CYTOTOXICITY AND FUNCTIONAL TOXICITY OF MEFLOQUINE AND THE SEARCH FOR PROTECTIVE COMPOUNDS

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Mefloquine hydrochloride is an antimalarial agent that has been used for the past 40 years. Numerous reports of neurological side effects have recently led the FDA to issue a strong warning regarding long-term neurological effects. This warning lead to the U.S. Army's Special Forces and other components to discontinue its use in July of 2013. Despite reported adverse side effects, mefloquine remains in circulation and is recommended to travelers going to specific Asian countries. Mefloquine has been used as a treatment for those already infected with the malaria parasite (blood concentrations ranging from 2.1 to 23 µM), and as prophylaxis (blood concentrations averaging 3.8 µM) (Dow 2003). The purpose of this study was to quantify Mefloquine's toxicity using spontaneously active nerve cell networks growing on microelectrode arrays in vitro and to identify compounds that alleviate or reduce toxic effects. The current literature on mefloquine toxicity is lacking electrophysiological data. These data will contribute to research on the mechanism of adverse side effects associated with mefloquine use.

Sequential titration experiments were performed by adding increasing concentrations of mefloquine solution to cultured neurons. Network responses were quantified and reversibility was examined. In each network, activity decreases were normalized as a percent of reference activity yielding a mean IC_{50} value of 5.97 ± 0.44 (SD) µM (n=6). After total activity loss, no activity was recovered with two successive medium changes. To test for network response desensitization resulting from sequential applications over 5-6 hr periods, one-point titrations at varying concentrations were conducted with fresh networks. These experiments yielded a single concentration response curve with an IC_{50} value of 2.97 µM. This represents a statistically

significant shift ($p < 0.0001$) to lower concentrations of mefloquine, demonstrating that sequential applications result in network desensitization.

After mefloquine exposures, cells were evaluated for irreversible cytotoxic damage. Over a 12-hour period under 6 µM mefloquine, process beading and granulation of somal cytoplasm were observed. At 8 µM mefloquine cell stress was apparent after only 10 minutes with major glial damage and process beading at 120 minutes.

In this study, quinolinic acid served as a neuroprotectant at 20 µM. There have been multiple studies on the endogenous concentrations of quinolinic acid and current literature is quite variable. Immunocompromised individuals have some of the highest blood levels of quinolinic acid (up to 20 µM). With 30 min pre-applications of quinolinic acid, the mefloquine IC_{50} value shifted from 5.97 ± 0.44 µM (n=6), to 9.28 ± 0.55 µM (n=3). This represents a statistically significant change to higher mefloquine concentrations and demonstrates neuroprotection.

TABLE OF CONTENTS

Page

I. Introduction and Background

Mefloquine remains the second most recommended antimararial agent only behind chloroquine (CDC) (Smithius et al. 2006). Mefloquine is unique and desirable because of its ability to protect against strains of malaria that have developed parasite resistance. However, mefloquine users report experiencing mild adverse side effects such as nausea, vomiting and headaches (Janowsky et al. 2014). A smaller subset of users report severe neurological side effects that can last for years after discontinued usage. These side effects include seizures, hallucinations, paranoia, and suicidal thoughts (Sturchler et al. 1990).

The current literature of mefloquine toxicity has large variability in concentrations from 2.1-23 µM (therapeutic dosage range) that cause side effects and to what extent neurological damage is reversible (Dow et al. 2003) (Table 8). The literature cites many receptors such as Serotonin (5-HT_{2a}); Dopamine; $GABA_A$; ACh; Adenosine A2a to be involved in the observed toxic phsychotrophic effects (Gale et al. 2004; Allison et al. 2011). The purpose of the study was to quantify the effects of mefloquine on spontaneous network activity, determine reversibility, establish the onset of cytotoxicity, and search for compounds that protect neurons from toxicity and try to elucidate mechanism of neurotoxicity.

Mefloquine Effectiveness and Structure

Mefloquine hydrochloride (Larium) has remained a popular antimalarial agent since its synthesis at the Walter Reed Army Institute of Research (DoD) in the 1970s. It was designed as a synthetic analogue of quinine. Numerous reports of neurological side effects have recently led the FDA to issue a black box warning. However, mefloquine is still used widely throughout the world because it is very effective against all five species of Plasmodium that contribute to malaria including *Plasmodium falciparum*, which has been developing a resistance to anti-malarial agents (Caridha et al. 2008). Despite serious side effects such as depression, general anxiety disorder, psychoses, convulsions, seizures, tinnitus, and movement disorders there are no quantitative data on mefloquine toxicity in the literature, and no explanation has yet been given for the observation that not all users report such side effects (Allison et al. 2011).

Mefloquine has four stereoisomers and is currently sold as a racemate of the (*R*,*S*)- and (*S*,*R*)-enantiomers by Hoffman-LaRoche, a Swiss pharmaceutical company. The enantiomers exhibit slightly different half lives and different severity of symptoms experienced by users (Bermudez et al. 2012). Essentially, it is two drugs in one (Figure 1). According to Bermudez, plasma concentrations “of the (–)-enantiomer are significantly higher than those for the (+)-enantiomer, and the pharmacokinetics of the two enantiomers are significantly different. The (+)-enantiomer has a shorter half-life than the (–)-enantiomer. According to some research the (+)-enantiomer is more effective in treating malaria, and the (–)-enantiomer specifically binds to adenosine receptors in the central nervous system, which may explain some of its psychotropic effects” (Bermudez et al. 2012).

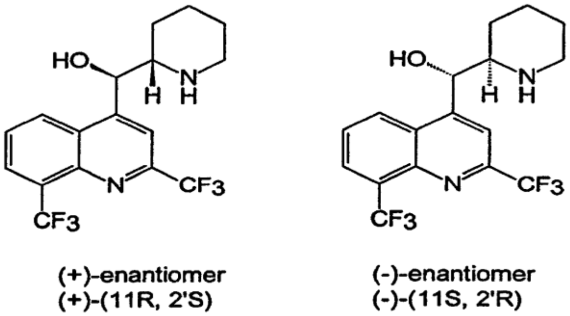

Figure 1. Enantiomers of mefloquine structures comprising present Larium products. These enantiomers contribute to the complexity of this medication.

Mefloquine Toxicity

As already mentioned, literature has cited many receptors such as Serotonin (5-HT$_{2a}$); Dopamine; GABA$_{A}$; ACh; and Adenosine A2a to be involved with the toxic effects that have been observed in mefloquine users (Allison et al. 2011). As a result of the variety of symptoms, it is likely that functional toxicity (i.e., changes in the electrical activity of neural tissue without causing cell death) and cytotoxicity depend on different mechanism. The most commonly accepted mechanism for cytotoxic changes is through the action of an ionophore (Caridha et al. 2008). This is similar in mechanism to ionomycin, which involves calcium entry and release from the smooth endoplasmic reticulum, leading to apoptosis or necrosis. Mefloquine integrates itself inside the cell membrane and leads to a disruption of ion homeostasis. With membranes themselves changing during mefloquine exposure, it is possible that receptors and their homeostatic activity could be altered. This variation in functional toxicity could contribute to the differences and similarities exhibited among mefloquine users.

Mefloquine Literature Comparisons

Many studies that have been conducted on quantifying mefloquine toxicity examined the different morphological changes in cells to determine concentration dependent damage to cells. In one such study, cultured cochlea from postnatal rats were treated with 35 and 50 µM mefloquine for 24 hours. Damage to auditory nerve fibers was evaluated through counting the number of nerve fibers projecting to the cochlear hair cells in basal and apical turns using a fluorescent microscope at 600x magnification. Damage to cochlear hair cells was assessed over 0.24 mm intervals along the entire length of the cochlea (Ding et al. 2013). At 35 µM there was significant loss of auditory nerve fibers and cochlear hair cells, and at 50 µM there was complete destruction of auditory nerve fibers and cochlear hair cells.

In another experiment, oxidative stress and dendritic degeneration of primary rat cortical neurons were evaluated. The oxidative stress markers , glutathione and F2- isoprostance, were quantified after a 24 hour exposure to 0, 1, 5, and 10 µM using mass spectrometry. Dendritic damage was evaluated using a confocal laser scanning unit (Hood et al 2009). There was a concentration dependent increase in both glutathione and F2-isoprostance starting at the lower concentration of 1 µM which indicated oxidative stress begins at low concentrations of mefloquine. There was a concentration dependent increase in dendritic spine damage and decrease in density at 5 and 10 µM mefloquine with an LD50 of 8.9 µM with reference to viable cells (Table 9). Morphological studies done in this thesis found beading of processes and deteoriation of cell bodies at 6 µM mefloquine over a 12 hour period, and 8 µM mefloquine over a 2 hour period (Figure 19 & 20). These qualitative comparisons with the literature show general agreement with data generated in this thesis.

One study using dissociated neurons from the substantia nigra of rats 6 to 17 days postnatal days (Zhou et al. 2006) quantified mefloquine toxicity through electrophysiological data. This study examined the effect of mefloquine on GABA-mediated receptor spontaneous inhibitory postsynaptic currents (sIPSCs) of dopaminergic neurons. An IC50 of 1.3 µM was reported which is very similar to the IC50 of 2.86 µM observed in the one-point titration section of this thesis (Figure 17).

The mefloquine paradox of why some users experience no side effects and other users report very severe side effects is part of what makes mefloquine so complex. In the last year, major organizations such as the CDC and U.S military have recognized mefloquine as a substance that can cause irreversible neurological damage to users. It is possible that some users are protected from this toxicity by endogenous compounds such as metabolites. More quantitative analysis of mefloquine toxicity is necessary to better understand the variation in neurological side effects.

II. Objectives and Hypothesis

The purpose of this study was to quantify the neurotoxicity of mefloquine using spontaneously active nerve cell networks on microelectrode arrays and to determine whether biochemical protection is possible.

Hypothesis 1: Mefloquine causes quantifiable concentration-dependent changes in network activity.

Hypothesis 2: At high concentrations, mefloquine causes irreversible cell damage (cytotoxicity).

III. Methods

Cell Culture and MEA Fabrication

All cultured networks were provided by the CNNS cell culture staff. Primary cultures of mouse cortical tissue grown on MEAs were used to investigate mefloquine toxicity and potential blockers of such toxicity. Timed pregnant mice (Balb-C/ICR mice) were obtained from the Harlan Spraque Dawley Corporation at gestation stage day 15. They were used the following day for the preparation of dissociated cell suspensions. Typically 10-14 embryos were delivered from the uterus. Each fetus was then decapitated in iced D1SGH (buffer) and frontal cortices were removed. This tissue was then minced with two sterile scalpel blades. After a short digestion in D1SGH containing papain, the tissues were then triturated in 5 mL DMEM, 5% horse serum, 2% B-27, and 8 µg vitamin C/mL. The tissue was then seeded onto a 4 mm^2 adhesion area in the center of a MEA. The cell culture steps are summarized in Figure 2. More detail of the culture procedure can be found in Gross (1994).

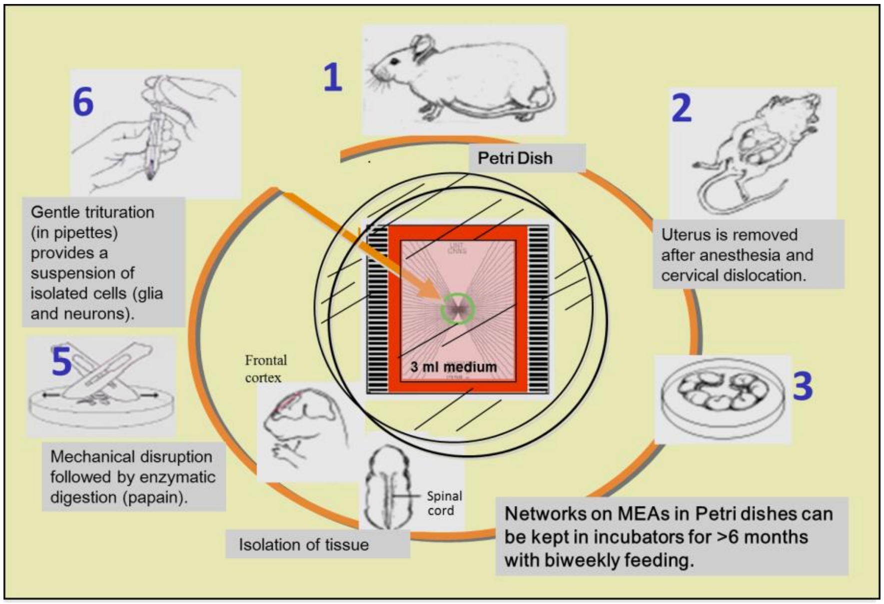

Figure 2. Summary of steps involved in the generation of primary cell cultures for growth on MEAs. All cultures were provided to the author by the CNNS culture staff.

Picture: CNNS archives (Dian/Gross).

After the first week cultures were fed biweekly with DMEM and 10% horse serum until the day of testing. If bacterial contaminations were present cultures were either treated with antibiotics (Gentamyacin) or were given several full medium changes. On the day of testing, the MEAs were integrated onto a recording chamber (Gross and Schwalm, 1994). After observing native activity for 30 to 60 minutes, a complete medium change was done to DMEM stock (DMEM without serum). The pH and osmolarity levels were maintained at 7.4 +/- .1 and 300-320 mOsmoles, respectively. Only mature (21 days or older) cultures were used for all pharmacological testing.

The microelectrode arrays were made in house by the CNNS according to methods defined formerly (Gross 1979)(Gross 1994)(Gross et al. 1985). Briefly, photoetched indium tin oxide (ITO)-sputtered glass plates were spin-insulated with methyltrimethoxysilane, cured, de-insulated at the electrode tips with laser shots, and electrolytically gold-plated to adjust the interface impedance to 0.8 - 1 MΩ at 1 kHz (Gross et al. 1985). The MEA insulation material is hydrophobic, and butane flaming through masks were used to activate the surface and generate a hydrophilic adhesion island (3 mm in diameter) centered on the MEA (Lucas 1986). Transparent ITO conductors allow extensive optical access to network morphology (Figure 3).

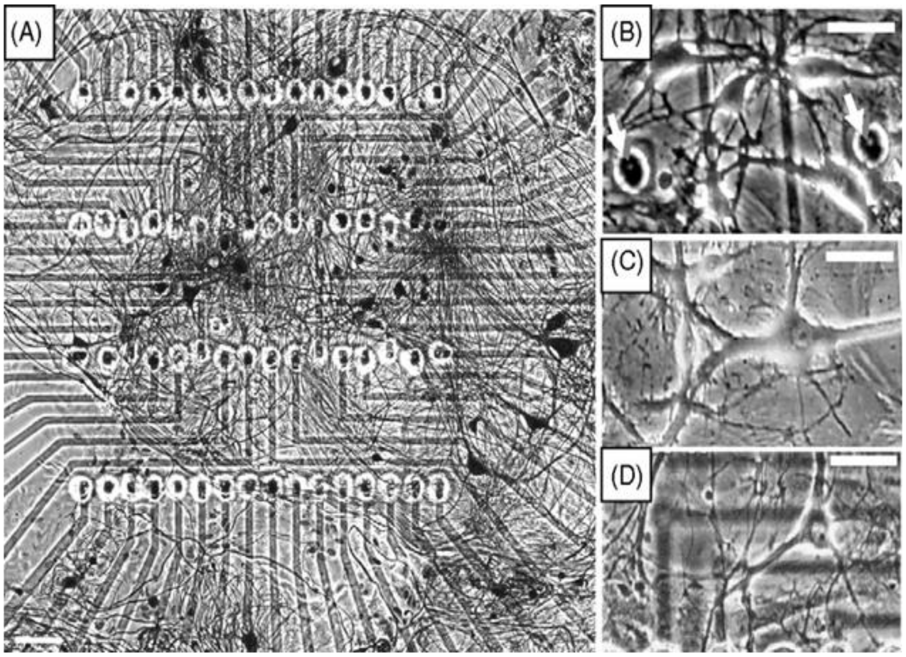

Fig. 2. Examples of neuronal circuits on microelectrode arrays. Transparent indium–tin oxide (ITO) conductors allow extensive optical access to the network morphology. (A) Neuronal network derived from murine spinal cord tissue, grown on the recording matrix of a 64-electrode array plate . (B–D) Living neurons on MEAs. Gold-plated, exposed ITO conductors are shown by arrows in (B). The ITO conductors are 8 mm wide and 1200 Å thick (all bars = 50 mm). Ref. 19.

Figure 3. Example of neuronal circuits on microelectrode arrays. Transparent indiumtin oxide (ITO) conductors allow extensive optical access to the network morphology. (A) Neuronal network derived from murine spinal cord tissue (92 days *in vitro*), grown on the recording matrix of a 64-electrode array plate (Bodian stained). (B-D) Living neurons on MEAs. Recording sites (gold-plated, exposed ITO conductors are shown by arrows in (B). The ITO conductors are 10 μm wide and 1200Å thick. bars = 40 μm. (CNNS Archives)

Recording Assembly and Data Analysis

The neuronal networks were maintained in a constant 2 mL bath of stock medium in the recording chambers. The assembly at the recording station consisted of an aluminum base plate that holds the MEA and a stainless steel chamber (Figure 4). Preamplifiers were placed on the microscope stage at either sides of the recording chamber and connected to the MEA by means of zebra strips (Fujipoly America Corporation, Carteret, NJ). The amplifier ground was connected to the stainless steel chamber confining the culture medium. A temperature probe was connected to the chamber to maintain a temperature of 37 ± 0.5 °C. To maintain pH of approximately 7.4, a gentle stream of 10% carbon dioxide in air was passed over the medium at about 10 mL/minute. This atmosphere is confined by a cap (Figure 4) that features a heated ITO window, which prevented condensation and allowed for continual optical observations. An infusion pump was used to compensate for slow water evaporation by injecting 60-70 micro liters of water per hour into the medium bath. This empirically determined water addition kept medium osmolarity at approximately 300-320 mOsmoles (Figure 4).

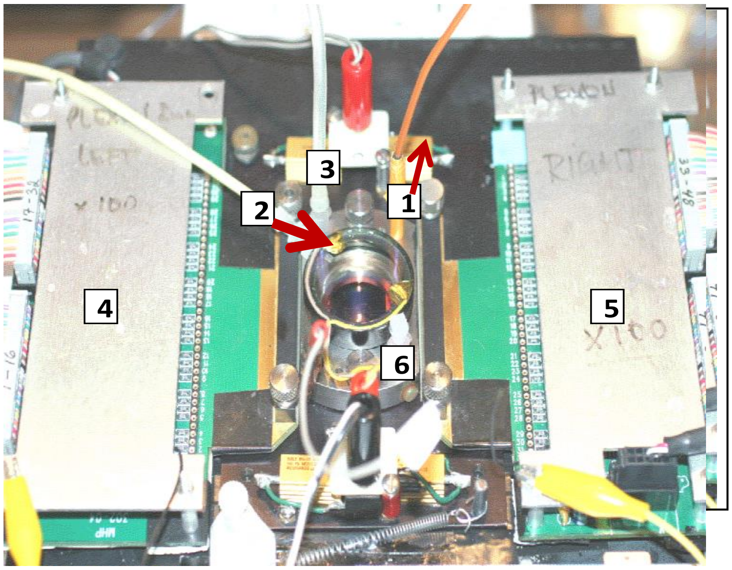

Figure 4. CNNS recording chamber set up. (1) Temperature probe that signals a power supply to maintain temperature at 37. (2) Gas line (10 % Co2 in air). (3) water line maintains osmolarity at 300-320 mOsmoles. (4 & 5) 32 channel pre-amplifiers that provide a 100x amplication and are connected to a second variable amplification unit. (6) Syringe ports for substance applications and medium changes.

The Plexon MNAP system digitized the analog action potentials at a frequency of 40 kHz (25 microsecond resolution). A Plexon template matching algorithm allowed selection of specific wave shapes representative of active neuronal components. Under optimal conditions, four different wave shapes can be separated on one channel in real time. Threshold crossing of templates provided a timestamp with a resolution of 25 microseconds. These time stamps were then used to calculate a variety of network activity variables ranging from total spike production to spatiotemporal pattern changes. A custom CNNS display program allowed the plotting of total or average network activity per minute. This very useful display made it possible to follow the evolution of network activity over long periods of time (Figure 5 & 6).

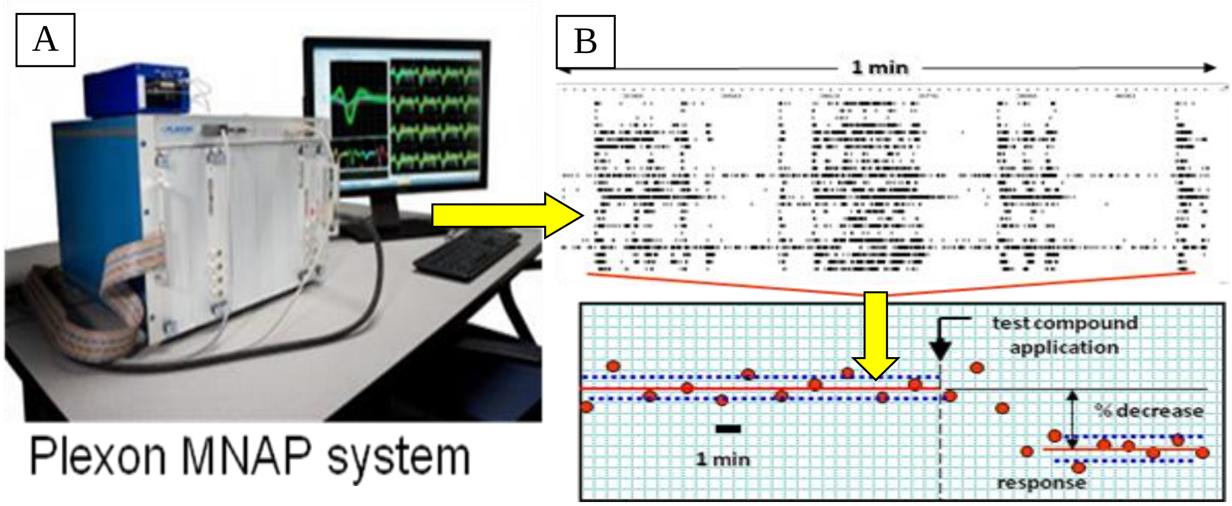

Figure 5. (A) Plexon MNAP system. (B) One minute timeframe of activity showing burst pattern and spiking from 25 wave-shape discriminated units. A custom CNNS program combines all activity during this one minute interval into a single data point. This simplification is effective for pharmacological studies.

The total system gain was set to 10,000. Single-unit activity was averaged or summed across the network to yield mean or total spike rate. All analyses were done with binned data (bin size of 60 seconds). Cultures were allowed to stabilize before any drugs were added (termed: reference activity or RA). The percent change in activity for each test substance at each drug concentration was always calculated relative to this 20 to 60 minute reference spontaneous activity. This procedure provided an internal normalization and allowed for effective comparisons across networks with different initial activity levels.

Statistics

All IC50 values obtained were generated from the program Origin 7.0 (OriginLab). This program does sigmoidal fits of the data plotted on a semi-log graph and calculates IC50 values, which are activity level decreases to 50% of the original reference activity for each individual experiment. All experiments were done with at least three different cultures (n =3)an n-value of 3. These data sets allowed for calculation of the average IC50 and standard deviation.

Individual concentration response curves for each a separate data set is preferred because it shows the spread of the data in terms of individual sigmoidal curves. However, pooled data concentration response curves were also in order to include incomplete or aborted concentration response curve experiments. The resulting IC50s were generally very similar but not identical (Table 3; Figure 15).

This study required the comparison of two means and the comparison of a mean with a hypothetical value. The comparison of means and their respective standard deviations (SD) was required for evaluation of concentration response curves generated in DMEM (n=6) versus DMEM with 20 μM quinolinic acid (n=3). Such a comparison required the use of a two tailed t-test after determining the data were distributed in a Gaussian fashion. In addition, the data were clearly independent as they were comprised of different networks, but involved the same methodology (i.e., sequential applications of mefloquine). All calculations were done with Graphpad QuickCalcs (http://www.graphpad.com/quickcalcs).

The statistical comparison of sequential and single point application cannot be done with a two-tailed t-test because the single point application generates only one concentration response curve. In this case, a one sample t-test was used, which compares the mean of a population with a specific value.

In both comparisons the p-values were highly significant (<0.0001), rejecting the null hypothesis that the populations were not different. In other words, quinolinic acid significantly increased the IC50 for mefloquine and, therefore, serves as a protectant. Single point applications significantly lowered the IC50 values, strongly suggesting network desensitization during sequential applications, which involves concentration and exposure time.

DMSO Control Experiments

As a result of mefloquine being insoluble in water, DMSO was used to prepare mefloquine solutions. DMSO control experiments were done to determine if the solvent alone could achieve network responses. Three such experiments are represented in Figure 6, which shows a small activity increase or decrease in with DMSO concentrations in the range of 1-4.5%. This effect was variable and was considered an application artifact. No systematic data corrections were considered necessary. At 5-6% DMSO, activity generally decreased and reached zero around 12% DMSO (Figure 6A). Mefloquine solutions were prepared in a range of concentrations so that experiments never exceed 3%.

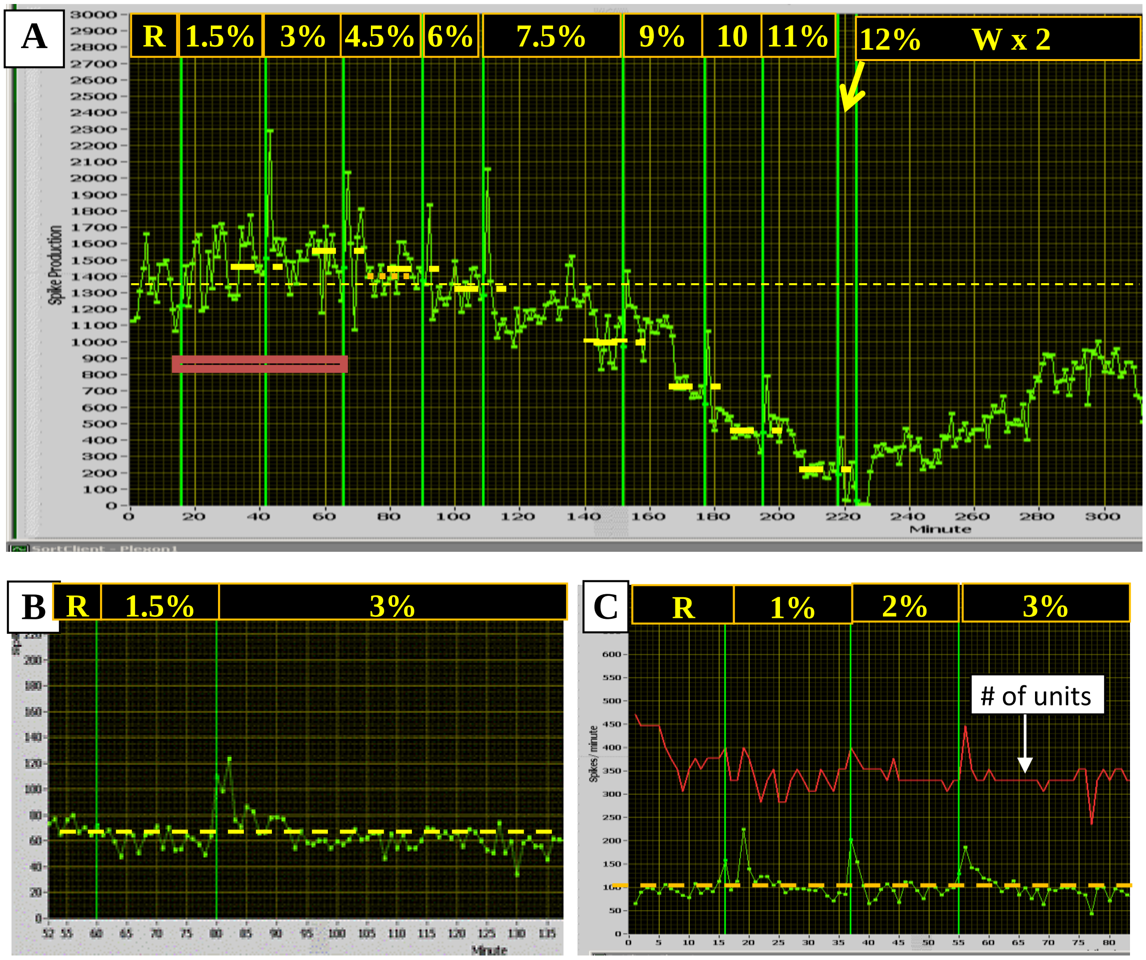

Figure 6. DMSO control experiments. (A) Sequential additions of DMSO ranging from 1.5 to 12 percent. Two medium changes (W x 2) recovered 64% of the initial activity. The thick dotted lines represent the newly formed plateaus of activity under the concentrations indicated. The thin long dotted line represents the original reference activity. The bar represents the experimental concentration range of DMSO used in mefloquine multiple step titrations. NOTE: in the concentration range of 1.5-4.5% DMSO there is a slight increase in activity. This increase does not occur all the time and was followed up with addition experiments shown above (B & C) Vernac displays of two DMSO control experiments reaching 3%. The thick yellow dotted line represents the reference activity. At 3%, little deviation from reference activity is observed in both experiments. Mefloquine concentrations never exceeded 1.4% DMSO. Panel A shows total activity whereas B and C plot average activity (total/number of active units). Panel C also includes the number of recorded units showing that this parameter remains relatively constant.

Application Methods

Most experiments were performed with successive applications of mefloquine in increasing concentration steps. Two application methods were compared. Method 1 involved the pipette addition directly to the medium bath followed by a mixing of the medium bath using a 3 mL syringe attached to the Luer port with gentle back and forth mixing. The pipette addition and mixing by withdrawing approximately 80% of the medium occurred within 10 seconds from one another (Figure 7).

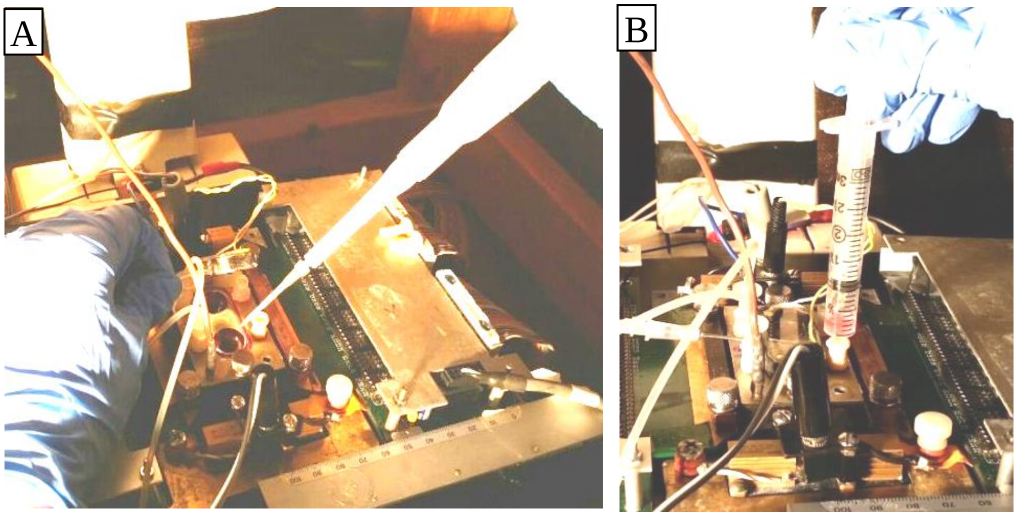

Figure 7. Application Method 1: Mixing in medium bath. (A) Direct application of mefloquine solution to the culture medium in recording chamber, followed within 10 seconds by mixing of medium using slow back and forth movement into a syringe (B).

Method 2 involved external mixing of the test substance in DMSO with bath medium in a syringe, vortexing, and re-introduction of the mixed medium to the chamber. In more detail, this method entailed syringe removal of 30% of the bath medium followed by an addition of 2-5 uL mefloquine in DMSO to the syringe tip with the syringe held vertical. The DMSO droplet sinks rapidly into the medium. This was followed with 3 seconds to mix the test solution with medium. Vortexing was used to break up the test solution. Air bubbles were then used to mix the solution in the syringe before pulling an additional 30% of bath medium into the syringe with subsequent mixing with air bubbles. This mixed test solution was then reintroduced into the remaining medium bath of the network (Figure 8).

The resulting network activity was allowed to stabilize for 30 minutes to establish a new activity plateau before subsequent repeat additions of mefloquine using the same method. Once activity was brought down to zero, two full medium changes were administered and the percent recovery relative to reference activity was calculated.

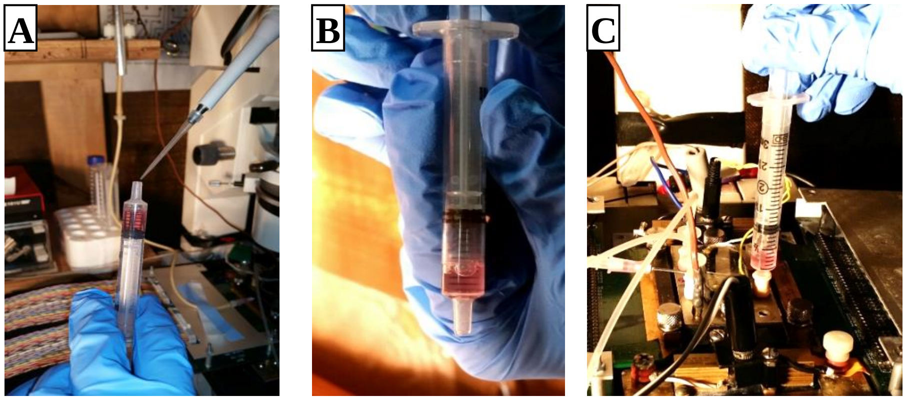

Figure 8. Application Method 2. (A) Pipette Tip transfer of mefloquine solution to extracted test medium via syringe. (B) The mixing of medium and solution with air bubbles (followed by vortexing). (C) Reintroduction of mixed solutions into CNNS chamber.

IV. Results

Repeatable Application Artifact (Method 1)

Mixing Method 1 was used first and then abandoned when it was realized that it gave inaccurate results. It is mentioned here to illustrate and emphasize that extreme care must be exercised when test substances, especially those that are dissolved in DMSO, are added to the bath medium. The repeatable artifacts are remarkably consistent which can lead to erroneous results. Test substances in DMSO sink to the bottom exposing networks to high concentrations even if mixing is initiated within seconds after application. During the initial investigation of toxicity, a pipette addition followed by syringe mixing was used to introduce the mefloquine solution into the test medium bath (Method 1). The resulting IC50 at 421 +/- 13 nM, was much lower than any therapeutic dose range of 2.1-23 µM in blood concentration level (Dow et al. 2003). DMSO was mixed with Black B dye, and introduced into a simulated medium bath (Figure 9). It was observed DMSO droplets sank rapidly to the bottom. Despite subsequent mixing in 3-5 seconds, networks were exposed to high concentrations of DMSO and test substance which contributed to the observed electrophysiological responses.

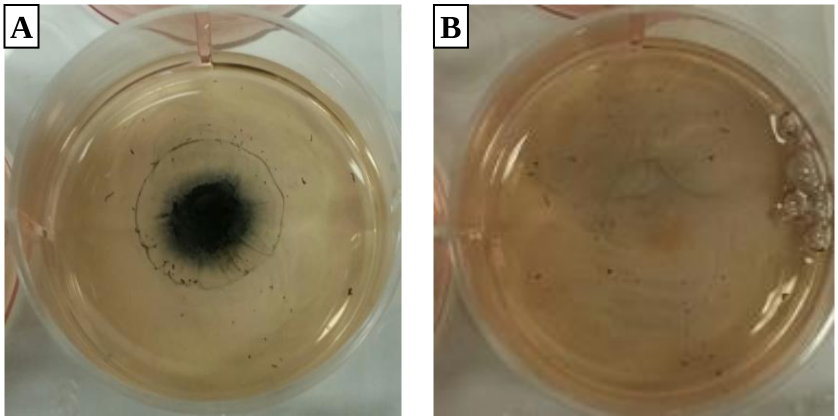

Figure 9. (A) Dyed DMSO in test medium to simulate dispersion patterns previous to mixing. The DMSO sinks rapidly to the bottom and spreads. (B) Dyed DMSO dispersion patterns after the mixing of medium and DMSO with a syringe (3 withdrawals, ~20 sec total). Although syringe mixing is fairly effective, cells at the bottom of a container are initially exposed to higher concentrations of test substance.

Dose Response Curves (Repeatable Artifact): Application Method "1"

Mefloquine exposure to neuronal networks caused a decrease in network activity in nanomolar concentrations. After a full application series of mefloquine, complete activity loss was achieved at 1 µM. Two successive medium changes did not recover any activity (Figure 10).

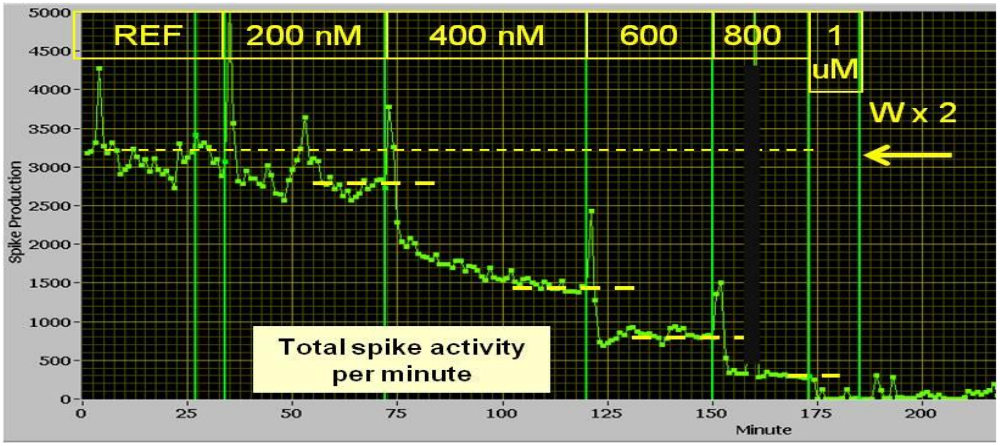

Figure 10. A Vernac display of a typical mefloquine experiment with successive decreases in activity under increasing concentration steps (200 nM- 1 uM). Two medium changes (W x 2) did not recover any activity. The thick dashed lines represent the newly formed plateaus of activity under the concentrations indicated. The thin long dashed line represents the original reference activity (Method 1).

Table 1- Summary of Mefloquine Experiments Using Method 1

Experiment	**Date**	**Unit***	**IC50 [nM]**
KH04	10/21/13	84	438
KH05	10/28/13	60	422
KH015	1/30/14	48	437
KH016	1/31/14	52	417
KH018	2/15/14	34	405
KH019	2/18/14	35	409
		Average	**421 ± 13**

* Unit refers to wave shape discrimination signals selected for recording

Data on mefloquine's functional toxicity showed it to be toxic at concentrations below the therapeutic dose. Using Method 1, mefloquine has an IC50 of 421 ± 13 nanomolar (nM), as shown in Table 1. Values were obtained through dose response curves with increasing concentration steps of 200 nM mefloquine (Figure 11 & Table 1).

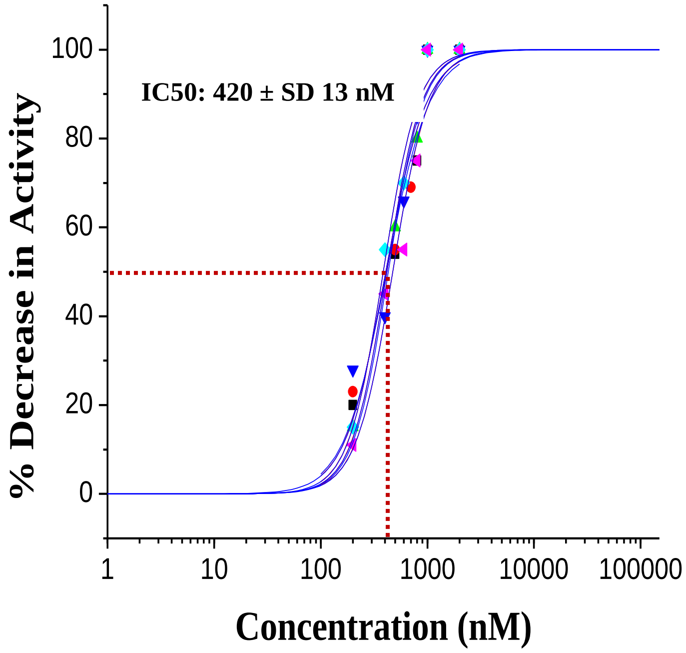

Figure 11. Concentration response curves of mefloquine titrations and IC50s (average 420 ± 13 nM; (n=6). Dotted lines represent the average concentration at 50% spike reduction.

Dose Response Curves: Application Method "2" (premixing in syringe)

A different application method was then examined that involved the removal of test medium into a syringe, the pipette tip introduction of DMSO solution into syringe collected medium, vortexing, further mixing of medium into the syringe, followed by reintroduction into the chamber (Method 2; previously described) (Figure 8). Both application methods resulted in stepwise decreases in activity of neuronal networks and had similar shifts in IC50s. As a result of application method comparison, Method 2 was used in all subsequent experiments because it provides much better mixing and yields physiologically relevant results.

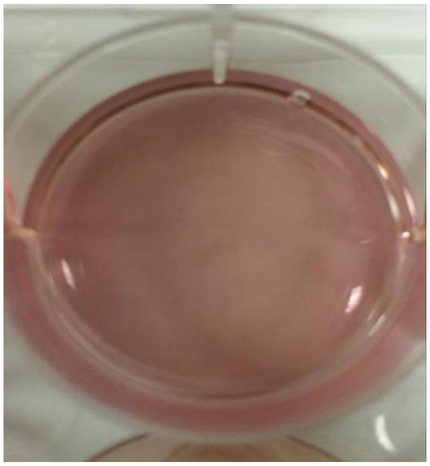

Figure 12. Dyed DMSO dispersion patterns after using Method 2. This procedure provides a uniform test substance concentration and eliminates the problem of over-exposure.

Using method 2, mefloquine additions show a slower, exponential decrease to response plateaus (Figure 13). These experiments yielded an IC50 of 5.97 ± 0.44 µM as shown in Table 2. The IC50 was achieved through dose response curves with increasing 2-3 um concentration steps of mefloquine (Figure 14). After the average IC50 was calculated for each experiment, pooled data concentration response curve was then used to calculate a single average curve (Table 3). The new IC50 was 5.67 µM, which was slightly lower than the average IC50 of 5.97 ± 0.44 µM (Figure 15). Through statistical analysis a p-value of .158 was achieved. This proves that there is no statistical significance between the IC50 values.

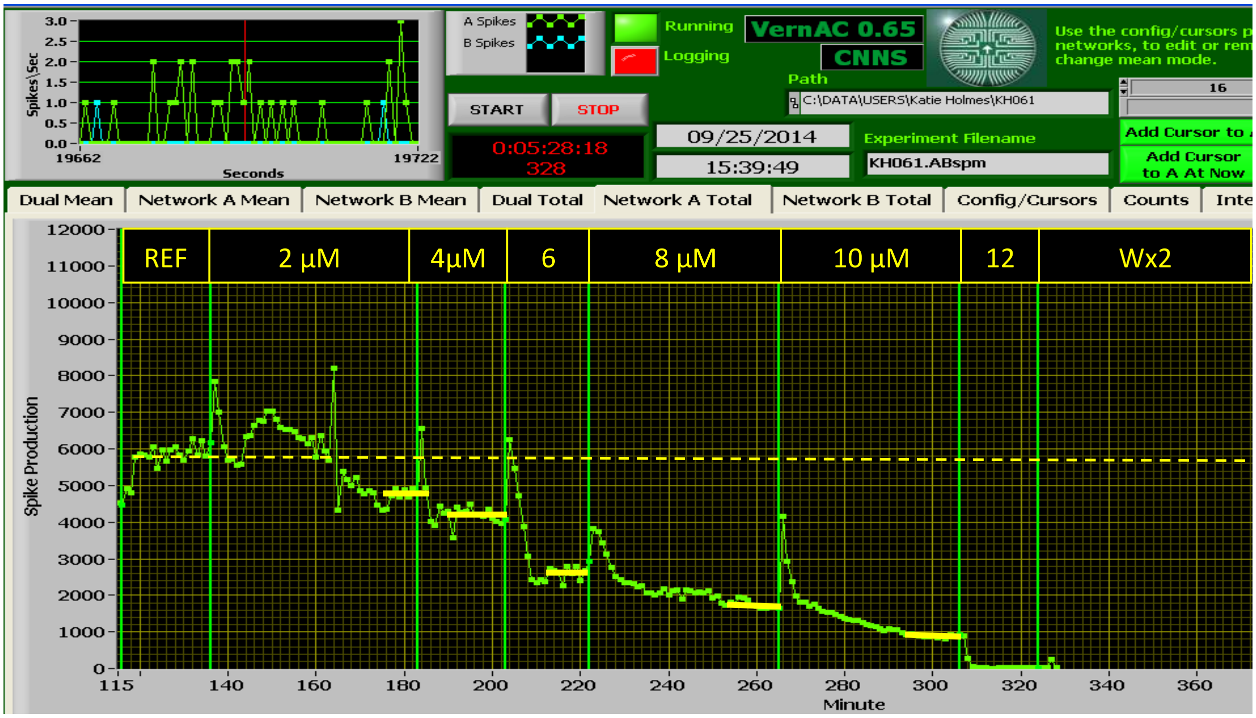

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Figure 13. A Vernac display of a typical mefloquine experiment with successive decreases in activity under increasing concentration steps (2 µM- 12 µM). Two medium changes (W x 2) did not recover any activity. The thick lines represent the newly formed plateaus of activity under the concentrations indicated. The thin long dashed line represents the original reference activity (Method 2).

Table 2. Summary of Mefloquine Experiments Using Method 2

Experiment	**Date**	**Units**	**IC50 [µM]**
KH061	9/25/14	24	5.93
KH062	9/26/14	44	5.88
KH064	10/2/14	41	6.65
KH065	10/9/14	57	5.40
KH066	10/10/14	62	5.70
KH084	1/18/15	32	6.30
		Average:	**5.97 ± 0.44**

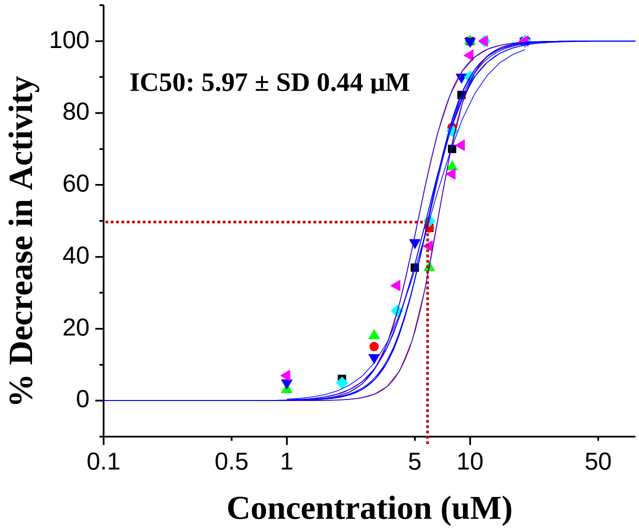

Figure 14- Concentration response curves of mefloquine titrations obtained with application Method 2. IC50s average 5.97 ± S.D 0.44 µM; (n=6). Individual IC50 values are listed in Table 2. Dotted lines represent the inhibitory concentration of 50% spike reduction.

Table 3. Pooled data for mefloquine experiments using Method 2

Concentration (µM)	**KH061**	**KH062**	**KH064**	**KH065**	**KH066**	**KH084**	**Avg. % Decrease**
0	0	0	0	0	0	0	**0**
1			3	5		7	**5**
2	6				5		**5.5**
3		15	18	12			**15**
4					25	32	**28.5**
5	37			44			**40.5**
6	48	48	37		50	43	**45.2**
8	70	76	65		75	63	**69.8**
9	85			90		71	**82**
10	100	100	100	100	90	96	**97.6**
12					100	100	**100**

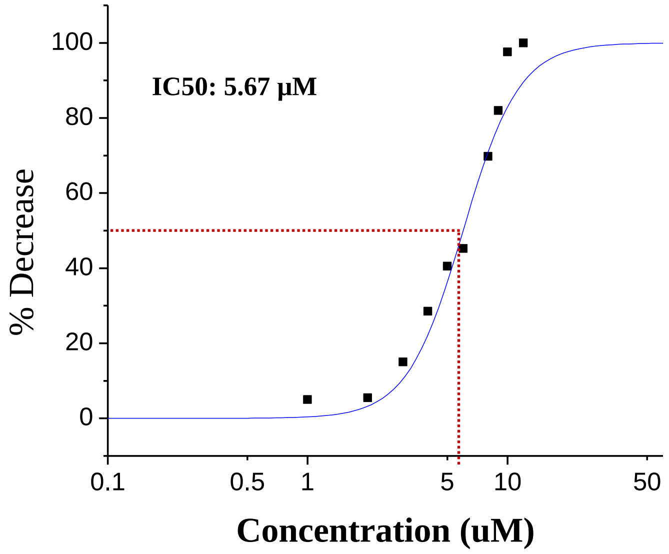

Figure 15. Concentration response calculated from of pooled data using all mefloquine experiments (Table 3). Pooled data IC50 of 5.67 µM was calculated. This is slightly lower than the average IC50 of 5.97 ± SD 0.44 (n=6) obtained from individually plotted concentration response curves. Dotted lines represent the inhibitory concentration of 50% spike reduction.

One-Point Titrations

To determine whether the IC50 values listed in Table 2 was influenced by dose (concentration x time) effects associated with sequential applications, single point applications were conducted using 13 networks. An example of a single point application at a concentration close to the established IC50 is shown in Figure 16. The application of 6.5 µM established a 100% decrease rather than the expected ~50% decrease in activity (Figure 16).

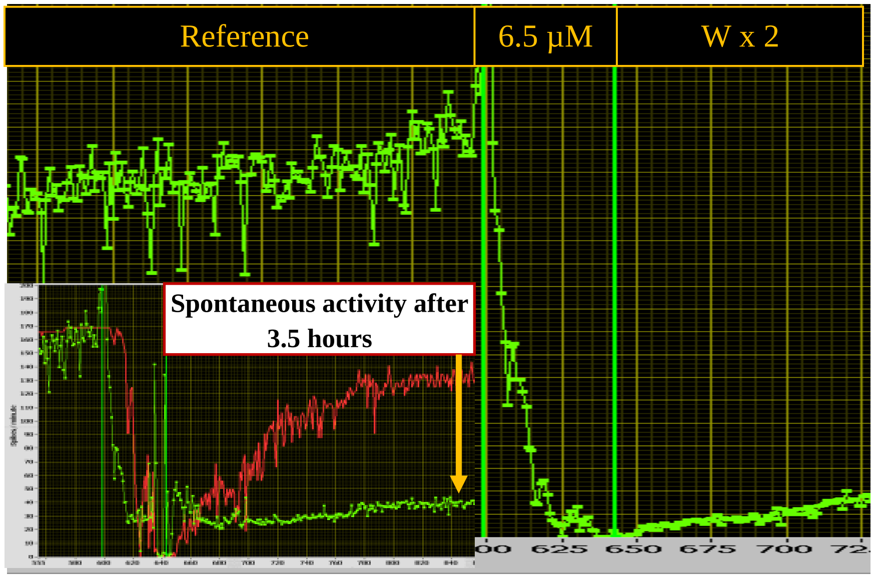

Figure 16. One point titration at 6.5 µM MEF (IC50 value) and minimal recovery after two successive washes (Wx2). The insert shows a longer time period (3.5 hours) with limited spike activity recovered. Active units recovered to 80% of reference (top trace): activity recovered to 25% (bottom trace).

The single applications ranged in concentration from 0.75 µM, resulting in 0% decrease in activity, to 8 µM, resulting in 00% decrease in activity (Table 4). The new single point IC50 was 2.86 µM (n=13) (Figure 17). When neuronal networks were exposed to mefloquine in successive concentration applications, the percent decreases in activity were less than when one large concentration was initially applied. For example, when applying mefloquine additions in a stepwise manner, an IC50 of 5.97 ± 0.44 was observed, but when one single addition of 6.5 µM was applied to networks there was a 100% decrease in activity and a new IC50 of 2.86 µM (n=13) was achieved (Figure 17). The two-tailed P value was less than 0.0001. Single point applications significantly lower the IC50 values, strongly suggesting network desensitization during sequential applications, which involves concentration and exposure time.

Table 4. List of single point single concentration

Experiment	Date	Conc. (µM)	% Decrease in Activity	% Recovery
KH083	1/14/15	**.75**	0	100
KH076	10/30/14	**1.5**	17	100
KH061	9/25/14	**2**	25	100
KH062	9/26/14	**2.5**	24.5	100
KH066	10/10/14	**2.5**	27	100
KH091	2/5/15	**3.5**	70	90
KH092	11/20/14	**4**	80	60
KH093	2/11/14	**4.5**	80	40
KH074A	10/28/14	**6**	100	32
KH074B	10/28/14	**6**	100	30
KH067	10/15/14	**6.5**	100	23
KH069	10/16/14	**6.5**	100	26
KH095	3/11/15	**8**	100	0

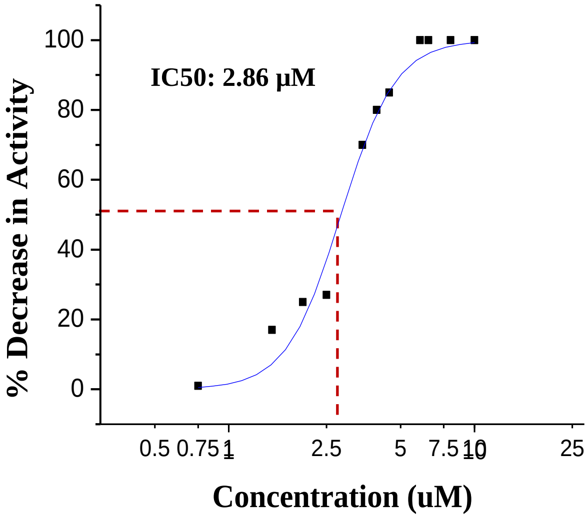

Figure 17. Concentration response curve of mefloquine single-point titrations. The resulting IC50 was shifted to lower concentration of 2.86 µM; (n=13). Dotted lines represent the inhibitory concentration of 50% spike reduction.

Recovery Analysis

Recovery after mefloquine application was analyzed both with multi-point titrations and single-point titrations. Multi-point titrations were conducted until network activity reached zero. After complete activity loss, two successive washes were applied and recovery was assessed. In five out of six experiments no recovery was observed in a 6-hour time frame. Single point titrations were added in varying concentrations from 0.75-8 µM and decreased reference activity was established. Two successive washes were applied within 1 hour and recovery overnight was recorded. Functional and cytotoxicity were based on percent activity decrease and percent recovery after two successive washes. It was observed that functional toxicity starts to occur around 1.5 µM of mefloquine and morphological signs of cytotoxicity begins to take place around 3.5 µM, having an accelerated decrease around 4-6 µM (Figure 18). No recovery was observed at 8 µM which is similar to the lack of recovery observed in sequential multipoint application at 10 µM. It must be noted here that cytotoxicity is defined here as a lack of full recovery after a minimum of 6 hours of observation.

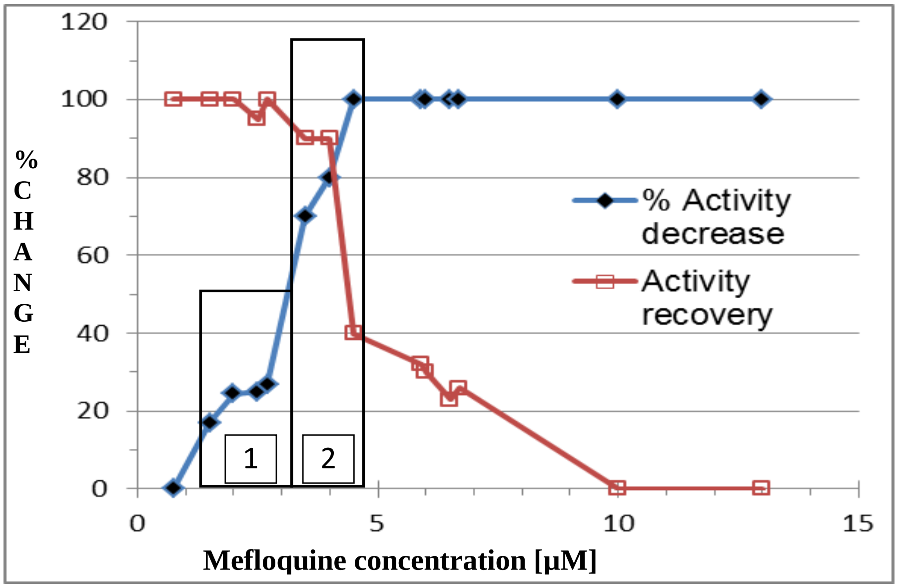

Figure 18. Functional and cytotoxicity based on percent activity decrease and percent recovery after two medium changes. Functional toxicity (box 1) starts at approximately 1.5 µM and precedes cytotoxicity (box 2). In the absence of quantitative cell death data, cytotoxicity may be considered irreversible functional toxicity. Data were derived from single network applications and are not biased by desensitization.

Morphological Observations:

Culture morphology was examined to determine irreversible cytotoxic damage to neuronal networks when exposed to mefloquine in various concentrations. In Figure 19, a single mefloquine application at 6 µM was added and cells were evaluated for 12 hours. During this time, process beading and cell deterioration were observed (Figure 19). A 6 µM addition causes a 100% decrease in activity and only a 30% recovery in activity. A similar experiment with an application of 8 µM mefloquine was applied to a different network. Cell stress is apparent after only 10 minutes. At 120 minutes glia cells were badly damaged and a previously smooth glial carpet became granular presumably via condensation of proteins (Figure 20).

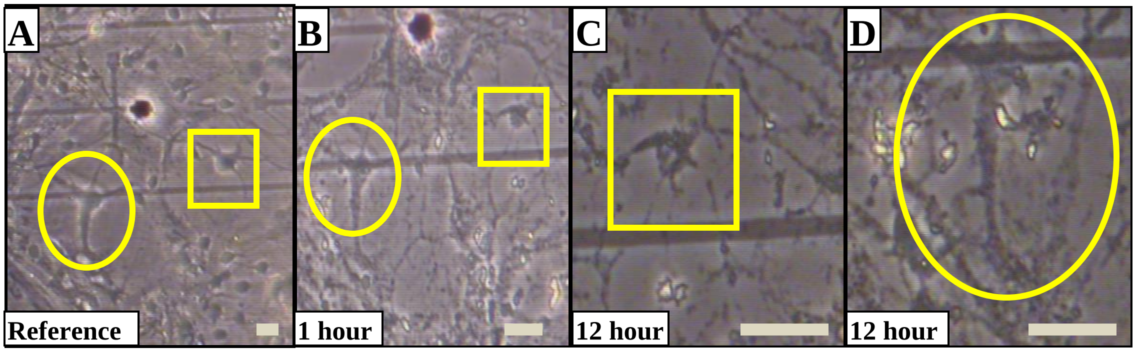

Figure 19. (A-D) Single mefloquine application at 6 µM (A) Reference; neuron 1- identified by circle; neuron 2- identified by square (B) 60 minutes after application showing cell swelling and beginning process beading (C-D) Magnified image of neurons 1 and 2 after 12 hours. NOTE: process beading and cell deterioration. Scale Bar= 30 µm.

Glial cells in Figure 20 showed rapid deteoriation with 10 minutes (see arrows). At 60 minutes under 8 µM mefloquine and most glial cells (presumably astrocytes) can no longer be identified. The neurons, although showing substantial stress and process retraction, may still be alive. After 120 minutes in the presence of mefloquine, two successive medium changes were applied. Glial recovery is observed 12 hours post wash and it maintained for the 24 hours.

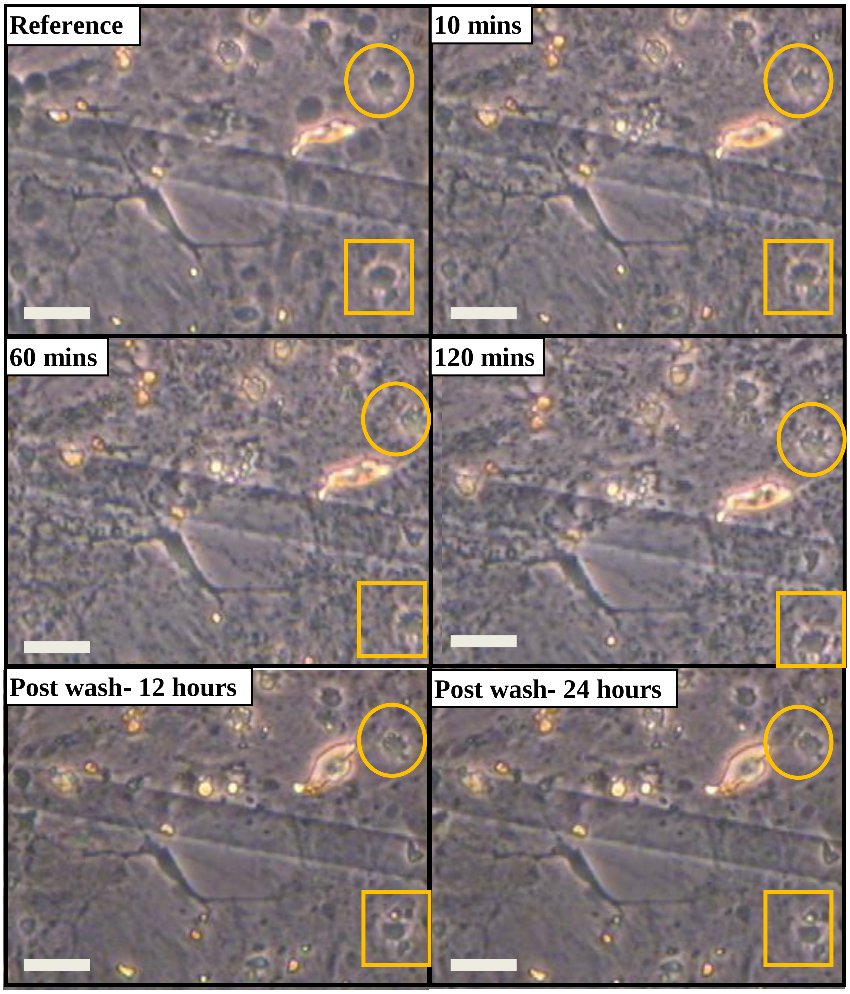

Figure 20. Gradual destruction of network components by 8 µM mefloquine. One neuron and surrounding cells (marked by a circle and square) are monitored for 120 minutes prior to two medium changes (wash), and then for 24 hours following the medium change. Both neurons and glia were affected. NOTE: recovery of glia carpet and cells after 12 and 24 hours post wash. Scale bar = 10 µm.

V. <u>Search for Protective Compounds-</u>

The search for protective compounds was limited to molecules that were part of normal metabolism. The rationale for this approach was based on observations that only some end users experience very serious side effects. This lead to the hypothesis that fluctuations of metabolites can be protective. Different compounds that were considered as possible candidates to act as endogenous neuroprotectants are listed in Table 5. This table describes the metabolic involvement and rationale as to why it might serve as a neuroprotectant in the presence of mefloquine.

Table 5. Potential endogenous neuroprotective compounds

Compound for Protection	**Metabolic Involvement**	**Rationale**
Picolinic Acid	Pproduced in the Kynurenine Pathway	Neuroprotectant (Clark et al. 2005) (Stone et al. 2012)
Quinolinic Acid	A direct precursor to NAD in the Kynurenine Pathway	Neuroprotectant (Chen et al. 2009) (Stone et al. 2002) (Kaneko et al. 2006)
Adenosine	Changes adenosine/glutamate balance more towards adenosine and this restores cell function	Neuroprotectant (Dechert et al. 1994)

Quinolinic Acid: Function and Metabolic Pathway

Quinolinic acid was the compound chosen to test for a protective effect. Quinolinic acid, a known NMDA agonist, is one of the main metabolites in the kynurenine pathway and increases in production at times of immune responses while aiding in the breakdown of toxins (Guillemin et al. 2012). This pathway involves the conversion of tryptophan to nicotinamide adenine dinucleotide (NAD) by six intermediate steps (Figure 21). Quinolinic acid is the precursor to the production of NAD in the presence of the enzyme quinolinate phosphoribosyl transferase (Chen et al. 2009) (Figure 21). NAD is a known neuroprotectant and prevents "axonal damage which is a major morphological alteration of the CNS that contributes to multiple neurological disorders" (Kaneko et al. 2006). In an experiment mentioned in the introduction, cochlear hair cells were treated with 35 and 50 µM mefloquine and damage to auditory nerve fibers was evaluated along with observing protective effects of NAD. At 35 µM there was a significant loss of auditory nerve cell fibers and cochlear hair cells, and at 50 µM there was a complete destruction of both auditory nerve cells and cochlear hair cells (Ding et al. 2013) (Table 9). It was observed that 5 mM NAD had no protective effect when compared to studies at both 35 and 50 µM mefloquine, but 20 mM NAD resulted in a decreased loss of auditory nerve cell fibers and cochlear hair cells. As a result of quinolinic acid being a precursor to NAD in the tryptophan pathway. The shift in IC5o to higher mefloquine concentrations in the presence of quinolinic acid might involve the protective effects of NAD shown by Ding et al.

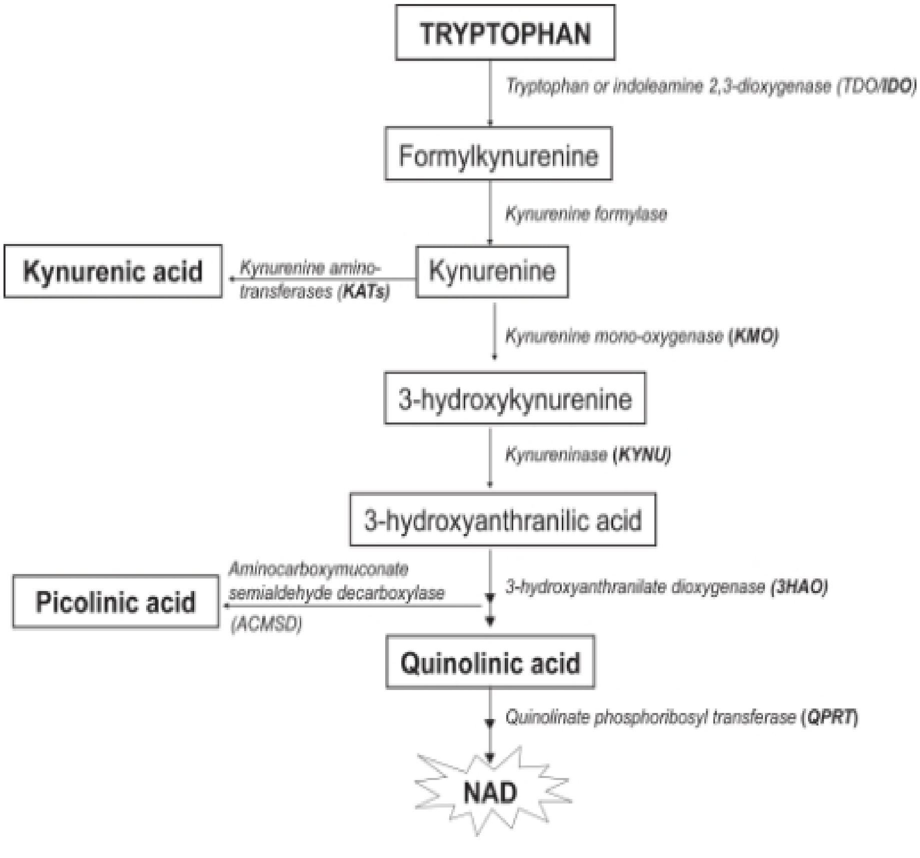

Figure 22. Metabolic pathway for NAD production. NOTE: quinolinic acid is the immediate precursor to NAD production.

Quinolinic Acid Toxicity

There have been multiple studies on the endogenous concentrations of QA and current literature is quite variable. It is known that some of the highest levels of QA (up to 20 µM) reside in immuno-compromised individuals. These levels are restricted to the brain tissue, while CSF levels remain at ~3.5 µM (Heyes et al. 2001). Quinolinic acid has previously been cited to be a neurodegenerative compound leading to excitotoxicity in the 75-150 nM range (Behan 2002). These articles describe chronic exposure whereas the experiments describes in this study involve the acute application and responses. Nonetheless, preliminary investigation of the excitotoxicity of quinolinic acid found that activity did not decrease until reaching concentrations of 375 µM, which decreased activity by only 22%. Complete activity loss was not achieved until reaching concentrations of 600 µM (Table 6; Figure 22). All quinolinic acid experiments used concentrations of 20 µM, which is far below acute toxicity.

Table 6. Summary of quinolinic acid experiments

Experiment	**Date**	**Units**	**Concentration (µM)**	**% Activity Loss**
DS017	12/26/13	50	40	0%
DS007	8/21/13	42	100	0%
DS008A	8/23/13	32	375	22%
DS008B	8/23/13	32	475	55%
DS008C	8/24/13	32	575	74%
DS012	9/17/13	47	600	100%

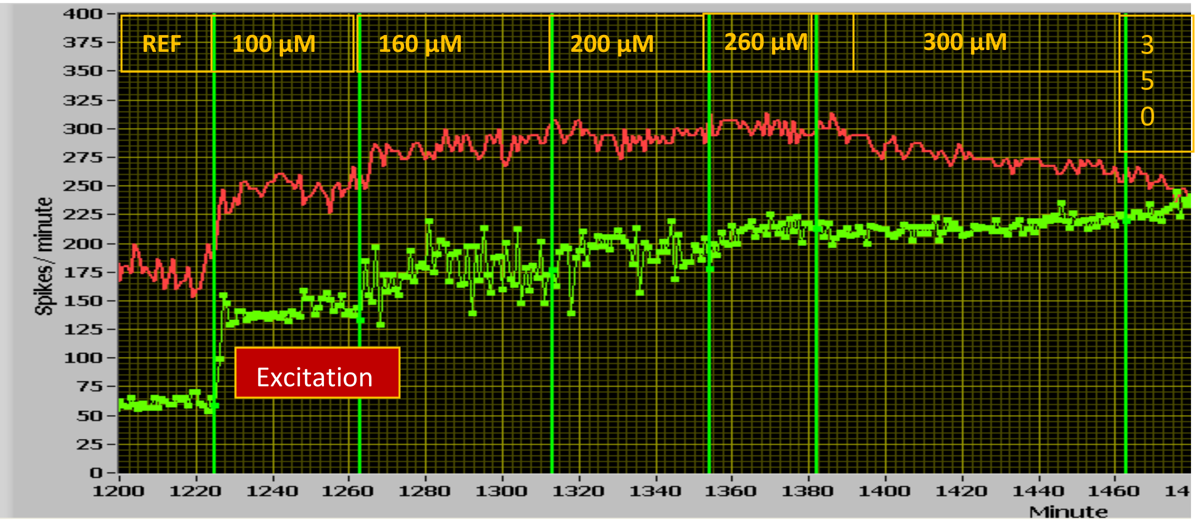

Figure 22. Successive applications of quinolinic acid showing excitation in activity under increasing concentration steps (100 µM- 350 µM). Such acute applications of quinolinic acid were not observed to be to cytotoxic until the concentration range of 450-550 µM (not shown above).

Dose Response Curves in the Presence of Quinolinic Acid

Quinolinic acid was added to networks 20 to 30 minutes previous to mefloquine exposures to establish a new reference activity. The concentration of quinolinic acid was held constant during experiments at 20 µM (Figure 23 & Table 7). This resulted in a shift of the IC50 from 5.97 ± 0.44 µM (n=6), (Table 3) to 9.28 ± 0.55 µM (n=3). This IC50 increase represents a 55% shift to higher mefloquine concentrations which reflects protection by quinolinic acid. The two-tailed P value is less than 0.0001. In other words, quinolinic acid significantly changed the IC50 values to higher concentrations of mefloquine and, therefore, serves as a protectant.

Table 7. Mefloquine experiments with quinolinic acid protection

Experiment	**Date**	**Units**	**QA Conc. (μM)**	**IC50 (μM)**
KH073	10/23/14	46	20	9.8
KH080	1/6/14	52	20	9.36
KH081	1/7/14	38	20	8.7
			Average:	**9.28 ± SD 0.55**

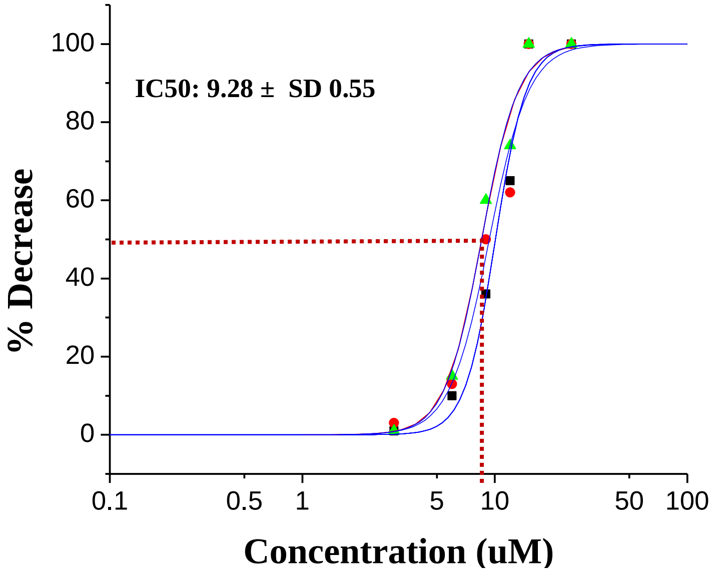

Figure 23. Shifted dose response in the presence of 20 μM Quinolinic Acid (average IC 50 ~ 9.28 ± SD 0.55 μM (n=3); (Table 8). Dotted lines represent the inhibitory concentration of 50% spike reduction.

Morphological Observations: Protection by quinolinic acid

Cell morphology studies were conducted to observe any protective effect that quinolinic acid might have on neuronal networks exposed to mefloquine. An IC50 of 5.97 ± 0.44 (n=6) was obtained from mefloquine sequential titrations while a statistically significant shifted IC50 of 9.28 ± 0.55 (n=3) was achieved when neuronal networks were pre-exposed to 20 μM quinolinic acid prior to any mefloquine additions. At 6 μM in the presence of quinolinic acid, there is deteoriation of some cells that occurs at 12 hours of exposure (Figure 24). When compared to an experiment held at the same mefloquine dose (concentration x time), but lacking the protection of quinolinic acid, there is less observed cell death. Without the protection of quinolinic acid there is complete cell destruction at 12 hours of 6 μM exposure (Figure 19). Another experiment shown in Figure 25 was carried out at the same dose as an experiment lacking the protection of quinolinic acid (Figure 20). Without the protection of quinolinic acid there is beading of processes and condensation of glial carpet at 8 μM. In the presence of quinolinic acid there is almost no observable cell damage. When comparing experiments held at the same dose, it was observed that less cell damage is achieved when neuronal networks are exposed to 20 μM quinolinc acid prior to any mefloquine additions indicating protective effects.

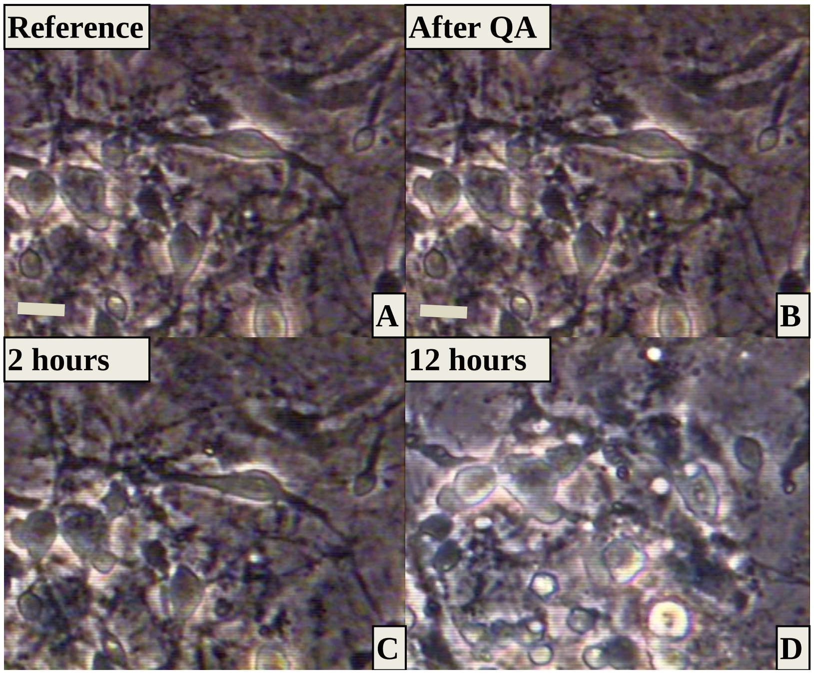

Figure 24. Protection in the presence of quinolinic acid. (A) Neurons before quinolinc acid or mefloquine additions. (B) Neurons after 20 mins exposure to 20 μM quinolinc acid. (C) Neurons after 2 hours exposure to 6 μM mefloquine. (D) The same neurons after 12 hours exposure to 6 μM quinolinc acid. NOTE: major disruption to cell morphology and retraction of dendritic processes. Damage is not as extensive as without the protection of quinolinc acid (Figure 19). Scale Bar: 20 um.

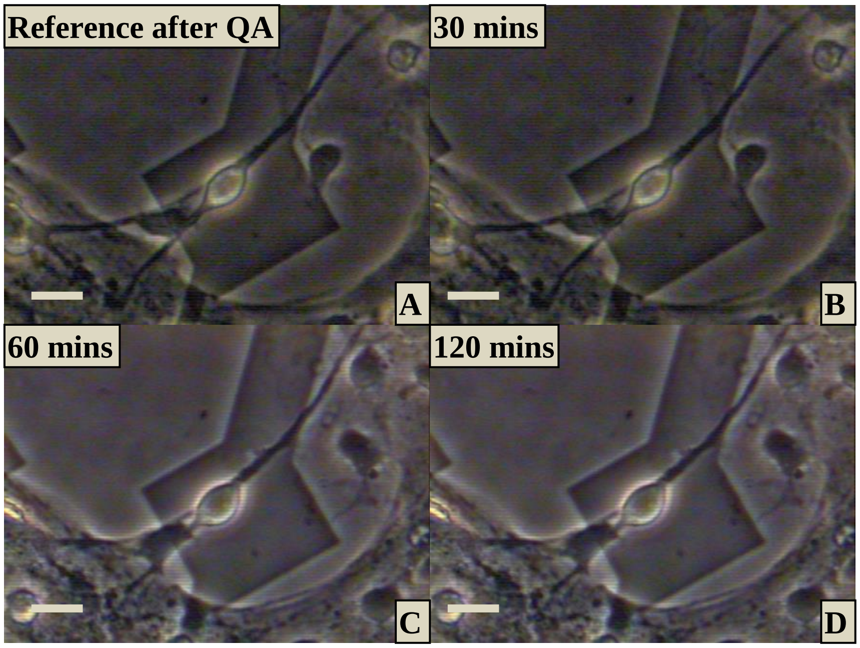

Figure 25. Protection of neuronal morphology by quinolinic acid. (A) Neuron after a 20 min exposure to 20 μM quinolinc acid. (B) Neuron after 30 min exposure to 8 μM mefloquine. (C & D) The same neuron after 60 and 12 min after 8 μM mefloquine exposure. A comparison to 120 min panel in Figure 20 shows substantially reduced morphological changes.

VI. Discussion

Mefloquine: Comparison to Clinical and Experimental Data

Mefloquine is a prophylactic antimalarial drug that is also used for malaria chemotherapy. Severe neurological symptoms requiring hospitalization occur in 1:10,000 patients undergoing chemoprophylaxis, and 1:200 to 1:1200 patients taking mefloquine as treatment for malaria (Dow et al. 2003). Milder neurological deficits are more common, being experienced by up to 25% of patients receiving chemoprophylactic doses when blood concentration levels reach around 3.8 µM. 90% of those receiving therapeutic doses have blood concentration levels between 2.1-23 µM (Table 8). When a mefloquine solution is mixed with DMSO and the effects observed using electrophysiological recordings, an IC50 of 5.97 ± 0.44 µM (n=6) was achieved through successive stepwise additions of mefloquine solution. These findings validate hypothesis number 1 showing there were quantifiable concentration-dependent changes in network activity. These decreases in activity are likely due to the inflow of calcium into the cell (Caridha et al. 2008) and triggered apoptosis. These experiments were done in DMEM without serum; no binding and potential protection was observed. This IC50 is found within the range of the therapeutic dose (Dow et al. 2003). However, it is higher than the chemoprophylaxis concentrations (Table 8). In addition, care must be exercised when comparing acute and chronic exposures, and when citing IC50 values. It is quite clear that therapeutic doses generally lie below the IC50 values.

In rat model experiments, mefloquine produced permanent and dose-dependent damage to viable neurons at an LD50 8.9 μM (Hood et al. 2011). These dose dependent lesions were demonstrated at a plasma concentration level of 5.6 µM (Table 9). Which are comparable to the concentrations of 6 µM mefloquine that led to cytotoxic damage at 12 hours in the present experiment (Figure 19). After these mefloquine exposures beading of processes and cell deteoriation was observed. There is substantial agreement between data presented in this study and published data in the literature with regard to concentration effects. Concentrations as low as 200 µM have been reported to destroy cochlear hair cells in postnatal rats. In contrast, concentrations as low as 1.3 µM have been reported having IC50 values when cultures are exposed to a single dose of mefloquine and sIPSCs were measured (Table 9). This is similar to the IC50 of 2.86 µM observed in the single point titrations in the present study.

Much of the current mefloquine data uses temporal morphological markers to evaluate toxicity. There have been limited electrophysiological data published on mefloquine toxicity (Table 10), and no data have previously been published on the reversibility of damage.

Table 8- Clinical Doses and Associated Side Effects

Author/Year	**Concentration**	**Model**	**Side Effects**
Dow et al. 2003	treatment- **2.1-23** µM- blood levels	in vivo- humans	**severe**- hospitalization, hallucinations, suicidal thoughts, headache, nausea, vomiting.
Dow et al. 2003	chemoprophylactic- **3.8** µM- blood levels	in vivo- humans	**mild**- headaches, nausea, vomiting, mood swings
Remington et al. 2009	chemoprophylactic- **1.5-3.3** µM- blood levels	in vivo- humans	**moderate**- headaches, nausea, vomiting, mood swings, violent thoughts
Remington et al. 2009	both- **21-34** µM- brain tissue	post-mortem- humans	death

Table 9. Experimental Doses of mefloquine and Associated Side Effects

Author/Year	**Concentration Range**	**Model**	**Effects/Observations**
Ding et al. 2013	35 or 50 µM	3 day postnatal rats; cochlear cells; cell counts 24 hours after exposures	**35 µM-** loss of auditory nerve fibers, moderate decrease in the number of hair cells **50 µM-** complete destruction of auditory nerve fibers and hair cells
Zhou et al. 2006	0-10 µM	6-17 day postnatal rats; sIPSC changes (spontaneous inhibitory postsynaptic currents)	Increased frequency of sIPSCs **IC50** of 1.3 µM
Hood et al. 2009	0-10 µM	Primary rat cortical neuron cultures Neurons were treated with oxidative stress markers (glutathione and F2- isoprostances) along with cell counts of spines per neuron and spine density	**LD50** 8.9 µM- viable neurons after 24 hour exposure Increase of glutathione and F2- isoprostances at 1 µM indicating oxidative stress at low end concentrations Significant decrease in spine frequency and density at 5 and 10 µM mefloquine

Repeatable DMSO Artifact

The observation that the test substance application method greatly influenced the pharmacological response is important. The gentle mixing of the bath medium by moving 80% of the medium into a syringe and back into the chamber within seconds after substance application to the medium bath is adequate for water soluble test substances, but is not adequate for DMSO aliquots. DMSO droplets form a layer over the network at the bottom of the chamber and mix very slowly with the medium. When performing the mefloquine experiments using this initial method (Method 1) there was an IC50 of 421 ± 13 nM (n=6). Using external mixing in a syringe with air bubbles must be supplemented with vortexing (Method 2) before re-introduction to the network. With this modified mixing method an IC50 of 5.97 ± .44 µM was achieved. This method is the most accurate because it exposes the cells to a more uniform mefloquine concentration and avoid overexposure to locally high, potentially toxic, concentrations.

Desensitization

The mechanisms of desensitization can vary for different compounds. Certain receptors can be responsible for desensitization (Meyerson et al. 2014) as well as through the action of a secondary messenger (Yang et al. 2009). Through sequential multipoint titrations, an IC50 of 5.97 ± 0.44 µM (n=6) was observed. Single point titrations that do not include exposure time yielded an IC50 of 2.86 µM. This change reflects a desensitization that is a function of concentration and time. The mechanism responsible for this effect is presently unknown, but must be considered when comparing acute and chronic exposures.

Quinolinic Acid Protection

Quinolinic acid has previously been reported as a neurodegenerative compound in the low nanomolar range (Stone et al. 2002). Despite numerous articles being published on its neurotoxicity, this study has been unable to replicate such excitotoxicity except at the much higher concentration of 600 µM. Quinolinic acid is produced endogenously and has been known to accumulate within the brain during times of immune response (Guillemin et al. 2012). Additionally, it is one of the steps in the breakdown of the essential amino acid tryptophan and is the direct precursor to NAD which is a known neuroprotectant (Ding et al. 2013). As a result of the continuous metabolism of tryptophan, quinolinic acid is actively converted to NAD. This accumulation of NAD could begin to explain why some users experience much mild effects while others only experience more serious side effects. Higher concentrations of quinolinic acid have been reported postmortem in individuals with HIV. The concentrations reach 20 µM in brain tissue and 3.79 µM in blood levels (Heyes et al. 2001). Through sequential multipoint titrations, an IC50 of 5.97 ± 0.44 µM (n=6) was observed. In the presence of quinolinic acid the IC50 was shifted to 9.28 ± 0.55 µM (n=3). Quinolinic acid significantly changed the IC50 values to higher concentrations of mefloquine and, therefore, served as a protectant.

Minimal morphological studies were conducted to confirm protection by quinolinc acid pre-exposure. This was caused by a low supply of usable cell cultures. Mefloquine was held at the same dose (concentration x time) as previous morphological studies in this thesis without the protection of quinolinc acid. With a pre-exposure of 20 µM quinolininc acid to networks, less extensive cell damage was observed within the same time frame. Combined with a shift in IC50 of 9.28 ± 0.55 µM (n=3) these alleviations in morphological damage can help confirm the observed neuroprotective effects that quinolinic acid has on neuronal network activity (Figure 24 & 25).

Areas for Improvement

Rapid glial responses that showed condensation of cytoplasm were surprising. In addition to neurons, the cultures normally contain astrocytes, oligodendrites, microglia, and endothelial cells. The limited analysis done so far shows shrinkage and crenation of what are assumed to be astrocytes (Figure 20), and condensation of cytoplasm in underlying flat cells.

Extensive immunohistological analysis would be necessary to complete this picture of cytotoxicity.

The further investigation of the irreversible cytotoxic damage caused by mefloquine exposures and the alteration of functional electrophysiological network responses could help to contribute to the understanding of what occurs in mefloquine users. To date, there have been very few studies that look at the changes in functional toxicity through quantitative electrophysiological recordings.

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