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Research Article

Studies on the Genotoxic and Mutagenic Potentials of Mefloquine

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Abstract

Purpose: *The detection of mefloquine mutagenicity has not been achieved by the use of Salmonella typhimurium his TA1535, TA1537 as tester strains. With the introduction of improved and more sensitive strains, it is of interest to evaluate the current mutagenic and genotoxic status of the drug. This study presents data on the in-vitro mutagenic and genotoxic potentials of mefloquine hydrochloride clinically used as an antimalarial agent.*
Method: *The mutagenicity potentials was investigated in the Escherichia coli WP_2 trp and WP_2 uvrA trp tester strains containing the plasmids, pEB017 and pKM101, and the Salmonella typhimurium TA97 containing pKM101. The genotoxicity potential was determined using the microscreen phage-induction assay.*
Results: *The presence of plasmids pEBO17 and pKM101 enhanced the detection of mutagenicity of mefloquine. Microsomal-activated mefloquine unequivocally elicited base-pair substitution mutagenicity. The genotoxicity test indicated that mefloquine was generally not genotoxic but was of the same potential mutagenicity as chloroquine phosphate.*
Conclusion: *Melfloquine hydrochloride exhibits base pair substitution mutagenesis, but not potentially genotoxic, even though it showed concentration dependent cytotoxicity. Its use as a last line antimalarial agent should still be encouraged.*

Key Words: Base-pair substitution, genotoxicity, mefloquine hydrochloride, mutagenicity, R-plasmid pEB017

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Introduction

The use of *Salmonella typhimurium his* TA1535, TA1537 etc. strains[1, 2] introduced a novel epoch in the rapid detection of mutagenic or carcinogenic chemical compounds. These strains, however, would not detect mefloquine - a quinoline-methanol antimalarial agent developed against chloroquine resistant *Plasmodium* sp) to be mutagenic. With the introduction of the improved and more sensitive *Salmonella typhimurium his* strains such as TA97, TA98, TA100, etc[3, 4] and other bacterial strains and bacteriophages, using more sensitive assay procedures to detect environmental genotoxic agents, it became necessary to reassess the mutagenicity and genotoxicity status of mefloquine. Mefloquine is one of the last line therapeutic drugs recently developed against the increasingly prevalent chloroquine – resistant *Plasmodium falciparium* parasite, a causative agent of chloroquine – resistant malarial fever.

The improved *Salmonella typhimurium his* TA97 strain is known to be very sensitive for the detection of frameshift mutagens[4]. *Escherichia coli trp* WP_2 tester strains also detect base-pair substitution mutagenesis[5, 6]. *Salmonella typhimurium his* TA97 was proposed as a replacement for TA1537[3, 4] and it also contained the plasmid pKM101 which had been reported to enhance error-prone DNA repair and mutagenesis[1, 7]. A novel plasmid pEB017[8] has also been reported to enhance bacterial mutagenesis[9] which was incorporated into the *E. coli trp* WP_2 tester strains as WP_2 *trp* (pEB017). The suitably modified protocol of the highly sensitive microscreen prophage induction assay earlier described[10, 11] was adapted for determining the genotoxicity potential of mefloquine. Microscreen phage induction assays detects potential genotoxins, mutagens and carcinogen which are as sensitive as the mutagenicity test of Ames *et al.*[2]. Activation of the compound through their prophage inducing activity in bacgteria has correlated well with their carcinogenic and genotoxic activities[12]. It measures the formation of prophage from a complex molecular events through the induction of the 'SOS' molecular response - a cellular reaction to DNA damage. The present study is designed to ascertain the mutagenicity and genotoxicity status of mefloquine using *Salmonella typhimurium his* TA97 and *E. coli trp* WP_2 strains.

Experimental

Bacterial Strains

The *Salmonella typhimurium his* TA97 strains contains R-plasmid pKM101[3, 4] and mediates frameshift mutagenesis on reversion from His^- to His^+ by a putative mutagen. The *Escherichia coli* WP_2 strains were WP_2 *trp uvrA*[5, 6], WP_2 (pKM101) *trp*[13] and WP_2 (pEB017) *trp*[8]. These *E. coli* strains respond to base-pair substitution mutagenesis by reverting from Trp^- auxotrophs to Trp^+ prototrophs. Bacteriophage – lambda lysogen *E. coli* WP_2s (lambda) F^-. *trp* and the indicator strains *E. coli* TH-008 (streptomycin resistant) were provided by Dr. Toby E. Rossman of the Institute of Environmental Medicine New York. Purified single colony isolates of all the tester strains were subcultured on nutrient agar slants and stored at 4°C.

Media

Nutrient broth No. 2 (Oxoid); nutrient agar (Oxoid); agar No. 1 (Oxoid); Davis and Mingioli (DM) 1950[14] salts solution; Vogel-Bonner minimal medium supplemented with 0.2% $^{w}\bullet_{v}$ glucose; *Salmonella* mutagenicity test plates were prepared as directed[4]. *E. coli trp* mutagenicity test plates were prepared with and without the inclusion of 20 mg/ml tryptophan. Tryptone medium (10 g of Bactone tryptone, 6 g of NaCl and 12 g of Bacto agar per litre of distilled water). Streptomycin (100 mg/ml) was added to the tryptone medium to select against the lysogen.

Drugs and biochemical

A stock solution (1 mg/ml) of mefloquine hydrochloride (obtained from Walter Reed Army Institute of Research, Washington, D.C., USA) was prepared and sterilised by filtration with bacteria proof membrane filters (Millipore, USA) and stored in an amber coloured bottle. Chloroquine phosphate, nalidixic acid (sodium salt), 2-nitrofluorene (used as positive controls to induce prophage) and reduced nicotinamide adenine dinucleolide phosphate (NADPH) type I) were obtained from Sigma-Aldrich (Gillingham-Dorset,UK).

Animals

Albino (Sprague-Dawley) rats weighing 130-200 g were injected peritoneally with phenobarbitone sodium (50 mg/kg) for seven consecutive days before they were killed. The livers of the rats were aseptically removed and pooled in a beaker containing TrisHCl (pH 7.5) at approximately 3 ml/g wet liver as earlier described[15].

Preparation of liver homogenate fraction (S-9)

The procedure of Ames *et al.*[2] was modified where necessary. The liver lobes were minced with sterile scissors and manually homogenized in a mortar and pestle that had been oven-sterilised and cooled to 4° C. The homogenate was centrifuged at 9000 *xg* for 20 min in an high speed 18 centrifuge (MSE, Sanyo, UK) at 4° C. The supernatant was carefully decanted into a sterile universal bottle and kept on ice. The liver extract (S-9) was immediately used for the mutagenicity tests after determining its protein concentration. The protein concentration was estimated using the method of Lowry et al.[16] with bovine serum albumin as standard.

Determination of minimum inhibitory concentration (MIC) of mefloquine

The MIC of mefloquine against the test bacterial strains was determined by the agar dilution protocol as previously described[13].

Mutagenicity testing

Mutatenicity testing was carried out by the plate incorporation method with and without activation using the method of Brusick *et al.*[17] was suitably modified as previously validated[15]. Sub-inhibitory concentrations of mefloquine (2.5 and 5 μg/ml) were added to tubes containing 2.5 ml of molten overlay agar (0.7% $^{w}/_{v}$ agar-agar) held at 45° C. An aliquot of 0.1 ml of undiluted, washed test culture were added to the tubes. The contents of the tubes were gently mixed and immediately poured onto the top of the prepared mutagenicity test agar plate and allowed to solidify. Positive control plates (containing 20 μg/ml tryptophan or histidine and no test agent) and negative control plates were also prepared. All plates were incubated at 37° C for 48 hr and the mean number of colonies growing per plate in triplicate experiments were recorded.

Activation was achieved in the plate incorporation method with NADPH and the enzyme preparation (S-9 mix). In this case, a sub-inhibitory concentration of mefloquine (0.1 ml of the undiluted test culture), 0.1 ml of NADPH 10mg/ml stock solutions, and 0.5 ml of the S-9 extract (3.13 ml/ml protein) at 4° C were mixed in sterile universal bottles and incubated at room temperature for 15 min. This mixture was added to 2.5 ml molten overlay agar (0.7%) held at 45° C and immediately poured on the top of the prepared mutagenicity test plates and the respective control plates allowed to solidify followed by the subsequent incubation at 37° C for 48 hr.

Tubes fluctuation test

The method of Green *et al*.[18] was suitably modified as earlier validated[15]. A fresh overnight washed test culture in 10 ml Davis & Mingioli liquid medium appropriately supplemented with either 20 μg/ml of histidine or tryptophan and 200 μg/ml of glucose was grown to log phase, harvested and washed free of the histidine or tryptophan supplement. The resuspended culture, 0.4ml (1×10^9 cfu/ml) in 10ml sterile distilled water was inoculated into 400 ml Davis & Mingioli basal salts containing glucose (200 μg/ml), biotin (5 μg/ml) and bromocresol purple indicator 12 μg/ml. The contents were thoroughly mixed and aseptically divided into 4 portions of 200ml as follows: 100 ml aliquot containing test concentrations of mefloquine (in duplicate); 100 ml aliquot containing additional concentrations of histidine or tryptophan (20 μg/ml); 100 ml aliquot containing no histidine, tryptophan or mefloquine. The contents of each flask were aseptically dispensed in 2 ml aliquot to 50 small sterile test tubes and incubated at 37° C. As from 72 hr of incubation, the tubes containing *His*$^+$ or *Trp*$^+$ revertants were turbid changing their colouration from purple to yellow and were recorded. Each duplicate test concentration of mefloquine was determined on at least two occasions.

For tubes fluctuation test with S-9 activation, the broth was supplemented minimal salts aliquot (above) contained, in addition to the test culture, 0.25 ml of the S-9 extract (3.13 mg/ml), protein (0.5 ml of 10mg/l NADPH) and the appropriate concentration of mefloquine.

Prophage Induction Assay

The microscreen prophage induction assay was performed as described by DeMarini *et al*.[11]. Sterile plastic 96-well microlitre plates (Corning, New York, USA) were used for the microsuspension assay. Briefly, the first well in a dilution series of the 96 well microlitre plates had 250 μl of supplemented Vogel-Bonner minimal salts containing 0.2% glucose and 20 μg/ml of tryptophan and 50 μl of either the mefloquine (100 μg/ml) or the control i.e., nalidixic acid and/or 2-nitrofluorene. The remaining wells had 150 μl of the medium with subsequent two-fold serial dilution of each well content down the column of each plate. Each well was inoculated with 75 μl (about 2×10^5 cells) of a resuspended log-phase culture of WP_2s[11]. In case of microsomal activation of the mefloquine, 25 μl of the prepared S-9 mix 100 μl of NADPH (10 mg/ml) were added to each well. Contents of each microlitre plate were mixed using a minimixer (Fisson plc, Loughborougb, UK), the lid was placed tightly on the plate, and the plates were incubated overnight at 30° C.

Assay for plaque formation

After incubation, the wells were scored for turbidity. Turbid wells indicated cell growth and clear wells indicated genotoxicity and/or inhibition of cell growth. The concentration of the lambda phage was determined by sampling the first three turbid wells. Whenever there was a clear well, samples were obtained from the first three wells adjacent to the last clear well. A sample (50 μl) from a well was diluted in 2.5 ml of the supplemented minimal medium and 100 μl of the dilution was added to about 2.5 ml of top agar (0.6% Bacto agar and 10 mM $MgSO_4$) along with 200 μl of log-phase indicator cells (TH-008), which had been grown in Oxoid No. 2 nutrient broth. The content of each tube was poured onto bottom agar made of tryptone medium. The plates were incubated overnight at 37° C and plaques were counted. The number of plaques from a dilution series of the microlitre plate that did not contain any test compound (control) was also determined.

Analysis of data

Scoring by Houk and DeMarini[10, 11] and DeMarini *et al*.[19] were adopted. Induced

plaque forming unit per plate (i.e., PFU/plate that reached the upper limit of the 99% confidence interval) represents an approximate three-fold increase over the background (tubes without any test compound or control). A dose-related increase of induced PFU/plate that reached or exceeded a three-fold increase in PFU/plate was considered a positive response. If a compound's response reached or exceeded the fold increase at only one dose, the result was scored as a weak positive.

Results

The plate incorporation tests showed that the repair-deficient strain, WP_2 *uvrA* responded positively to activated mefloquine when compared with the negative response with WP_2 *trp,* which are DNA-repair proficient. Strains TA97, WP_2 *trp* (pEB017), WP_2 (pKM101) – all bearing mutator *R*-plasmids – responded positively to activated mefloquine mutagenicity (Table 1). It would also be seen from the tube fluctuation tests that microsomally activated mefloquine elicited base-pair substitution mutation with the tester strains bearing mutator *R*-plasmids (Table 2). The activated mefloquine elicited prophage induction to the same level as chloroquine phosphate (Table 3).

Mefloquine hydrochloride was dose dependently bactericidal against all the test bacterial strains with a minimum inhibitory concentration (MIC) of 20 μg/ml except against the test host strains that had R-plasmids and the MIC was 40 μg/ml. The fluctuation mutagenicity tests were generally more sensitive than the plate incorporation method probably because of the greater diffusion of mefloquine molecules in liquid medium than in solid medium (Tables 1 and 2). Mutagenicity tests indicated that activation of mefloquine hydrochloride produced mutagenic products that mediated base-pair substitution mutation in the *E. coli* WP_2 *trp* (pEB017) tester strains. The *S. typhimurium his* TA97 strains also mediated a weak frameshift mutation but mefloquine mutagenicity potential was most remarkable as a basepair substitutions mutagen.

Discussion

The effect of plasmid pEB017 in demonstrating enhanced induction of reversion of *E. coli* WP_2 *trp* from Trp^- to Trp^+ compared to the enhanced reversion induction effect of the well known plasmid pKM101 in the isogenic *E. coli* WP_2 *trp* indicated the superiority of pEB017 to pKM101 in *E. coli* host strains. It was also observed that there was a relative decreased number of revertants by higher concentration of mefloquine, thus indicating the toxicity of mefloquine hydrochloride (Table 2) hence induced revertants were obtained at 2.5 μg/ml of mefloquine hydrochloride but not at 5.0 μg/ml. It could therefore be suggested that compounds that are bactericidal and mutagenic have an optimum concentration that induced mutations, beyond the critical level, the bacterial tester strains reversion response and cell survival would be impaired. This phenomenon was earlier observed in nitrofurantoin and chlorpromazine hydrochloride mutagenicity[15, 20].

Our data also showed that the presence of *R*-mutator plasmids (pKM101 and pEB017) enhance detection of activated mefloquine as a base-pair mutagen. The positive response of the repair deficient tester strains, WP_2 uvrA, without *R*-plasmids to activated mefloquine showed possible mediation of DNA damage repair enzymes in the observed base-pair mutagenic events. Generally, WP_2 uvrA strains are sensitive determinanats of base-pair substitution mutagens[3]. The observed negative activity in WP_2 strains could be attributed to non-penetration or weak penetration of the bacterial cells by mefloquine. Possible facilitation of entry of the activated mefloquine into the bacterial tester strains are indicated by the positive response with activated mefloquine in the presence of mutator plasmids.

Table 1: Revertant colonies in mefloquine reversion tests using the Plates Incorporation Tests Methods

Organisms	Negative control plates	Test plates containing 2.5µg mefloquine/ml	Positive control plates	Significance
E. coli WP_2 *trp*	3	2	500	
E. coli WP_2 *trp* (microsomal activation)	2	2	480	
E. coli WP_2 *uvrA trp*	1	3	205	
E. coli WP_2 *uvrA trp* (microsomal activation)	2	15	200	$P < 0.01$
E. coli WP_2 *trp* (pEB017)	3	4	200	
E. coli WP_2 *trp* (pEB017) (microsomal activation)	1	12	220	$P < 0.01$
E. coli WP_2 *trp* (pKM101)	2	3	480	
E. coli WP_2 *trp* (pKM101) (microsomal activation)	1	11	470	$P < 0.01$
S. tryphimurium his TA97	3	5	320	
S. tryphimurium his TA97 (microsomal activation)	1	18	430	$P < 0.01$

Table 2: Turbid tubes in Fluctuation test with mefloquine

Organisms	Positive control per total tubes	Negative control per total tubes	50 tubes containing mefloquine 5 µg/ml	Significance
E. coli WP_2 *trp*	50	2	2	
E. coli WP_2 *trp* (microsomal activation)	50	2	6	$P < 0.01$
E. coli WP_2 *uvrA trp*	50	0	1	
E. coli WP_2 *uvrA trp* (microsomal activation)	50	1	7	$P < 0.01$
E. coli WP_2 *trp* (pEB017)	50	0	0	
E. coli WP_2 *trp* (pEB017) (microsomal activation)	50	2	9	$P < 0.01$
E. coli WP_2 *trp* (pKM101)	50	3	1	
E. coli WP_2 *trp* (pKM101) (microsomal activation)	50	3	12	$P < 0.01$
S. tryphimurium his TA97	50	0	0	
S. tryphimurium his TA97 (microsomal activation)	50	0	8	$P < 0.01$

The genotoxicity test indicated that mefloquine hydrochloride was not potentially genotoxic but concentration dependent cytotoxic. This could be seen in the inhibition of bacterial growth in wells at concentrations of 5 µg/ml whereas the other wells containing serial double dilutions supported bacterial growth. This observation agreed with the observed bactericidal action of 5 µg/ml mefloquine hydrochloride in the plate incorporation mutagenicity test. This study therefore corroborates the earlier report of

Davidson *et al*.[21] that mefloquine weakly binds to DNA. The genotoxicity potentials of mefloquine favourably compared with the negative control (without the test or positive compounds) that contained only the growth medium. PFU/plate obtained from the mefloquine wells contrasted with the positive control wells containing nalidixic acid and 2-nitrofluorene (Table 3), thus indicating that mefloquine hydrochloride was not genotoxic. Considering the earlier results of Schupbach[22] that indicated mefloquine to be non-mutagenic in the now obsolete tester strains, the results of this study constitute an instance where the use of an improved tester strain (i.e., containing a novel mutator plasmid[9]) and/or the use of a particular species of bacterial tester strain enabled the detection of a compound as a mutagen that was earlier documented to be non-mutagenic. Since mefloquine hydrochloride exhibits base-pair substitution mutagenesis and is not strictly genotoxic, and remains a very effective therapeutic antimalarial agent, its use as a last line antimalarial agent should still be encouraged.

Table 3: Induction of prophage lambda by mefloquine hydrochloride, nalidixic acid and 2-nitrofluorene

	Sample Well A	Sample Well B	Sample Well B
Control (no chemical)	1	1	1
Mefloquine hydrochloride	2.1	1.5	1.1
Mefloquine hydrochloride (microsomal activation	2.5	1.5	1.1
Chloroquine phosphate	2.5	1.5	1.0
Nalidixic acid	5.5	6.4	5.9
2-nitrofluorene	5.8	8.8	7.5

Values indicate the ratio of proportion of PFU/plate in the control (no chemical) to the test compound (mefloquine hydrochloride) and the positive controls – nalidixic acid and 2-nitrofluorene

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