Mutagenesis vol.14 no.6 pp.613–619, 1999

Antimutagenic effects of centchroman—a contraceptive and a candidate drug for breast cancer in multiple mutational assays

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Centchroman (CC), a non-steroidal oral contraceptive and a candidate drug for breast cancer, has been reported to exhibit partial to complete remission of lesions in 40.5% of breast cancer patients. The potent anti-oestrogenic activity, negligible side-effects and anti-breast cancer activity of CC prompted us to evaluate the antimutagenic effects of this compound in a bacterial mutagenicity assay and CHO/HPRT and AS52/GPT mutation assays *in vitro* and *in vivo* in female Swiss albino mice as measured by both sister chromatid exchange (SCE) and chromosome aberrations (CA) against three known positive mutagen compounds, dimethylbenz[*a*]anthracene (DMBA), cyclophosphamide (CP) and mitomycin C (MMC). Antimutagenicity assays in *Salmonella* strains TA97a, TA100, TA98 and TA102 were carried out against commonly used known positive mutagens, sodium azide, 4-nitro-*o*-phenylenediamine, cumine hydroperoxide, 2-aminofluorene and danthron. A significantly reduced number of bacterial histidine revertant colonies was observed in the plates treated with 0.1, 1, 5 and 10 μg/plate CC and a positive compound when compared with bacterial plates treated with the respective positive compound alone. Ethyl methanesulfonate (EMS), a commonly used positive mutagen for CHO/HPRT and AS52/GPT gene mutation assays, was used for antimutagenicity assay in these cells. CC exhibited protective effects against the mutagenicity of EMS in these two mammalian cell mutation assays, CHO/HPRT and AS52/GPT. In the *in vivo* studies, pretreatment with CC reduced DMBA-induced SCE and CA and CP- and MMC-induced CA when compared with the group treated only with the positive compounds. These results indicate that CC can reduce the mutagenic effects of known genotoxic compounds.

Introduction

Centchroman (CC), a non-steroidal oral contraceptive, has been developed by the Central Drug Research Institute, Lucknow, India, and has been successfully marketed in India for the last several years (Figure 1). The contraceptive efficacy of this compound has been attributed to its multiple hormonal properties (Anand and Ray, 1977; Kamboj *et al*., 1977; Kamboj, 1979). Both acute and chronic toxicity studies in mice, rats and rhesus monkeys showed that CC did not produce any toxic manifestations (Mukherjee *et al*., 1977; Mishra *et al*., 1989). It was found to be non-genotoxic in bone marrow cells of female mice (Giri and Khan, 1995). Oestrogen antagonists have been used in the treatment of breast cancer. CC has been reported to be responsible for partial to complete remission of lesions in 40% of breast cancer patients (Mishra *et al*., 1989). They concluded that CC can be a useful agent for the treatment of advanced breast cancer in both males and females (Mishra *et al*., 1989). The Central Drug Research Institute, Lucknow, India, has also organized phase III clinical trials of breast cancer patients with CC. Out of 171 breast cancer patients, 14 showed >50% response, 55 showed <50% response, 31 showed evidence of stable disease and 71 patients failed to respond to the therapy (Central Drug Research Institute, 1991, 1995).

We have been testing the *in vitro* and *in vivo* genotoxic and antimutagenic effects of different drugs and chemicals in multiple test systems (Giri *et al*., 1992; Giri and Lu, 1995; Giri, 1996, 1997; Philipose *et al*., 1997). Considering the potent anti-oestrogenic activity, negligible side-effects and the anti-breast cancer activity of CC we recognized the need to evaluate the antimutagenic effects of CC in multiple test systems. In the present paper we have evaluated the antimutagenic effects of CC in a mutagenicity assay in *Salmonella*, the CHO/HPRT and AS52 assays and sister chromatid exchange (SCE) and chromosome aberration (CA) assays in mice against known positive compounds dimethylbenz[*a*]anthracene (DMBA), cyclophosphamide (CP), mitomycin C (MMC) and ethyl methanesulfonate (EMS).

Materials and methods

Animals

Inbred strains of female Swiss albino mice (*Mus musculus*), 10–12 weeks old, weighing 25–30 g, and Charles River male rats of 150–175 g were used for these experiments. They were kept five per cage with husk bedding, were fed standard rodent pellet diet (Gold Mohar; Lipton India Ltd, Chandigarh, India) and water *ad libitum*. The light cycle was 12 h light and 12 h dark. Room temperature and relative humidity conditions were 28 ± 2°C and 60 ± 5%, respectively.

Chemicals

5-Bromodeoxyuridine (BrdU) tablets (50 mg each) were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). CP, DMBA, MMC, dimethyl sulphoxide (DMSO) colchicine, biotin, histidine, NADP, glucose 6-phosphate, crystal violet, ampicillin trihydrate, agar, sodium azide (SA), 4-nitro-*o*-phenylenediamine (NPD), EMS, cumine hydroperoxide (CH), 2-aminofluorene (AF), danthron (DN) and sodium ammonium phosphate were purchased from Sigma Chemical Co. (St Louis, MO). The test chemical CC was synthesized, purified and supplied by the Division of Medicinal Chemistry, Central Drug Research Institute, Lucknow, India.

Bacterial strains

Salmonella tester strains TA97a, TA100, TA98 and TA102 were used for Ames bacterial mutagenecity and antimutagenicity assays. These strains were provided by Dr Bruce N. Ames (Biochemistry Division, University of California, Berkeley, CA).

Bacterial mutagenicity and antimutagenicity assays

Standard plate incorporation tests. Plate incorporation tests were performed as previously described (Maron and Ames, 1983). In the plate incorporation

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assay the test chemical CC was dissolved in DMSO and different concentrations (0.1, 1, 5, 10, 50, 100 and 1000 μg/plate) of this test chemical were used for the mutagenicity assay. The plates were inverted within 1 h and placed in a dark vented incubator at 37°C for 48 h. Similar experiments were carried out for positive mutagens (NPD for TA97a and TA98, SA for TA100 and CH for TA102) and solvent controls (DMSO). Four plates were used for each concentration tested and for both positive controls (mutagens) and a solvent control. After 48 h incubation of the culture plates the revertant colonies on the test plates were counted. The presence of a background lawn on all the plates was confirmed. A similar experiment was also carried out using liver homogenate (S9) fractions. The positive mutagen 2-AF was used for strains TA97a, TA100 and TA98 and DN was used for strain TA102 in the +S9 experiments. So two sets of experiments were carried out with and without S9 mix for all the tester strains.

Antimutagenicity assay. Antimutagenicity assay of CC in a plate incorporation assay was carried out against known positive compounds in TA97a, TA100, TA98 and TA102. In this case both CC (different concentrations as mentioned above) and the respective positive compounds were added to the top agar for the antimutagenicity assay. The rest of the procedure was as described above in the plate incorporation test. Antimutagenicity assay of CC was also carried out using rat liver S9 in strains TA97a, TA100, TA98 and TA102.

In vitro CHO/HPRT and AS52/GPT assays

K1BH4 and AS52, a subclone and a derivative of the CHO-K1 cell line, respectively, were used for selection of $HPRT^-$ and GPT^- mutants. K1BH4 cells have a karyotype of 20 chromosomes with an active X chromosome on which the *hprt* gene is located (Hsie *et al*., 1975, 1986). AS52 cells, derived from K1BH4 with an *hprt* gene deletion, contain a single stable copy of a transfected *Escherichia coli gpt* gene located on an autosome (Tindall *et al*., 1984, 1986), presumably chromosome 6 (Michaelis *et al*., 1992; K.L.Michaelis, personal communication). Cells were cultured in F12 medium containing 5% dialysed fetal calf serum (F12FCM5).

To decrease the background of $HPRT^-$ mutants, K1BH4 cells were cultured for 48 h in F12FCM5 medium supplemented with 100 μM hypoxanthine, 10 μM aminopterin and 10 μM thymidine (HAT medium). To decrease the background of GPT^- mutants, AS52 cells were grown in F12FCM5 medium supplemented with 250 μg/ml xanthine, 25 μg/ml adenine, 5 μM thymidine, 3 μM aminopterin and 10–20 μg/ml mycophenolic acid (MPA medium) for 48 h (An and Hsie, 1992). After 48 h recovery in F12FCM5 medium, 10^5 K1-BH4 cells or 2.5×10^4 AS52 cells were plated in a 60 mm dish in 3 ml F12FCM5 medium. After another 24 h, the cells were treated with Dulbecco's phosphate-buffered saline (PBS) or CC (10 or 20 μg/ml) in the presence or absence of EMS (200 or 400 μg/ml) (see Tables I and II for study design). Cells were then grown in F12FCM5 medium for 7 days to allow full expression of $HPRT^-$ and GPT^- mutant phenotypes (Hsie *et al*., 1975; Tindall *et al*., 1984). Depending on the extent of cell survival after mutagen treatment, one or two subcultures were performed during this period. At the end of the full expression period 2×10^5 cells from the cultures in each original 60 mm dish were plated in each of three 100 mm dishes to select for HPRT or GPT mutants in hypoxanthine-free F12FCM5 medium containing 10 μM 6-thioguanine.

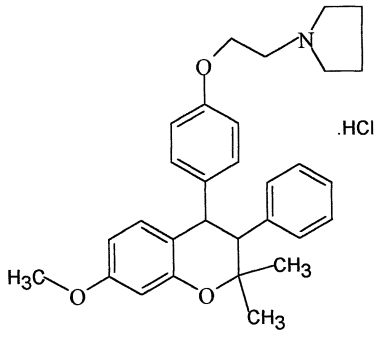

Fig. 1. Structure of centchroman.

Aliquots of 200 cells were plated in triplicate in 60 mm dishes in the same medium without 6-thioguanine to determine the cloning efficiency of the cells in the absence of the selective agent, 6-thioguanine. After 7 days incubation the mutant colonies were fixed, stained and counted. Mutant frequency is calculated by dividing the total number of mutant colonies by the total number of cells selected (1×10^6) corrected for cloning efficiency and is expressed as mutants/10^6 clonable cells (Hsie *et al*., 1981).

Chromosome aberrations (CA) assay

For CA analysis CC was suspended in distilled water with gum acacia (2%) and was gavaged (0.25–0.3 ml suspension depending on body weight) to three sets of five animals each at the rate of 10, 20 and 40 mg/kg, respectively, for 7 days. A group of five animals were gavaged with an equal volume of distilled water only with 2% gum acacia for 7 days. On day 7, 30 min after the last dose of CC or distilled water (for control only), the positive compound DMBA was gavaged at a rate of 50 mg/kg body wt in corn oil to all mice. Two sets of 20 female mice were also used for a similar experiment for two other positive control mutagens, CP and MMC. Three doses of CC (10, 20 and 40 mg/kg) were gavaged to two sets of 15 mice each as described above. CP was gavaged at the rate of 20 mg/kg body wt to all of the 20 mice and MMC was gavaged at a rate of 1.5 mg/kg to another set of 20 mice. For CA analyses, 22 h after treatment with the positive compounds animals were injected (i.p.) with colchicine (2 mg/kg) and 2 h later they were killed by cervical dislocation. Bone marrow chromosomes were prepared and stained with Giemsa following the method of Preston *et al*. (1987). All the slides were coded and 100 well-spread metaphase cells were scored per animal for CA. A total of 500 metaphase cells were scored for each set of positive control treated groups and also for positive control treated groups pretreated with different doses of CC. Mitotic index (MI) was scored from 1000 cells/animal and is expressed as a percentage. CA were scored following the guidelines of the World Health Organization (1985) and Preston *et al*. (1987). The aberration frequencies per cell for chromatid and chromosome types were calculated. Gaps were recorded and not included either as percent aberrant cells or as the frequency of aberrations per cell (Sharief *et al*., 1986).

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 anaesthesia following the methodology of McFee *et al*. (1983) for *in vivo* SCE study and cell replication kinetic analysis. CC was suspended in distilled water with gum acacia (2%) and was gavaged (0.25–0.3 ml suspension depending on body weight) to three sets of five animals each at a rate of 10, 20 and 40 mg/kg for 7 days. A group of five animals was gavaged with an equal volume of distilled water with 2% gum acacia for 7 days. On day 7, immediately after the last dose of CC and distilled water (for controls only), BrdU tablets were implanted into each mouse. Thirty minutes after tablet implantation the positive compound DMBA was gavaged at 50 mg/kg body wt in corn oil to all mice. Another set of 20 female mice was also used in a similar experiment with another positive control compound, CP. Three doses of CC were gavaged to 15 mice as described above. CP was gavaged at the rate of 10 mg/kg body wt to all the 20 mice 30 min after tablet implantation. For SCE analysis, colchicine (4 mg/kg) was injected (i.p.) 22 h after BrdU tablet implantation. The rest of the procedure was as described above (Preston *et al*., 1987). Slides were prepared and chromosomes were differentially stained by the fluorescence-plus-Giemsa technique (Perry and Wolff, 1974; World Health Organization, 1985). All slides were coded and 30 second division metaphase cells (40 ± 2 chromosomes) per animal were scored for SCE frequencies, i.e. a total of 150 cells were scored from five animals in each set of positive

Table I. The protective effects of centchroman on the frequency of *hprt* mutants induced by EMS in CHO-K1BH4 cells

EMS			CC10			CC20		
0	E200[a]	E400[b]	0	E200[c]	E400[d]	0	E200[e]	E400[f]
2.50 ± 3.50	225.00 ± 48.10	455.50 ± 7.80	6.00 ± 8.50	192.00 ± 8.50	281.00 ± 8.50	28.50 ± 2.10	159.00 ± 12.00	205.0 ± 1.40

All data are expressed as mutant frequency in mutants ± SD per 10^6 clonable cells from two independent experiments. A significant difference ($P < 0.51$ and $P < 0.31$) for treated groups c and d, respectively, from untreated control group a was found using Fisher's protected least significant difference procedure. Similarly, a significant difference ($P < 0.03$ and $P < 0.01$) for treated groups e and f, respectively, from untreated control group b was observed. EMS, ethyl methanesulfonate; CC, centchroman; E200 and E400, 200 and 400 μg/ml EMS; CC10 and CC20, 10 and 20 μg/ml centchroman.

control treated groups and from the positive control treated groups pretreated with different doses of CC. Randomly selected metaphase cells (100/animal) were scored for replicative index (RI) analysis following the method of Ivett and Tice (1982).

Statistical analysis

Mutagenicity and antimutagenicity results for the different concentrations of chemicals with bacteria were compared with the negative and positive controls by Dunnett's multiple comparison with controls (Dunnett, 1955). SCE, CA, MI and RI results were also compared with their respective positive controls by Dunnett's multiple comparison with controls and the level of significance is given in the respective tables. Results of the CHO/HPRT and AS52/GPT assays were compared with the respective controls by Fisher's protected least significant difference procedure.

Results

Mutagenicity and antimutagenicity assays in Salmonella

Tables III and IV show the results of mutagenicity and antimutagenicity assays in *Salmonella* strains TA97a, TA100, TA98 and TA102. CC did not show any mutagenic effect in any of the tester strains described above. In strains TA97a and TA100 toxicity was induced at 50 μg/plate both with and without S9 mix. Antimutagenicity assays in both strains showed ~10–30% protective effects of CC against NPD and SA at the concentrations of 1, 5 and 10 μg/plate without S9 mix and an ~12–25% protective effect was observed against AF at concentrations of 1, 5 and 10 μg/plate with S9 mix (Tables III and IV). In strain TA98 toxicity was also observed at a concentration of 50 μg/plate, at which a statistically significant decrease in revertant colonies was observed. Similarly, ~15–28% protective effects of CC in strain TA98 were observed at concentrations of 0.1, 1 and 10 μg/plate without S9 mix and ~15–45% protective effects were observed at concentrations of 0.1, 1 and 10 μg/plate with S9 mix (Tables III and IV). As expected, CC did not show any mutagenic effects in strain

Table II. The protective effects of centchroman on the frequency of the *gpt* mutants induced by EMS in CHO-AS52 cells

EMS			CC10			CC20		
0	E200[a]	E400[b]	0	E200[c]	E400[d]	0	E200[e]	E400[f]
18.00 ± 2.80	271.50 ± 9.20	498.00 ± 4.20	34.00 ± 4.20	228.00 ± 1.40	327.50 ± 16.30	79.50 ± 2.10	182.50 ± 0.70	264.50 ± 38.90

All data are expressed as mutant frequency in mutants ± SD per 10^6 clonable cells from two independent experiments. A significant difference ($P < 0.10$ and $P < 0.05$) for treated groups c and d, respectively, from untreated control group a was found using Fisher's protected least significant difference procedure. Similarly, a significant difference ($P < 0.04$ and $P < 0.07$) for treated groups e and f, respectively, from untreated control group b was observed.

Table III. Mutagenic and antimutagenic effects of centchroman (CC) in the plate incorporation test using *Salmonella* strains TA97a, TA100, TA98 and TA102 without S9

Chemical (CC μg/plate)	Mutagenicity assay (no. revertants/plate, mean ± SD[a])	Chemical (CC μg/plate)	Antimutagenicity assay (no. revertants/plate, mean ± SD[a])
TA97a			
Solvent control	129.25 ± 24.78	NPD (20 μg /plate)	1039.50 ± 37.25
1	136.25 ± 24.76	NPD + 1	925.25 ± 37.20[b]
5	140.50 ± 9.33	NPD + 5	845.50 ± 40.00[b]
10	130.50 ± 11.68	NPD + 10	730.30 ± 45.50[b]
50	81.50 ± 3.37[b]	NPD + 50	635.50 ± 50.00[b]
100	47.00 ± 20.78[b]	NPD + 100	555.20 ± 36.30[b]
1000	Toxic	NPD + 1000	Toxic
TA100			
Solvent control	195.00 ± 16.39	SA (1.5 μg/plate)	1214.75 ± 98.65
1	181.20 ± 28.79	SA + 1	1068.50 ± 37.53[b]
5	207.25 ± 13.60	SA + 5	836.75 ± 63.68[b]
10	188.25 ± 20.95	SA + 10	860.75 ± 51.80[b]
50	169.25 ± 13.95[c]	SA + 50	837.50 ± 16.52[b]
100	134.40 ± 23.96[b]	SA + 100	785.50 ± 93.94[b]
1000	Toxic	SA + 1000	Toxic
TA98			
Solvent control	39.75 ± 6.18	NPD (20 μg/plate)	1019.75 ± 68.91
0.1	36.25 ± 6.13	NPD + 0.1	863.50 ± 40.00[b]
1	39.00 ± 4.69	NPD + 1	768.75 ± 76.35[b]
10	36.25 ± 4.50	NPD + 10	737.00 ± 47.67[b]
50	27.75 ± 7.80[a]	NPD + 50	730.00 ± 132.48[b]
100	17.75 ± 4.57[b]	NPD + 100	450.75 ± 49.61[b]
1000	Toxic	2-AF + 1000	Toxic
TA102			
Solvent control	267.25 ± 26.01	CH (100 μg/plate)	1170.50 ± 68.39
0.1	270.50 ± 21.62	CH + 0.1	1027.75 ± 25.46[b]
1	262.00 ± 18.13	CH + 1	976.50 ± 26.34[b]
10	246.75 ± 26.41	CH + 10	904.75 ± 28.39[b]
50	237.75 ± 21.86	CH + 50	627.00 ± 45.58[b]
100	176.00 ± 56.98[b]	CH + 100	429.50 ± 73.71[b]
1000	Toxic	CH + 1000	Toxic

[a]Mean ± SD of four plates. Results of each concentration were compared with the positive control by Dunnett's test.
[b]$P < 0.01$.
[c]$P < 0.02$.

Table IV. Mutagenic and antimutagenic effects of centchroman (CC) in the plate incorporation test using *Salmonella* strains TA97a, TA100, TA98 and TA102 with S9

Chemical (CC μg/plate)	Mutagenicity assay (no. revertants/plate, mean ± SD[a])	Chemical (CC μg/plate)	Antimutagenicity assay (no. revertants/plate, mean ± SD[a])
TA97a			
Solvent control	132.50 ± 15.35	2-AF (10 μg/plate)	955.50 ± 56.00
1	145.25 ± 20.00	2-AF + 0.1	840.25 ± 45.50[b]
5	148.00 ± 18.30	2-AF + 1	822.50 ± 50.00[b]
10	116.50 ± 20.50	2-AF + 10	710.00 ± 38.50[b]
50	69.00 ± 17.50[b]	2-AF + 50	525.00 ± 35.00[b]
100	50.50 ± 10.00[b]	2-AF + 100	175.00 ± 32.50[b]
1000	Toxic	2-AF + 1000	Toxic
TA100			
Solvent control	182.00 ± 17.50	2-AF (10 μg/plate)	1055.50 ± 37.00
1	197.50 ± 20.30	2-AF + 0.1	890.25 ± 50.50[b]
5	200.50 ± 30.00	2-AF + 1	810.50 ± 35.25[b]
10	169.25 ± 22.50	2-AF + 10	750.25 ± 33.70[b]
50	150.25 ± 36.00[c]	2-AF + 50	715.35 ± 52.50[b]
100	110.00 ± 16.20[b]	2-AF + 100	335.50 ± 60.00[b]
1000	Toxic	2-AF + 1000	Toxic
TA98			
Solvent control	39.25 ± 6.34	2-AF (10 μg/plate)	1079.50 ± 71.76
0.1	37.00 ± 9.59	2-AF + 0.1	921.25 ± 24.21[b]
1	35.00 ± 6.32	2-AF + 1	780.75 ± 36.56[b]
10	36.00 ± 7.25	2-AF + 10	590.50 ± 26.25[b]
50	28.75 ± 6.94[c]	2-AF + 50	503.00 ± 86.77[b]
100	18.25 ± 3.30[b]	2-AF +100	359.25 ± 23.08[b]
1000	Toxic	2-AF + 1000	Toxic
TA102			
Solvent control	301.50 ± 28.44	DN (30 μg/plate)	794.25 ± 36.50
1	274.25 ± 27.73	DN + 0.1	642.25 ± 15.90[b]
1	294.25 ± 23.71	DN + 1	598.25 ± 62.73[b]
10	274.50 ± 20.40	DN + 10	566.50 ± 59.96[b]
50	247.25 ± 32.62[b]	DN + 50	540.75 ± 53.45[b]
100	189.25 ± 27.73[b]	DN + 100	442.50 ± 43.44[b]
1000	Toxic	DN + 1000	Toxic

[a]Mean ± SD of four plates. Results of each concentration were compared with the positive control by Dunnett's test.
[b]$P < 0.01$.
[c]$P < 0.02$.

TA102. Toxicity was induced at 100 μg/plate. In strain TA102 an ~12–47% protective effect of CC was observed at concentrations of 0.1, 1, 10 and 50 μg/plate without S9 mix and ~19–32% protective effect was observed at concentrations of 0.1, 1, 10 and 50 μg/plate with S9 mix (see Tables III and IV).

Mutagenicity and antimutagenicity in CHO/HPRT and AS52 assays

Toxicity. CC exhibits slight toxic effects in both CHO-K1BH4 and CHO-AS52 cells. CC shows a slight protective effect against the toxic effect of EMS (200 or 400 μg/ml). The protective effect of CC is more pronounced at 20 than at 10 μg/ml (data not shown).

*Mutagenicity and antimutagenicity assay*s. We tested the antimutagenic effects of CC in the CHO/HPRT and AS52/GPT assays. We found that in the CHO/HPRT assay, CC is not mutagenic at a concentration of 10 μg/ml but showed a slight mutagenic effect at high dose (20 μg/ml). EMS (200 and 400 μg/ml) exhibits a dose-dependent increase in induced mutant frequency and treatment of cells with CC (10 and 20 μg/ml) reduces EMS (200 and 400 μg/ml) mutagenicity. CC at 20 μg/ml exhibits a higher protective effect than at 10 μg/ml. CC showed a 15–38% protective effect against EMS at 10 μg/ml and a 29–55% protective effect at 20 μg/ml (see Table I).

Similar results were found in the AS52/GPT assay. CC exhibits a slight mutagenicity at these doses, EMS shows a dose-dependent mutagenicity and CC exhibits antimutagenic activity against EMS mutagenicity. CC exhibits a 16–34% protective effect against EMS mutagenicity at 10 μg/ml and a 33–47% protective effect at 20 μg/ml (Table II).

Antimutagenic effects in vivo in mice

Table V shows the results of the antimutagenicity assay of CC as measured by CA against three positive compounds, DMBA, CP and MMC, in female mice when fed orally. A statistically significant decrease in CA was observed in the DMBA-treated group pretreated with CC at a dose of 20 mg/kg for 7 days when compared with the group which received only DMBA. In this case the protective effect was ~36%. CC showed a very weak protective effect against CP. A weak (5% level) but statistically significant decrease in CA was observed in the CP-treated group pretreated with CC at a dose of 10 mg/kg for 7 days when compared with the group that received only CP. The protective effect was only 19%. In the MMC-treated series, a significant decrease in CA was observed in all three sets of MMC-treated groups pretreated with 10, 20 and 40 mg/kg CC when compared with the group which received only MMC. The protective effects of CC in these MMC-treated groups were ~30–55%. A significant decrease in MI was observed at the highest dose in all CC-treated groups, indicating a slight cytotoxic effect of CC at this dose (see Table V).

Table VI show the results of the antimutagenicity assay of CC as measured by SCE against two positive compounds,

Table V. Antimutagenic effects of centchroman against the known positive mutagens DMBA, cyclophosphamide and mitomycin C as measured by chromosome aberrations

Treatment (mg/kg)	Gap[a]	Aberrations/cell[b]		Aberrant cells (%, mean ± SD)[c]	Mitotic indices (mean ± SD)[c]
		Chromatid type	Chromosome type		
DMBA (50 mg/kg)	103	0.224	0.044	24.00 ± 3.39	2.23 ± 0.48
Centchroman (mg/kg)					
10 + DMBA (50 mg/kg)	115	0.346	0.038	22.80 ± 2.28	1.68 ± 0.79
20 + DMBA	81	0.162	0.032	15.20 ± 3.76[d]	1.45 ± 0.45[e]
40 + DMBA	130	0.208	0.044	21.60 ± 4.39	1.22 ± 0.53[e]
Cyclophosphamide (20 mg/kg)	74	0.544	0.032	31.40 ± 3.36	1.91 ± 0.59
Centchroman (mg/kg)					
10 + CP (20 mg/kg)	81	0.376	0.018	25.20 ± 4.90[e]	1.59 ± 0.38
20 + CP	67	0.460	0.026	28.80 ± 3.27	1.40 ± 0.43
40 + CP	77	0.490	0.022	29.80 ± 2.38	1.42 ± 0.32[e]
Mytomycin C (1.5 mg/kg)	49	0.130	0.026	12.60 ± 2.30	2.19 ± 0.53
Centchroman (mg/kg)					
10 + MMC (1.5 mg/kg)	14	0.064	0.014	7.40 ± 1.82[d]	2.48 ± 0.38
20 + MMC	13	0.046	0.012	5.60 ± 2.07[d]	2.07 ± 0.58
40 + MMC	22	0.072	0.016	8.80 ± 1.64[e]	1.32 ± 0.53[e]

[a]Total chromatid and chromosome gaps at each dose were recorded but not included as aberrations/cell.
[b]Total number of aberrations (chromatid or chromosome type)/total number of cells scored per dose group. Results are of five animals (100 cells/animal).
[c]Results at each dose were compared with those of the control using Dunnett's multiple comparison with control.
[d]$P < 0.01$.
[e]$P < 0.05$.

Table VI. Antimutagenic effects of centchroman against known positive mutagens DMBA and cyclophosphamide as measured by sister chromatid exchanges

Treatment (mg/kg)	SCE/cell of five animals	SCE/cell (mean ± SD)[a]	Replicative indices (mean ± SD)[a]
DMBA (50 mg/kg)	14.0,15.8,14.0,15.3,15.1	14.84 ± 0.80	1.86 ± 0.05
Centchroman (mg/kg)			
10 + DMBA(50 mg/kg)	12.7,13.8,11.9,12.4,14.7	13.10 ± 1.16	1.73 ± 0.09
20 + DMBA	13.2,13.2,11.2,11.7,12.9	12.45 ± 0.91[b]	1.72 ± 0.07[b]
40 + DMBA	16.9,13.7,13.4,13.5,15.9	14.67 ± 1.64	1.76 ± 0. 4[b]
Cyclophosphamide (10 mg/kg)	24.5,26.4,26.6,25.4,27.2	26.01 ± 1.06	1.75 ± 0.07
10 + CP (10 mg/kg)	25.8,28.9,26.5,28.6,29.5	27.86 ± 1.61	1.71 ± 0.06
20 + CP	23.9,26.0,31.7,24.2,20.7	25.31 ± 4.07	1.61 ± 0.05[b]
40 + CP	27.2,30.6,35.5,31.1,27.9	30.47 ± 3.28	1.60 ± 0.07[b]

[a]Mean ± SD of five animals. Results at each dose were compared with the respective positive control using Dunnett's test.
[b]$P < 0.02$.

DMBA and CP. As observed in the CA study, a statistically significant protective effect was observed in DMBA-treated series pretreated with 20 mg/kg CC when compared with DMBA alone. No statistically significant differences were observed in CP-treated series pretreated with any of the doses of CC when compared with the CP-alone group. A significant decrease in RI was observed in both DMBA- and CP-treated groups pretreated with 20 and 40 mg/kg CC when compared with the respective positive compounds alone (see Table VI).

Discussion

The reported onco-protective effect of CC on breast cancer (Mishra *et al*., 1989; Central Drug Research Institute, 1991, 1995) and its potent anti-oestrogenic activity with negligible side-effects has prompted us to examine the antimutagenic effects of this compound against known positive mutagens in multiple test systems. Chemically induced cancers are primarily due to the ability of the chemical to damage DNA and to induce CA, SCE and gene mutations. These biomarkers for DNA damage can be used to assess the antigenotoxic potential of a chemical. Our main objective was to investigate whether racemic centchroman, having antimutagenic effects and with an anti-cancer profile similar to tamoxifen (TM), could replace the latter for toxicity reasons. CC has been resolved into *l*- and *d*-enantiomers (Salman *et al*., 1986), of which the *l* form is biologically active (see Figure 1). Work is also in progress to study whether *l*-centchroman has better antimutagenic properties or whether the pharmacologically inert *d* form has any advantage in reducing mutagenicity. These are the aims for our future research. In the present study we report the antimutagenic effects of racemic centchroman.

CC is not mutagenic in *Salmonella* strains TA97a, TA98, TA100 and TA102 in plate incorporation assays either with or without S9 activation (see Tables III and IV). It reduces the mutagenic activities of the known mutagens SA, NPD, AF, CH and DN. The overall protective effects of CC in the antimutagenicity assay in these strains were ~10–47%. These protective effects of CC may be due to a direct interaction of these mutagens with CC. Initially we also tried to see whether we could increase the protective effect in the antimutagenicity assay of CC using preincubation tests for TA97a and TA100. However, the protective effects were almost the same as observed in the plate test for these two strains (data not presented). Thus, we decided to study the antimutagenic effects of CC only in the plate incorporation test. CC also exhibits antimutagenic activities in both the CHO/HPRT and AS52/GPT assays.

We conducted the *in vivo* antimutagenicity studies of CC in female Swiss albino mice based on the dose recommended for human breast cancer patients in clinical trials at the Central Drug Research Institute (1991). The best effective human dose converted to a mouse dose was ~20 mg/kg body wt. Thus, we selected one lower (10 mg/kg) and one higher (40 mg/kg) dose relative to this effective dose (20 mg/kg) for our *in vivo* studies in mice. We have chosen CA and SCE for *in vivo* antimutagenicity assay since these are the two cytogenetic end-points which detect the genotoxic potential of a chemical. We have reported earlier that the soya-derived phyto-oestrogen genistein has a significant protective effect against the known breast cancer inducing compound DMBA (Giri and Lu, 1995) as measured by SCE and DNA adduct formation. Genistein is a weak oestrogen (Eldridge, 1982; Stob, 1983; Price and Fenwick, 1985) that may also act as an anti-oestrogen by competing with endogenous oestrogens for oestrogen receptors (Martin *et al.*, 1978; Verdeal *et al*., 1980). Like genistein, CC is a weak oestrogen and also acts as an anti-oestrogen. Our results of *in vivo* CA assay showed a significant protective effect of CC in a DMBA-treated group pretreated with CC (20 mg/kg) and a MMC-treated group pretreated with all three doses of CC (10, 20 and 40 mg/kg) in mice. Pretreatment with CC also reduced SCE induced by DMBA. The most effective dose of CC was 20 mg/kg body wt for both the CA and SCE assays. It is interesting to note that the maximum protective effects of CC were observed at the dose of 20 mg/kg, which was very close to the human dose in clinical trials with human breast cancer patients (Central Drug Research Institute, 1991, 1995). CC showed a very weak protective effect against the CP. Since CC has an onco-protective effect (Mishra *et al*., 1989), it may also be effective only against the cancer inducing compounds DMBA and MMC. The onco-protective effects as reported earlier may be attributed, in part, to the ability of CC to reduce the levels of genetic damage induced by known carcinogenic compounds.

CC inhibits both the MI and RI in CA and SCE studies. The extents of inhibition, while small, were statistically significant and most likely biologically relevant since the RI was calculated based on the presence of metaphase chromosomes, which requires cell cycle progression. Any compound that severely blocks cell cycle progression would produce few metaphase cells for scoring. Thus, RI estimation based on metaphase cells tends to underestimate the ability of a compound to inhibit replication. These data suggest that CC inhibits cell replication.

In summary, we show that CC reduces revertant colonies in the Ames assay and mutagenic effects in the CHO/HPRT and AS52/GPT assays induced by the known positive compounds NPD, SA, 2-AF, CH, DN and EMS *in vitro*. It also reduces both CA and SCE induced by DMBA and CA induced by MMC. The mechanism responsible for the reduction in DMBA-induced CA and SCE and MMC-induced CA is currently unknown. CC is structurally similar to the non-steroidal anti-oestrogen TM (Ray *et al*., 1994). Ferlini *et al*. (1997) reported that TM can induce apoptosis and has some antiproliferative activity in human breast cancer cells. Thus, the protective activities of CC against the known positive mutagens in the present study may be due to induction of apoptosis by CC, which leads to the elimination of cells damaged by these mutagens from the cell population. Determination of the antimutagenic effects of both the enantiomers of CC is in progress and will be reported later. Determination of induction of apoptosis by CC and its enantiomers in MCF-7 and CHO cell lines is also in progress, to better understand the exact mechanism of action of this drug. Since CC is a candidate drug for breast cancer treatment, more information is required on the mechanism of action of this drug in mammalian systems.

Acknowledgements

The authors are grateful to the Department of Science and Technology, Government of India, for sanctioning the centchroman research project by A.K.G., sanction no. SP/SO/B-53/95, and also providing the Junior Research Fellowship to A.M. The authors express their appreciation to Dr Bruce Ames, University of California at Berkely, for supplying *Salmonella* strains.

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Received on June 9, 1999; accepted on August 12, 1999