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Evaluation of Genotoxicity of Three Antimalarial Drugs Amodiaquine, Mefloquine and Halofantrine in Rat Liver Cells

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ABSTRACT : **The genotoxic effect of antimalarial drugs amodiaquine (AQ), mefloquine (MQ) and halofantrine (HF) was investigated in rat liver cells using the alkaline comet assay. AQ, MQ and HF at concentrations between 0-1000 μmol/L significantly increased DNA strand breaks of rat liver cells dose-dependently. The order of induction of strand breaks was AQ > MQ > HF. The rat liver cells exposed to AQ and HF (200 and 400 μmol/L) and treated with (Fpg) the bacterial DNA repair enzyme that recognizes oxidized purine showed greater DNA damage than those not treated with the enzyme, providing evidence that AQ and HF induced oxidation of purines. Such an effect was not observed when MQ was treated with the enzyme. Treatment of cells with catalase, an enzyme inactivating hydrogen peroxide, decreased significantly the extent of DNA damage induced by AQ, and HF but not the one induced by MQ. Similarly quercetin, an antioxidant flavonoid at 50 μmol/L attenuated the extent of the formation of DNA strand breaks by both AQ and HF. Quercetin, however, did not modify the effects of MQ. These results indicate the genotoxicity of AQ, MQ and HF in rat liver cells. In addition, the results suggest that reactive oxygen species may be involved in the formation of DNA lesions induced by AQ and HF and that, free radical scavengers may elicit protective effects against genotoxicity of these antimalarial drugs.**

Key words : **Amodiaquine, mefloquine, halofantrine, genotoxicity, DNA damage, malaria, reactive oxygen species, antimalarials**

Abbreviations

DMSO, dimethyl sulfoxide; FCS, foetal calf serum; FPG, formamidopyrimidine glycosylase; MES, 4-morpholinoethane sulphonic acid; SDS, sodium dodeocyl sulphate, AQ, amodiaquine; MQ, mefloquine; HF, halofantrine; CAT, catalase; ROS, reactive oxygen species.

Introduction

The incidence of malaria has been estimated to be in excess of 300 million cases per year worldwide and over two billion people in more than 100 countries are at risk of malaria (Aseri, 2005).There are four species of *Plasmodium* that cause human malaria. The most virulent of these is *P. falciparum* which kills more than 1 million people a year, with 90% of these cases occurring in children under 5 years mostly in the tropical and subtropical region of the world (Cowman, 1995; Jeffress and Fields, 2005). Over the years, management of malaria has relied on vector control of the Anopheles mosquito and on chemotherapy and chemoprophylaxis. Treatment of malaria however is becoming more complicated partly because of the incidence of multidrug resistant strains of *P. falciparum.*

Amodiaquine (AQ), 4-aminoquinoline used widely for treatment and prophylaxis of malaria, has been considered to be an effective and cost efficient alternative to chloroquine and sulphadoxine which have been proved to be ineffective due to the problem of drug resistance (Graupner *et al.*, 2005). Mefloquine (MQ), a quinoline methanol and halofantrine (HF), a phenanthrene methanol are two most recent antimalarial agents which require fewer doses for efficacy and compliance (Leo *et al.*, 1996). Although, resistance to MQ is already a problem in South-East Asia and is threatening to spread to other regions (Fontanet *et al.*, 1993), AQ, MQ and HF have

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been incorporated into the artemisinin-based combination therapy (ACT), a new regimen for the cure of malaria recently approved by the World Health Organization.

Biochemical studies have associated AQ with hepatotoxicity (Farombi, 2000) inhibition of dug metabolizing enzymes (Murray, 1984) and alteration of enzymic and non-enzymic antioxidant status of rats (Farombi, 2000). MQ and HF have also been shown to interfere with drug oxidation (Halliday *et al.*, 1995; Sukhumanan *et al.*, 1990, Farombi et al., 2000) as well as induction of lipid peroxidation in rats (Farombi *et al.*, 2000). AQ was demonstrated to induce prophage in tester strains of *Escherichia coli* (*E. coli*) D21 and D22 (Fasunon and Uwaifo, 1989). Subsequently, the mutagenic and genotoxic effects of AQ were demonstrated in in vivo sister chromatid exchange and chromosome aberration assay in bone marrow cells of mice (Chatterjee *et al.*, 1998).

Since these drugs are widely used world wide for the treatment of malaria and particularly in the Sub-Saharan Africa and South-East Asia, we have investigated in the present study the genotoxicity of AQ, MQ and HF in single-cell gel electrophoresis or the comet assay using DNA stand breaks as end points. Additionally, we assessed the role of oxidative DNA damage using DNA repair enzymes formamidopyrimidine-DNA glycosylase (Fpg) and evaluated the underlying mechanisms of the genotoxic action of the drugs, by exploring the ability of quercetin, an antioxidant flavonoid and catalase an enzyme that splits hydrogen peroxide to water to mitigate the DNA damaging effects of AQ, MQ and HF. The comet assay is suitable for this study because it has many applications including measurement of chemically induced DNA damage, prediction and monitoring of the genotoxic potential mutagenic and carcinogenic substances (Hartman and Speit, 1995).

Materials and Methods

Chemicals

Amodiaquine (AQ; Camoquine, Parke-Davies, Senegal), Mefloquine (MQ; Lariam®, Roche) and halofantrine (HF; Halfan®, Smith Kline Beecham) were obtained from the University College Hospital, University of Ibadan, Nigeria. Bovine serum albumin, Triton X-100, Dimethyl sulfoxide (DMSO) and hydrogen peroxide (H_2O_2) were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 medium, foetal calf serum (FCS) (Gibco BRC, Scotland, UK), agarose type I-A and type VII, triton X-100 and trizma base. Lymphoprep ™-1.077 was purchased from Nycomed, Norway and YOYO-1 from molecular probes, The Netherlands. Sodium dodeocyl sulphate (SDS) was from Aldrich Chem. Co. Steinheim, Germany.

Cell isolation

The livers isolated from rats were rinsed in ice cold phosphate buffered saline (PBS), blotted on paper towel, weighed, immersed in liquid nitrogen and stored at −80°C. Nuclear DNA from liver cells was isolated according to the procedure of Thein *et al.*, (2000). Briefly, 1 g of liver was placed in stainless steel cylindrical sieve (0.5 cm in diameter, mesh size 0.4 mm). The sieves were placed in each 2 ml of ice-cold merchants medium (0.14 M NaCl, 1.47 mM KH_2PO4, 2.7 mM KCl, 8.1 mm Na_2HPO4, 10 mM Na_2EDTA, pH 7.4). The tissue was disrupted with the aid of a plastic plunger. The homogenate was filtered through a 53 μm nylon mesh to remove the cellular debris and was kept on ice and in a dark container.

Cell treatment

AQ, MQ and HF were added to the suspension of hepatocytes to give a final concentration in the range of 0-1000 μmol/L for 30 min at 37°C along side with untreated control samples. For the treatment of cells with AQ, MQ and HF (200 and 400 μmol/L), the experiment included a positive control, which was hydrogen peroxide at 100 μmol/L for 5 min at 4°C. In experiment with quercetin incubation with AQ, MQ and HF was preceded by the addition of the compounds to the cells. The cells were incubated for 30 min in a dark incubator with quercetin (50 μmol/L) along side with untreated controls. After incubation, the cells were washed and incubated with AQ, MQ and HF. Quercetin was dissolved in DMSO. The final concentrations of DMSO and ethanol were < than 1% and did not affect the assays (data not shown).

Comet assay

The comet assay was carried out as described previously (Farombi *et al.*, 2004). The freshly prepared rat liver cells were embedded in 0.75% low melting agarose in PBS. Aliquots were placed on microscope slides that had been previously coated with 1% normal-melting point agarose and dried. The gels were left to solidify for 10 min and thereafter lysed by immersion in

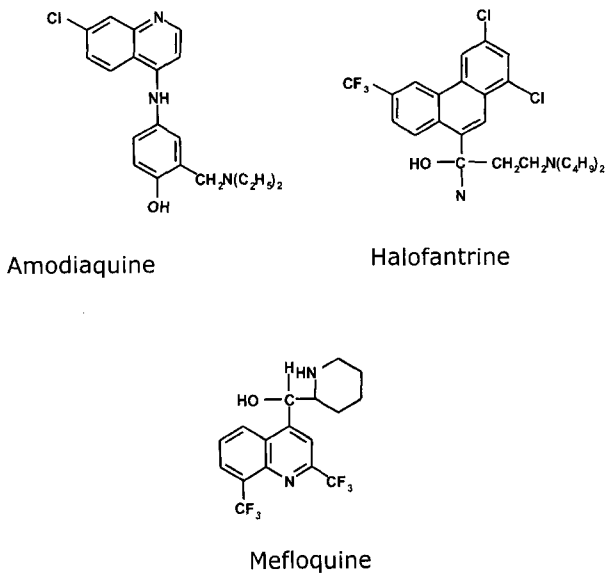

Fig. 1. Structures of Amodiaquine (AQ), Mefloquine (MQ) and Halofantrine (HF).

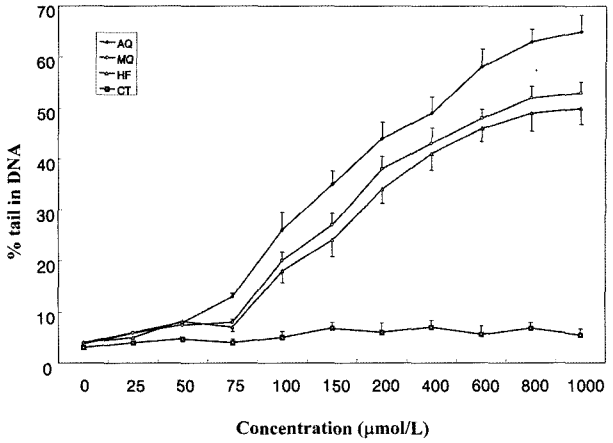

Fig. 2. DNA damage (strand breaks) measured as the percentage of tail DNA in the alkaline comet assay in rat liver cells treated with Amodiaquine (AQ), Mefloquine (MQ) and Halofantrine (HF) (0-1000 μmol/L) compared with untreated cells (control). Values are mean ± standard deviation of 5 determinations.

lysis solution (2.5 M NaCl, 0.1 M Na_2 EDTA, 10 mM Tris-HCl, 1% Triton X-100, pH 10). After lysis, all slides were then placed in 260 mm wide horizontal electrophoresis tank with 0.3 M NaOH and 1 mM Na_2EDTA buffer (pH 13) and left for 40 min at 4°C. Electrophoresis was conducted for 25 min at 25 V, 300 mA. The slides were washed three times for 5 min with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) before staining with 50 μl YOYO-1.

Fluorescently stained nucleoids were quantitated using image analysis (Komet 3.0, Kinetic Imaging Ltd., Liverpool, UK) as the percentage fluorescence in the tail (representing the fraction of DNA in the comet tail). Cells were classified in different categories according to their degree of damage: minimum: 0-10% tail DNA, low: 10-25% tail DNA, medium: 25-40% tail DNA, high: 40-75% tail DNA and maximum: > 75% tail DNA.

Formamidopyrimidine glycosylase (Fpg) treatment

To examine the levels of oxidized purines, the nucleoids after lysis were washed twice with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0) for 5 min. The nucleoids were then digested with formamidopyrimidine glycosylase (Fpg) (recognizing altered purines including 8-oxo guanine) or with enzyme buffer alone as control (Collins *et al.*, 1997) and incubated for 45 min at 37°C. Following electrophoresis, fluorescently stained nucleoids were quantitated as described above.

Catalase treatment

The freshly prepared rat liver cells embedded in 0.75 % low melting agarose in PBS were placed on microscope slides and lysis was carried out as described above. After lysis and washing with buffer, 50 μl of AQ, MQ or HF at concentration of 200 or 400 μM with 250 U/ml catalase was placed on agarose, covered with a cover slip and incubated for 1 hr at 37°C. The slides were electrophoresed, stained and quantitated as indicated above.

Statistics

The data were analyzed by one-way analysis of variance (ANOVA) with subsequent post-hoc comparison of the mean values using the Dunnett's test. The SAS statistical package (release 6.12) (SAS Institute, Carey, NC was used and a P- value equal to or less than 0.05 was considered statistically significant.

Results

AQ, MQ and HF at concentrations between 0-1000 μmol/L significantly increased the percentage tail in DNA of the cells dose-dependently (Fig. 2). AQ

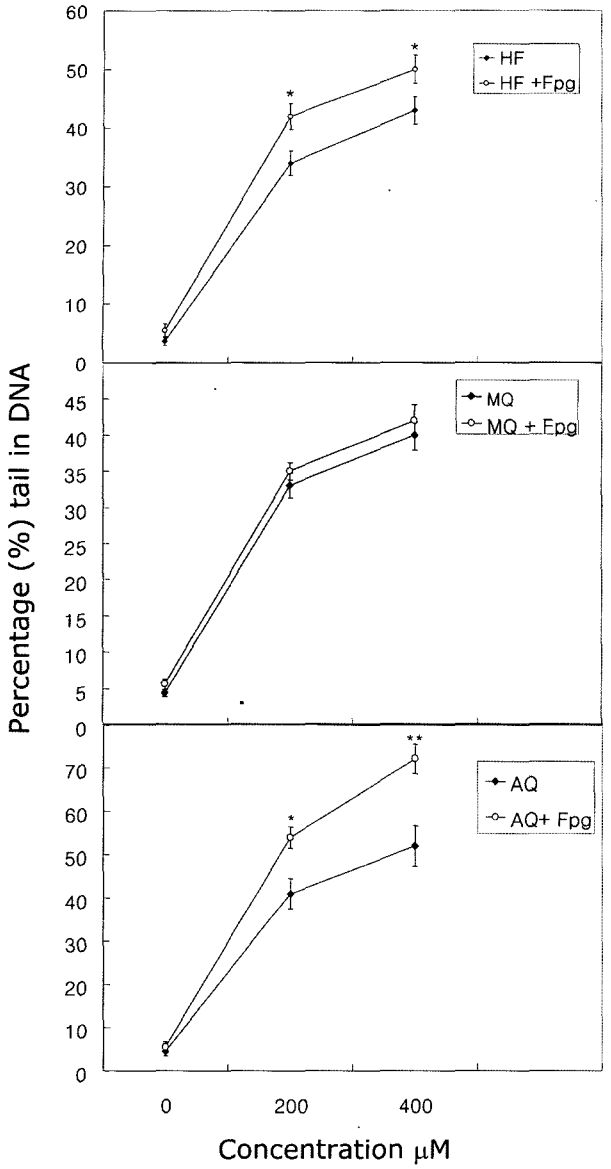

Fig. 3. DNA damage in rat liver cells exposed to Amodiaquine (AQ), Mefloquine (MQ) and Halofantrine (HF) at 37°C with post treatment with formamidopyrimidine-DNA glycosylase (FPG). DNA damage was measured as Percentage of tail DNA. SB is strand breaks; Values are mean ± standard deviation of 5 determinations. *$p < 0.05$; **$p < 0.01$ refers to difference between AQ or HF treated rat liver cells preincubated with or without FPG

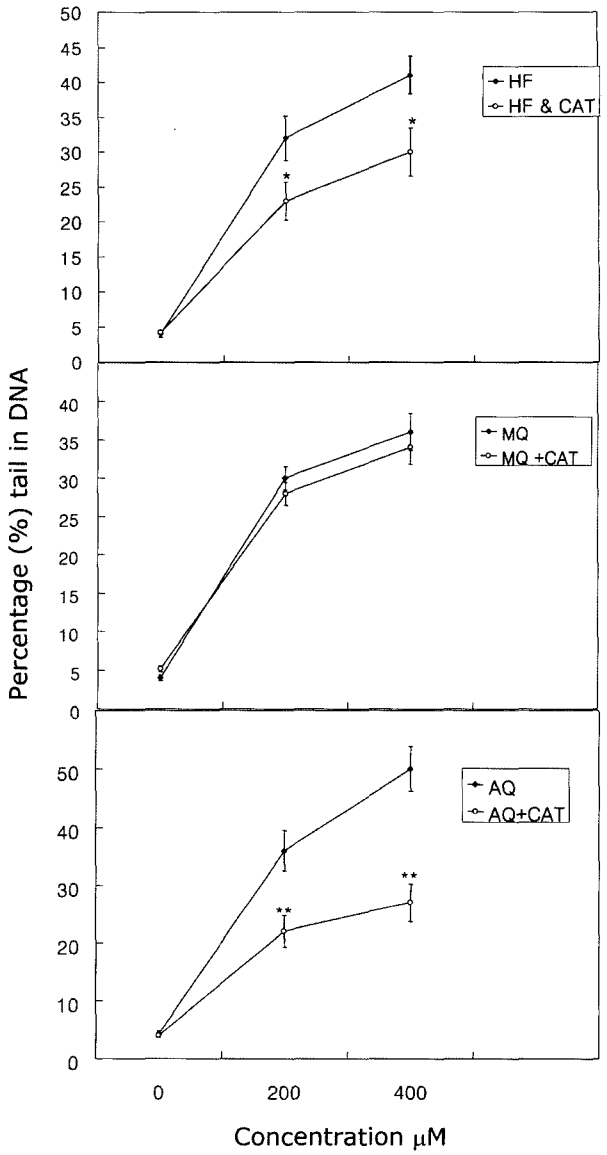

Fig. 4. DNA damage in rat liver cells exposed to Amodiaquine (AQ), Mefloquine (MQ) and Halofantrine (HF) at 37°C with post treatment with catalase (CAT) at a concentration of 250 U/ml. DNA damage was measured as Percentage of tail DNA. SB is strand breaks; Values are mean ± standard deviation of 5 determinations. * $p < 0.05$; **$p < 0.001$ refers to difference between AQ or HF treated rat liver cells incubated with or without CAT

appears to induce strand breaks more than MQ and HF. At concentrations of 600 μmol/L, percentage tail in DNA for AQ, MQ and HF were 58 ± 2.5, 48 ± 3.1 and 46 ± 2.4 compared with control (5.6 ± 2.2). However, at concentrations up to 75 μmol/L, the levels of DNA damage were not high for the three drugs.

Fig. 3 shows DNA damage in rat liver cells exposed to 200 μM and 400 μM AQ, MQ and HF at 37°C with post treatment with the bacterial DNA repair enzyme Fpg that recognizes oxidized purine. While rat liver cells exposed to AQ and HF and treated with Fpg endonuclease showed greater percentage tail in DNA than those not treated with the enzymes (Fig. 3) suggesting the presence of oxidative DNA damage

elicited by drugs, cells treated with MQ and the enzyme did not show significant changes in strand breaks compared with cells treated with MQ alone. The cells exposed to 100 μmol/L of H_2O_2 for 5 min at 4°C with post treatment with Fpg endonuclease contained significant levels of strand breaks and oxidized purines (data not shown)

DNA damage in rat liver cells exposed to AQ, MQ and HF after pretreatment 250 U/ml of catalase (CAT) is presented in Fig. 4. Treatment of cells with CAT significantly decreased the AQ and HF-induced strand breaks whereas the enzyme did not significantly affect the level of DNA damage induced by MQ ($p > 0.05$) suggesting that MQ may not cause DNA damage via mechanisms involving reactive oxygen species.

The ability of quercetin, a naturally occurring flavonoid antioxidant to affect DNA damage induced by AQ, MQ and HF in the rat liver cells was examined. Fig. 5 shows DNA damage in the cells exposed to AQ, MQ and HF and pre-incubated for 30 min at 37°C with quercetin (50 μmol/L).Quercetin significantly ($P < 0.001$) decreased strand breaks of cells exposed to AQ and HF at concentrations of 200 and 400 μM. Quercetin did not exert any effect ($P > 0.05$) on strand breaks in cells exposed to MQ at any of these concentrations.

Discussion

AQ, MQ and HF are commonly used antimalarial agents for chemotherapy and chemoprophylaxis of malaria particularly in South East-Asia and the tropics where malaria is endemic inspite of the multi drug resistance strains of *P. falciparum* that have emerged world wide in recent times. Earlier reports have indicated AQ to induce prophage in *E. coli* D21 and D22 (Fasunon and Uwaifo, 1989). Subsequently, Chatterjee *et al.* (1998) demonstrated the mutagenic and genotoxic effects of AQ in in vivo sister chromatid exchange and chromosome aberration assays.

In this study, our data indicated that AQ, MQ and HF induce DNA strand breaks in rat liver cells. It was shown that both AQ and HF except MQ oxidized purine base in the cells and that specific antioxidants could mitigate their DNA damaging effects, thus underlying the mechanism of the genotoxic action of the drugs. The observed increase in the comet percentage tail DNA might possibly be due to the induction of DNA strand breaks and /or the formation

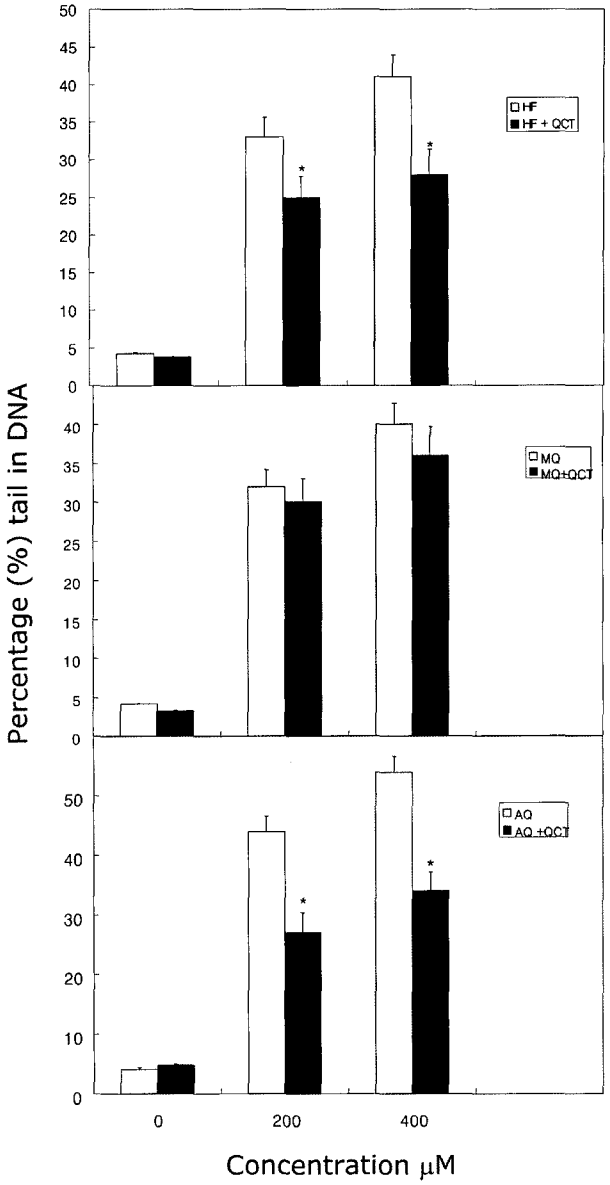

Fig. 5. Effect of quercetin (QCT) on rat liver cells treated with Amodiaquine (AQ), Mefloquine (MQ) and Halofantrine (HF) for 30 min at 37°C. DNA damage was measured as Percentage of tail DNA. SB is strand breaks Values are mean ± standard deviation of 5 determinations. *$p < 0.05$ refers to difference between AQ or HF treated rat liver cells incubated with or without QCT.

of abasic sites, which can be transformed into strand breaks in the alkaline comet assay.

The results revealed the oxidative DNA damaging effect of AQ and HF in rat liver cells. Treatment of the cells with Fpg, an enzyme recognizing mainly 2,6-diamino-4-hydroxyl-5-N-methyl formamido pyrimidine and 7,8 dihydro-8-oxo-2'deoxyguanine (8'-oxoG) (Collins

et al., 1993) introduced strand breaks to DNA of AQ and HF treated cells and elicited a pronounced increase in DNA damage in the cells indicating that the drugs induced formation of oxidized purine thus providing evidence for the oxidative damage to DNA caused by the drugs. Such an effect was not observed in cells treated with MQ suggesting that it did not induce oxidized purines.

Our results show that reactive oxygen species (ROS) might play a role in the observed DNA-damaging activity of AQ and HF. Catalase, an antioxidant enzyme with high affinity for hydrogen peroxide decreased the DNA strand breaks induced by AQ and HF indicating that the formation of ROS causing DNA damage may originate from hydrogen peroxide. The present data is consistent with earlier observation of decreased catalase enzymatic activity in rats following treatment with AQ (Farombi, 2000). Catalase however did not alter the DNA damaging activity of MQ which is consistent with its inability to induce oxidized purines.

Furthermore, a protective effect of quercetin against DNA-damaging effect of AQ and HF was observed while such protection was not observed with MQ. The results further confirm the involvement of free radicals in the genotoxicity of AQ and HF. Chemical compounds including drugs that produce oxidants have been reported to induce potentially mutagenic DNA damage, for example, by direct action of ROS on DNA, or indirectly via aldehydic lipid peroxidation degradation products (Collins, 1999). Therefore, antioxidant protection against oxidant challenge may therefore decrease the rate of mutation and hence help prevent diseases (Middleton *et al.,* 2000). Quercetin within the concentration used in this study has been shown to protect against ROS-mediated DNA damage by other chemical compounds. Nakayama *et al.* (1993) showed that quercetin protects against growth inhibitory effect of ROS in Chinese hamster cells. Also quercetin protected human lymphocytes against nickel chloride-induced DNA damage in human colonic cells (Blasiak *et al.,* 2002 a), and against DNA damage induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in human lymphocytes (Blasiak *et al.,* 2002 b).

The results obtained indicate that AQ and HF induce DNA damage by mechanisms different from that of MQ though MQ appears to induce more strand breaks than HF but not more than AQ. AQ is a 4- amino-quinoline possessing lone pairs of electrons and has been shown to form AQ-quinone imine intermediate (Maggs *et al.,* 1987). HF contains an aliphatic alkyl amino side chain, a positively charged nitrogen atom and a trifluoromethyl phenanthrene nucleus. Phenanthrenes have been shown to be converted to fjord region epoxide by microsomal cytochrome P450 (Seidel *et al.,* 1995) which may be further converted to radical species. These features may play specific roles in AQ and HF-induced ROS mediated DNA damage. Moreover, Livertoux *et al.* (1996) proposed factors that could enhance the one-electron reduction potential of several xenobiotics and increase their ability to generate radicals. Specifically, compounds related to quinones, nitroaromatics and nitroheterocyclics and iminiums have been shown to have higher reduction potential and produce oxygenated free radicals (Livertoux *et al.,* 1996). Both AQ and HF show one or more of these properties. Although, MQ being a quinoline methanol also contains quinoline nucleus and is expected to mediate damage also possibly by ROS formation as it induced lipid peroxidation in rats (Farombi *et al.,* 2000; Farombi *et al.,* 2001). This differential effect suggests that while MQ induces oxidative damage to membrane lipids it may induce damage to DNA by a different mechanism. Further studies to elucidate the mechanisms of genotoxicity of these drugs are needed.

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