cells, although it would not have been possible to observe such an effect in the present study, since we investigated only the spontaneous action of mefloquine on ER calcium homeostasis.

Mefloquine's disruption of ER calcium homeostasis may contribute to the acute neurotoxicity of the drug, since the concentration-response for these effects are superimposable over the same time frame (5 minutes). However, the toxicity of the drug is somewhat time-dependent, since the magnitude of its effect is greater after twenty minutes exposure as compared to five minutes. We also investigated mefloquine's effects in calcium-containing medium and found that the drug induces a sustained secondary rise in cytoplasmic calcium originating from an external source. Thapsigargin blocked both the initial and secondary rises in cytoplasmic calcium induced by meflo-

quine and partially reversed the neurotoxicity of the drug. These observations are suggestive of two things. Firstly, the trigger for the sustained elevation in cytoplasmic calcium may occur at the level of the ER, and secondly, this effect may also contribute substantially to the neurotoxicity of mefloquine. However, the molecular basis for these two events is unclear. It is possible that mefloquine induces store-mediated capacitative calcium entry [see reference [17]]. However, it is unclear why thapsigargin, which also depletes the ER calcium store, would not produce a similar effect. Our laboratory is currently investigating the possibility that known inhibitors of store-mediated calcium entry (e.g. non hydrolysable GTP analogs and SKF96365) might also block the effects of mefloquine.

Mefloquine use has been associated with two broad classes of neurological disorders: (i) central and peripheral nervous system disorders including headache, dizziness, vertigo and seizures and (ii) psychiatric events including insomnia, anxiety, affective and major disorders [2]. It is well known and has been accepted for some time that prolonged disruptions in Ca^{2+} homeostasis can result in impairment of neuronal function and cell death [18,19]. Impairment or loss of neurons in specific regions of the brain might be manifested in symptoms reported to have occurred in patients treated with mefloquine. For example, the vertigo reported by some patients might be attributable to neuronal death in the inferior cerebellum [20], whereas symptoms of fear/anxiety might arise as a consequence of the death of neurons in the amygdala [21]. Mefloquine-induced neurotoxicity in the limbic system might be responsible for reported disturbances in emotion [22].

Table 3: Accumulation of mefloquine in the brain.

Animal/Case	Mefloquine Brain Concentration (μM equivalent)	Mefloquine Plasma Concentration (μM)	Brain/plasma ratio	Reference
Rats (50 mg/kg daily)	90	7.2	12.5	[23]
Human case 1 (p.m.)	52	1.7	30.1	[24]
Human case 2 (p.m.)	32–51	?	-	[24]
Mean of three human cases 3–5 (p.m.)	27*	Postmortem redistribution?	-	[25]

* Data calculated from literature data on basis of the free-base mefloquine molecular weight of 378.4.

Mefloquine accumulates to plasma concentrations of 3.8 and 2.1–23 μM after administration at prophylactic dose and therapeutic dosing respectively [3,4]. Therefore, only at the time of peak plasma concentrations in a few patients, do mefloquine plasma concentrations approach the threshold necessary ($> 10 \mu\text{M}$) to induce any of the effects observed in the present study. However, in human post-mortem cases, mefloquine has been found to accumulate in the brain relative to plasma by as much as 30-fold, with absolute, equivalent concentrations exceeding 50 μM (Table 3). These observations have been confirmed in rats, where the accumulation was found to be 12.5-fold with absolute, equivalent concentrations exceeding 90 μM (Table 3). Thus, mefloquine apparently crosses the blood-brain barrier and accumulates to sufficient levels to include those concentrations found to disrupt neuronal calcium homeostasis and ER function *in vitro*. The possibility that a disruption of neuronal calcium homeostasis or ER function may be responsible, at least in part, for the clinical neurotoxicity of mefloquine is worthy of further investigation.

Conclusion

Mefloquine was found to disrupt calcium homeostasis in rat neurons by mobilizing the ER calcium pool and inducing a sustained elevation of cytoplasmic calcium originating from an external source. Mefloquine also induced a characteristic ER stress response. The effects were observed at physiologically relevant concentrations and may contribute to the acute neurotoxicity of the drug *in vitro*.

Competing Interests

None declared.

Acknowledgements

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References

- Phillips-Howard PA and ter Kuile FO: **CNS adverse events associated with antimalarial agents: Fact or fiction?** *Drug Safety* 1995, **12**:370-383.
- Schlagenhauf P: **Mefloquine for malaria chemoprophylaxis 1992–1998** *J Travel Med* 1999, **6**:122-123.
- Kollaritsch H, Karbwang J, Wiedermann G, Mikolasek A, Na-Bangchang K and Wernsdorfer WH: **Mefloquine concentration profiles during prophylactic dose regimens** *Wien Klin Wochenschr* 2000, **112**:441-7.
- Simpson JA, Price R, ter Kuile F, Teja-Isavatharm P, Nosten F, Chongsuphaisiddhi T, Looareesuwan S, Aarons L and White NJ: **Population pharmacokinetics of mefloquine in patients with acute falciparum malaria** *Clin Pharmacol Ther* 1999, **66**:472-84.
- Lee HS and Go ML: **Effects of mefloquine on Ca^{2+} uptake and release by dog brain microsomes** *Arch Int Pharmacodyn Ther* 1995, **331**:221-231.
- Go ML, Lee HS and Palade P: **Effects of mefloquine on Ca^{2+} Uptake by crude microsomes of rabbit skeletal muscle** *Arch Int Pharmacodyn* 1995, **329**:255-271.
- Dow GS: **Effect of sample size and P-value filtering techniques on the detection of transcriptional changes induced in rat neuroblastoma cells by mefloquine** *Malaria J* 2003, **2**:4.
- Mengesdorf T, Althausen S, Oberndorfer I and Paschen W: **Response of neurons to an irreversible inhibition of endoplasmic reticulum Ca^{2+} -ATPase: relationship between global protein synthesis and expression and translation of individual genes** *Biochem J* 2001, **356**:805-812.
- Racay P, Kaplan P and Lehotsky J: **Control of Ca^{2+} homeostasis in neuronal cells** *Gen Physiol Biophys* 1996, **15**:193-210.
- Morgan AJ and Jacob R: **Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane** *Biochem J* 1994, **300**:665-672.
- Berridge MJ: **The endoplasmic reticulum: a multifunctional signaling organelle** *Cell Calcium* 2002, **32**:235-249.
- Kaufman RJ: **Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls** *Genes Dev* 1999, **12**:1211-1233.
- Pahle HK and Baeuerle PA: **The ER-overload response: activation of NF- κB** *TIBS* 1997, **22**:63-67.
- Koenig ML, Sgarlat CM, Yourick DL, Long JB and Meyerhoff JL: **In vitro neuroprotection against glutamate-induced toxicity by pGlu-Glu-Pro-NH(2) (EEP)** *Peptides* 2001, **22**:2091-2097.
- Vahey M, Nau M, Jogodzinski J, Yalley-Ogunro M, Taubman N, Micjeal N and Lewis M: **Impact of viral infection on the gene expression profiles of proliferating normal human peripheral blood mononuclear cells infected with HIV-RF** *AIDS Research and Human Retroviruses* 2002, **18**:179-192.
- Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, Koromila A and Wouters BG: **Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2 α** *Mol Cell Biol* 2002, **22**:7405-7416.
- Putney JW, Broad LM, Braun FJ, Lievrement JP and Bird GSJ: **Mechanisms of capacitance calcium entry** *J Cell Sci* 1999, **12**:2223-2229.

18. Choi DW: **Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage** *TINS* 1988, **11**:465-469.
19. Hartley DM, Kurth MC, Bjerkness L, Weiss JH and Choi DW: **Glutamate receptor-induced 45Ca^{2+} accumulation in cortical cell cultures correlates with subsequent neuronal degeneration** *J Neurosci* 1993, **13**:1993-2000.
20. Brust JCM: **Circulation of the brain** *In Principles of Neuroscience* Edited by: Kandel ER, Schwartz JH, Jessell TM. New York: McGraw-Hill; 2000:1302-1316.
21. Kandel ER: **Disorders of mood: Depression, mania and anxiety disorders** *In Principles of Neuroscience* Edited by: Kandel ER, Schwartz JH, Jessell TM. New York: McGraw-Hill; 2000:1209-1226.
22. Iverson S, Kupferman I and Kandel ER: **Emotional states and feelings** *In Principles of Neuroscience* Edited by: Kandel ER, Schwartz JH, Jessell TM. New York: McGraw-Hill; 2000:1209-1226.
23. Baudry S, Pham YT, Baune B, Vidrequin S, Crevoisier C, Gimenez F and Farinotti R: **Stereoselective passage of mefloquine through the blood-brain barrier in the rat** *J Pharm Pharmacol* 1997, **49**:1086-90.
24. Pham YT, Nosten F, Farinotti R, White NJ and Gimenez F: **Cerebral uptake of mefloquine enantiomers in fatal cerebral malaria** *Int J Pharmacol Ther* 1999, **37**:58-6.
25. Jones R, Kunsman G, Levine B, Smith M and Stahl C: **Mefloquine distribution in postmortem cases** *Forensic Sci Int* 1994, **68**:29-32.

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