Comparative mutagenic and genotoxic effects of three antimalarial drugs, chloroquine, primaquine and amodiaquine

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Comparative mutagenic and genotoxic effects of three antimalarial drugs, chloroquine, primaquine and amodiaquine, were assessed in the Ames mutagenicity assay (in strains TA97a, TA100, TA102 and TA104) and in vivo sister chromatid exchange (SCE) and chromosome aberration (CA) assays in bone marrow cells of mice. These are the most commonly used antimalarial drugs available at present throughout the world. The results of the bacterial mutagenicity assays showed a very weak mutagenic effect of all three drugs in Salmonella strains TA97a and TA100 both with and without S9 mix and in TA104 only with S9 mix. The results of the in vivo SCE and CA assays indicate that these three drugs are genotoxic in bone marrow cells of mice.

Introduction

Chloroquine (CHQ), primaquine (PRQ) and amodiaquine (AMQ) are the quinoline derivatives used in medicine as antimalarial drugs (Henry, 1973). CHQ is the most commonly used antimalarial drug at present in different parts of the world. CHQ is reported to induce teratogenic effects in rats (Sharma and Rawat, 1989). It is deposited in tissues and can cross the placenta (McChesney and McAnliff, 1961; McChesney et al., 1967; Linquist, 1973). CHQ forms intercalated complexes with DNA (Cohen, S.N. and Yielding, 1965; Sternglanz et al., 1969; Waring, 1970) and acts as an inhibitor of DNA synthesis and repair (Yielding et al., 1970; Wirchard et al., 1972; Michael and William, 1974; Field et al., 1978). PRQ has a limited use because of its toxic effects. PRQ is known to produce methemoglobinemia (Cohen, R.J. et al., 1986) and to cause acute haemolysis in patients deficient in glucose 6-phosphate dehydrogenase (Reever et al., 1992). AMQ has an action similar to CHQ. It is as effective as CHQ against CHQ-sensitive and some CHQ-resistant strains of Plasmodium falciparum.

CHQ and PRQ have been reported to be weakly mutagenic in Salmonella typhimurium (Ames and Whitfield, 1966; Obaseikun-Ebor and Obasi, 1986; Mars et al., 1987; Thomas et al., 1987) and in Bacillus subtilis (Kadotani, 1984). Mutagenic effects of CHQ were also reported in Salmonella strains using the fluctuation assay by Schupbach (1979) and Cortinas de Nava et al. (1983) and in strains TA1537, TA1538, TA98 and TA100 by Espinoza-Aguirre et al. (1989). Mars et al. (1987) observed weak mutagenic effects of PRQ in TA1537 with or without S9 and no mutagenic effects in TA100, regardless of the presence or absence of S9. PRQ was reported to induce mutagenic effects in TA97 both with and without S9 (Ono et al., 1994). Both CHQ and PRQ were mutagenic in TA100 without S9 (Shubber et al., 1986) and non-mutagenic in TA102 (Ono et al., 1994).

CHQ is reported to induce sex-linked recessive lethals in Drosophila (Xamena et al., 1985) and micronuclei and sister chromatid exchange (SCE) in Chinese hamster ovary cells (Raj and Heddle, 1980). A significant increase in SCE in both Chinese hamster lung fibroblast (V79) and rat hepatocyte cells (H4) in vitro were also reported by Shubber et al. (1986). Interaction of CHQ with sodium nitrite and mercuric chloride in the induction of chromosome aberrations (CA) was reported by Grisvila and Takahashi (1994). Although there are some publications on the mutagenicity of these drugs, reports on their genotoxic effects are relatively scarce. There are no publications on in vivo SCE in mice for any of the three drugs and in vivo CA for PRQ and AMQ. We have tested the in vivo mutagenic and genotoxic effects of different environmental chemicals and drugs (Gir et al., 1989, 1992; Giri, 1996, 1997; Philipose et al., 1997). Considering the widespread use of these drugs, mainly in the tropical regions of the world, we felt the need to extend the in vitro mutagenicity assay and in vivo genotoxicity assays of these drugs. In the present study, mutagenic and genotoxic effects of these three antimalarial drugs were tested employing the Ames mutagenicity assay in strains TA97a, TA100, TA102 and TA104 and in vivo SCE and CA assays in bone marrow cells of mice.

Materials and methods

Animals

Inbred strains of Swiss albino male mice (Mus musculus), 10–12 weeks old, weighing 25–30 g, were received from the Division of Laboratory Animals, Central Drug Research Institute (Lucknow, India) and Charles River male rats of 150–175 g were received from the animal house of the Indian Institute of Chemical Biology (Calcutta, India). They were kept four per cage with husk bedding, were fed standard rodent pellet diet (Gold Mohar, Lipton India Ltd, Chandigarh, India) and water ad libitum. The lighting cycle was 12 h light and 12 h dark. Room temperature and relative humidity conditions were 28 ± 2°C and 60 ± 5%, respectively. Mice were used for both SCE and CA assays and rats were used for rat liver homogenate (S9) preparations for mutagenicity assay.

Chemicals

5-Bromodeoxyuridine (BrdU) tablets (50 mg each) were purchased from Boehringer Mannheim Biochemicals (Germany). Cyclophosphamide, mitomycin C, dimethyl sulfoxide, colchicine, biotin, histidine, NADP, glucose 6-phosphate, crystal violet, ampicillin trihydrate, agar, sodium azide, 4-nitro-o-phenylenediamine (NPD), 2-aminofluorene (2-AF), cumine hydroperoxide and sodium ammonium phosphate were purchased from Sigma Chemical Co. (St Louis, MO). Crotonaldehyde was purchased from Aldrich (Milwaukee, WI). Chloroquine, primaquine and amodiaquine were also purchased from the Sigma Chemical Co. (St Louis, MO).

Bacterial strains

Salmonella typhimurium strains TA97a, TA100, TA102 and TA104 were used for the Ames mutagenicity assay. These strains were provided by Dr Bruce X. Ames (Biochemistry Division, University of California, Berkeley, CA). The reasons for choosing these four strains are as follows. TA97a detects frameshift

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mutagens while TA100 detects base pair mutagens. TA97 was replaced by TA97a. The mutagenic specificity of TA97 is similar to that of the hisC307 mutation in TA1537 but, because TA97 also has a second hotspot of alternating GC base pairs near a run of cytosines, it is sensitive to some of the mutagens that revert TA1538 and TA98. Thus, we did not include TA98 in this experiment (Maron and Ames, 1983). TA102 contains AT base pairs at the site of mutation (determined by DNA sequence analysis), in contrast to the other Salmonella tester strains that detect mutagens damaging GC base pairs. This strain differs from previous tester strains in that the mutation has been introduced into a multicopy plasmid, so that ~30 copies of the mutant gene are available for back mutation (Levin et al., 1982). Salmonella strain TA104 detects a variety of naturally occurring carbonyl compounds as direct-acting mutagens (Marnett et al., 1985) as well as some hydroquinones (Hakura et al., 1996). Some of the antimalarial drugs are metabolized to hydroquinones, so we also used TA102 and TA104 in addition to TA97a and TA100 for the mutagenicity assay of these three antimalarial drugs.

Bacterial mutagenicity assay

Preparation of the S9 fraction. The procedure of Garner et al. (1972) was used for the preparation of rat liver homogenate (S9 fraction). Since Aroclor 1254 was not available in our laboratory and 0.1% phenobarbital gave satisfactory results in our S9 fraction prepared from rat liver, we used phenobarbital as an enzyme inducer for our S9 preparation. Rats were fed 0.1% phenobarbital in their drinking water for 7 days. On day 6 no food was provided for these rats. The next day they were killed for preparation of the S9 fraction. All steps were performed at 0-4°C with cold and sterile solutions and glassware.

S9 fraction was prepared following the method of Maron and Ames (1983). S9 fraction was distributed in 2 ml aliquots in small sterile plastic tubes, quickly frozen and stored at -80°C.

Standard plate incorporation assay. The plate incorporation test was performed following the method of Maron and Ames (1983) for all strains. The test chemicals, CHQ, PRQ and AMQ, were dissolved in distilled water and different concentrations (0.1, 1.0, 10, 100, 1000, 5000 and 10,000 μg/plate) of these chemicals were tested for mutagenicity. The plates were inverted within 1 h and placed in a dark vented incubator at 37°C for 48 h. Positive controls (20 μg/plate NPD for TA97a, 1.5 μg/plate sodium azide for TA100, 100 μg/plate crotonaldehyde for TA102 and 100 μg/plate crotonaldehyde for TA104) and negative controls (distilled water) were run concurrently in all experiments. Four plates were used for each concentration tested and for both positive and negative controls. After 48 h incubation the revertant colonies on all plates were counted. The presence of a background lawn on all plates was confirmed. For experiments with S9 mixture 2-AF was used as the positive control for both the TA97a and TA100 strains. In our present experiments we obtained a maximum response of the positive control with 1% S9 mixture, therefore, 1% liver homogenate was used throughout the study.

Selection of dose for in vivo study

The LD50 of these three drugs i.p. in mice were not available in the literature. In the initial animal experiment a group of five mice were injected i.p. with 200 mg/kg CHQ. Out of five animals, three died within 24 h of this i.p. treatment. To evaluate the effectiveness of CHQ dose, the highest doses selected for the SCE and CA studies were 50 and 100 mg/kg body wt, respectively and other doses were serial dilutions of these highest doses. So the highest i.p. dose of CHQ in the CA study was close to 25% of the LD50.

In vivo sister chromatid exchange (SCE) assay

Paraffin-coated (~80% of the surface) BrdU tablets (50 mg each) were implanted s.c. in the flank of mice under diethyl ether anesthesia following the methodology of McFee et al. (1983) and Sharief et al. (1986) for the in vivo SCE study and cell replication kinetics analysis. The test chemicals were administered as a single i.p. injection 1 h after tablet implantation. Three doses (12.5, 25 and 50 mg/kg) of the three drugs were injected i.p. in distilled water to different groups of mice. Four mice were used for each dose of the test chemicals and the controls. Negative control mice were injected with 100 μl distilled water, while mitomycin C was used as a positive control at a dose of 1 μg injected i.p. The positive control (20 μg/plate crotonaldehyde for TA102 and 100 μg/plate crotonaldehyde for TA104) and negative controls (distilled water) were run concurrently in all experiments. Four plates were used for each concentration tested and for both positive and negative controls. After 48 h incubation the revertant colonies on all plates were counted. The presence of a background lawn on all plates was confirmed. For experiments with S9 mixture 2-AF was used as the positive control for both the TA97a and TA100 strains. In our present experiments we obtained a maximum response of the positive control with 10% S9 mixture, therefore, 10% liver homogenate was used throughout the study.

Results of the Ames mutagenicity and the CA studies were 50 and 100 mg/kg body wt, respectively and other doses were serial dilutions of these highest doses. So the highest i.p. dose of CHQ in the CA study was close to 25% of the LD50.

Table I. Number of revertants induced by chloroquine, primaquine and amodiaquine in the Salmonella plate incorporation test using TA97a with or without S9

<table>
<thead>
<tr>
<th>Chemicals (μg/plate)</th>
<th>Revertants/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Revertants/plate</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>S9</strong></td>
</tr>
<tr>
<td>Solvent control</td>
<td>120.75 ± 10.72</td>
</tr>
<tr>
<td>(100 μl distilled water)</td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>124.25 ± 6.08</td>
</tr>
<tr>
<td>1</td>
<td>155.75 ± 13.99*</td>
</tr>
<tr>
<td>10</td>
<td>186.50 ± 15.07*</td>
</tr>
<tr>
<td>100</td>
<td>150.50 ± 11.03*</td>
</tr>
<tr>
<td>1000</td>
<td>132.25 ± 12.28</td>
</tr>
<tr>
<td>5000</td>
<td>117.50 ± 6.45</td>
</tr>
<tr>
<td>10 000</td>
<td>Toxic</td>
</tr>
<tr>
<td>Primaquine</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>125.00 ± 10.80</td>
</tr>
<tr>
<td>1</td>
<td>149.50 ± 13.03*</td>
</tr>
<tr>
<td>10</td>
<td>184.00 ± 22.06*</td>
</tr>
<tr>
<td>100</td>
<td>156.75 ± 9.50*</td>
</tr>
<tr>
<td>1000</td>
<td>Toxic</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>118.00 ± 9.83</td>
</tr>
<tr>
<td>1</td>
<td>129.50 ± 9.11</td>
</tr>
<tr>
<td>10</td>
<td>173.75 ± 16.91*</td>
</tr>
<tr>
<td>100</td>
<td>112.44 ± 13.46</td>
</tr>
<tr>
<td>10 000</td>
<td>Toxic</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
</tr>
<tr>
<td>NPD (20 μg/plate)</td>
<td>1202.25 ± 50.18</td>
</tr>
<tr>
<td>2-AF (10 μg/plate)</td>
<td></td>
</tr>
</tbody>
</table>

~S9, without metabolic activation; +S9, with metabolic activation.
Mean ± SD of four plates. Results for each concentration were compared with the solvent control by Dunnett's multiple comparison with control:

*p < 0.01; **p < 0.05.

1982). The replicative index was calculated as follows: RI = (1M1 + 2M2 + 3M3) = 100

Chromosome aberrations (CA) assay

For CA analysis four doses (12.5, 25, 50 and 100 mg/kg) of the three drugs were dissolved in distilled water and injected i.p. in a volume of 100 μl/mouse. Four mice were used for each group or the control. Negative control mice were injected with an equal volume of distilled water, while positive control animals received 25 mg/kg cyclophosphamide. After 2 h the animals were injected with colchicine (2 mg/kg) and 2 h later they were killed by cervical dislocation. Bone marrow was expelled from the femur with 0.1% phenobarbital. After hypotonic treatment (0.075 M KCl at 37°C for 20 min, cells were fixed three times with methanol-acetic acid (3:1). Slides were prepared for bone marrow chromosomes and the slides stained with Giemsa (Presston et al., 1987). All slides were coded and 100 well-spread metaphase cells were scored per animal for CA. So a total of 400 metaphase cells were scored for each dose and for the controls. Mitotic index (MI) was scored from 100 cells/animal and was expressed as a percentage. CA was scored following the guidelines of the World Health Organization (1985) and Preston et al. (1987). The chromatid- and chromosome-type aberration frequencies per cell were calculated. Statistical calculations were carried out on percent aberrant cells. Gaps were recorded and not included in percent aberrant cells (Sharief et al., 1986; Giri et al., 1989).

Statistical analysis

Results of the Ames mutagenicity and the in vivo SCE and CA assays were analysed using Dunnett's multiple comparison with control and the level of significance has been given in the respective tables (Dunnnett, 1955). All data were transformed prior to the Dunnett's test.

Results

Tables I-IV are summaries of the results of the Ames mutagenicity assay in Salmonella typhimurium strains TA97a, TA100, TA102 and TA104. After CHQ, PRQ and AMQ treatment a weak but significant increase in revertant colonies
was observed at some concentrations in strains TA97a and TA100, both with and without metabolic activation (see Tables I and II). However, these compounds showed less mutagenic effects when S9 was present. In strain TA102 no significant differences in the revertant colonies were observed at any of the concentrations tested for CHQ and PRQ, either with or without S9 mix. A weak but significant increase in revertant colonies was observed at certain concentrations of AMQ both with and without S9 mix (see Table III). In the case of strain TA104, a weak but significant increase in revertant colonies was observed at certain concentrations of all three drugs when S9 mix was added (see Table IV). Since a positive control for TA102 and TA104 with S9 was not available to us, it was not possible to show a positive control result for these strains with S9.

Table V and VI represent a summary of the results of SCE and CA induced by CHQ, PRQ and AMQ after in vivo exposure of mice. A significant increase in SCE was observed at all three doses for all three drugs tested. No significant changes in RI were observed for any of the three doses tested for any of the drugs. As CA is less sensitive than SCE, a higher dose (100 mg/kg) was selected for the CA than for the SCE (50 mg/kg) study. Chromatid- and chromosome-type aberrations per cell are presented (Preston et al., 1987) and percentage aberrant cells is used for statistical calculations (Sharief et al., 1986). CHQ and AMQ showed a significant increase in the percentage of CA at the highest dose tested when compared with the solvent control. In the case of PRQ,
The results of the mutagenicity assay indicate that these drugs are very weak direct-acting mutagens in *Salmonella* strains TA97a and TA100. In both strains the compound was less mutagenic when S9 was present. Our results of weak mutagenic effects of both CHQ and PRQ fully support earlier observations reported by several authors (Ames and Whitfield, 1966; Xamena et al., 1985; Obaseiki-Ebor and Obasi, 1986; Shubber et al., 1986; Marrs et al., 1987; Thomas et al., 1987). The non-mutagenic effects in TA102 are in agreement with observations by Obaseiki-Ebor and Obasi (1986) and Ono et al. (1994) reported for CHQ and PRQ, respectively. No reports are available on the mutagenic effects of AMQ. In the present study, AMQ shows a weak but significant mutagenic effect in all four *Salmonella* strains used in this experiment. In these mutagenicity assays both AMQ and PRQ show more toxic effects than CHQ in all strains. Both CHQ and AMQ persist at high concentrations (with respect to the concentrations of time in the human system, even after discontinuation of therapy. Moreover, CHQ has been shown to interact with DNA and form an intercalated complex (Cohen,S.N. and Yielding, 1966; Xamena et al., 1985; Obaseiki-Ebor and Obasi, 1986; Shubber et al., 1986; Marrs et al., 1987). The non-mutagenic effects in TA102 are in agreement with observations by Obaseiki-Ebor and Obasi (1986) and Ono et al. (1994) reported for CHQ and PRQ, respectively. No reports are available on the mutagenic effects of AMQ. In the present study, AMQ shows a weak but significant mutagenic effect in all four *Salmonella* strains used in this experiment. In these mutagenicity assays both AMQ and PRQ show more toxic effects than CHQ in all strains. 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1965; Waring, 1970). This indicates that CHQ might induce frameshift mutations by shifting the reading frame.

Mutagenicity results for these three drugs with S9 mix in strain TA104 indicate that the metabolic products of these drugs are weakly mutagenic in this strain. TA104 detects a variety of naturally occurring carbonyl compounds as direct-acting mutagens (Marnett et al., 1985). CHQ in the course of its metabolism is mainly converted to monodesmethyl chloroquine, with small amounts of bisdesmethylochloroquine (Goodman and Gilman, 1980; Martindale, 1996). The metabolite bisdesmethylochloroquine is a carboxylic acid derivative and may be responsible for the weak mutagenic effects in TA104 with S9 mix. AMQ, which belongs to the same group of compounds as CHQ, probably produces identical types of metabolic products as CHQ which are responsible for the weak mutagenic effects in TA104 with S9 mix. Hakura et al. (1996) reported that some hydroquinones (e.g., 1,4-dihydroxy benzene) showed significant mutagenic effects in strain TA104. In the course of metabolism, the 6-methoxy group of PRQ is reduced to a hydroxy group. A second hydroxy group is added in the 5 position and the resultant compound is converted to a quinonimine by way of the 5,6-quinone derivative of the parent compound (Goodman and Gilman, 1980). Thus, this 5,6-quinone derivative may be responsible for the weak mutagenicity of PRQ in TA104.

All three drugs are rapidly and almost completely absorbed from the gastrointestinal tract. They are extensively localized in the tissues of kidney, liver, lung and spleen and are strongly bound by melanin-containing cells, such as those in the eyes and skin. In these tissues they become concentrated in lysosomes, particularly in such cells as those of the liver parenchyma. From all these sites they are slowly excreted and metabolized and may be present in tissues for months or even years after discontinuation of therapy (Bruce-Chwatt, 1986). The recommended dose for both CHQ and AMQ is ~10 mg base/kg followed by 10 mg base/kg at 24 h and 5 mg base/kg at 48 h orally for uncomplicated malaria caused by Plasmodium vivax and Plasmodium ovale and 3.5 mg base/kg by i.m. or s.c. injection every 6 h for 24 h for severe malaria. The dose of PRQ is ~0.25 mg base/kg/day for 14 days in order to obtain a radical cure (White and Breman, 1998). After oral administration of the equivalent of 300 mg CHQ base as a tablet, a mean peak plasma concentration of 76 ng/ml has been obtained after a mean of 3.6 h in healthy adults (Martindale, 1996). In the case of PRQ, a mean blood level of 250 ng/ml is reached within 1 h of ingesting a single dose of 45 mg PRQ base (Bruce-Chwatt, 1986). Our in vivo genotoxicity studies of these drugs were carried out by i.p. administration, so the i.m. and s.c. human therapeutic doses of CHQ mentioned above can only be partially compared with our results. The recommended CHQ dose i.m. or s.c. per day as mentioned by White and Breman (1998) is ~980 mg CHQ base per adult human of ~70 kg weight. In our present study the minimum effective doses of CHQ to induce SCE and CA were 12.5 and 100 mg/kg, respectively, in the case of mice. Considering the surface area ratio of mice to humans (Ghosh, 1984), the effective doses that might induce SCE and CA in humans will be ~96.97 and 775.8 mg, respectively, which is much less than the presently recommended daily dose of CHQ via the i.m. or s.c. route. Moreover, human doses are recommended for several days. Considering the mutagenic and genotoxic effects observed by us in a single i.p. injection study, more attention should be given to restricting the use of these drugs for longer periods of time, since they are accumulated in different organs.

Thus the overall results of the Ames mutagenicity and the SCE and CA assays in mice indicate that these three antimalarial drugs are very weakly mutagenic in strains TA97a, TA100 and TA104 and are capable of inducing significant SCE and CA in bone marrow cells of mice. Our results of a significant increase in SCE at all three doses and in CA at the highest doses induced by all three drugs fully support the observations of Raj and Heddle (1980) and Shubber et al. (1986) for CHQ and PRQ in CHO cells in vitro. PRQ induced CA at the two higher doses (50 and 100 mg/kg) compared with CHQ and AMQ, which induced CA only at the highest dose (100 mg/kg). The increase in CA at the highest dose of PRQ was ~4-fold, compared with 2- to 3-fold for CHQ and AMQ. These observations are in accordance with the known toxicity of PRQ over CHQ and AMQ as reported by several authors. Taking into consideration the risk to humans due to the widespread use of these quinoline derivatives as antimalarial drugs, these findings suggest the necessity of more in vivo genotoxicity and carcinogenicity studies for these drugs.

References


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Received on February 2, 1998, accepted on May 18, 1998