Antineoplastic and cytogenetic effects of chlorpromazine on human lymphocytes *in vitro* and on Ehrlich ascites tumor cells *in vivo*

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The inhibitory effect of phenothiazines in tumor growth and cancer cell proliferation *in vitro* and *in vivo* has been established. These reports motivated us to investigate the genotoxic, cytotoxic, and cytostatic potential of chlorpromazine, alone or in combination with mitomycin C, *in vitro* and *in vivo.* Sister chromatid exchange levels were assessed providing a quantitative index of genotoxicity. In-vitro studies were performed on human lymphocyte cultures and in-vivo studies involved Ehrilch ascites tumor (EAT) cells. An antitumour study was also conducted on the survival time and the ascitic volume in EAT-bearing Balb/C mice. The combination of chlorpromazine plus caffeine and mitomycin C exerted cytostatic and cytotoxic actions in human lymphocytes. The combination of chlorpromazine plus mitomycin C exerted cytostatic and cytotoxic actions in EAT cells, significantly increased the survival span of the mice inoculated with EAT cells, and suppressed the expected tumor growth increase. The findings of this basic study illustrate that high chlorpromazine concentrations increase chemotherapeutic effectiveness of mitomycin C. Chlorpromazine concentrations within the observed human plasma concentration range need to be tested along with antineoplastic agents *in vitro* for its synergistic action so as to evaluate a potential clinical application. Further investigation including other phenothiazines, biological systems, and cancer models is required. *Anti-Cancer Drugs* 20:746–751

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Introduction

Chlorpromazine (CPZ) is one of the oldest representatives of the phenothiazine–thioxanthene group of antipsychotic agents and is given particular attention as newer agents can be compared and contrasted with it [1]. Phenothiazines possess strong inhibitory effects on various molecules involved in carcinogenesis and tumor growth *in vitro* and *in vivo* [2–8]. It has been illustrated that they exert their antiproliferative activity in a concentration-dependent manner [9,10]. Other studies have found that they promoted programmed cell death in human neuroblastomas and rat C6 glioma cells and in primary mouse brain tissue [11].

Administration of CPZ and other phenothiazines such as thioridazine can induce cytotoxicity [12–14] and cause cell division delays [15,16]. CPZ, combined with caffeine (CAF) and antineoplastic agents, such as melphalan, bleomycin, or chlorambucil, enhanced the antitumour effect of these antineoplastic agents in human lymphocytes and murine L1210 leukemia cells [14,17]. Lee *et al.* [18] reported the synergistic action of pentamidine, an antiparasitic agent, and CPZ, and their inhibitory effect on tumor cell proliferation *in vivo*. Hoshi *et al.* [19] reported the synergistic action and antitumor activity of psychotropic drugs, including CPZ, with cyclophosphamide, in Ehrlich ascites tumor (EAT) sarcoma in ascites form.

Although phenothiazines have been extensively studied in in-vitro models, studies in in-vivo systems and cancer models are inadequate. All these prompted us to conduct the present study aiming at the following: (i) study the effect of CPZ, as a representative of the phenothiazine group, on sister chromatid exchange (SCE) levels, mitotic index (MI), and cell kinetics in human lymphocytes in combination with mitomycin C (MMC) (a well-known antineoplastic agent), (ii) investigate the genotoxic and cytostatic effects of CPZ on EAT cells pretreated *in vivo* with MMC, and (iii) examine the antitumor activity of CPZ in combination with MMC in mice inoculated with EAT cells, by observing and recording survival time and suppression of ascitic volume.

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The combination of in-vitro and in-vivo studies concerning SCEs and cell proliferation kinetics in different cell lines could be positively correlated with efficient in-vivo antitumour activity [20–24].

Methods

In-vitro experiments

Heparinized blood samples were obtained from six healthy individuals, none of whom were under medical treatment or was a smoker. Cultures of peripheral lymphocytes were prepared in universal containers by adding 11 drops of whole blood to 5 ml of chromosome medium B (Biochrom KG, Germany). These were incubated at 37°C for 96 h. For SCE observations, the cultures were first treated with CPZ (3 or 9 μg/ml) followed by MMC (2 or 10 ng/ml) and CAF (120 μg/ml) (a known inhibitor of certain DNA repair mechanisms) (Sigma Chemicals, Germany) at 20 h. For the differential staining of sister chromatids, 5-bromodeoxyuridine (5-BrdU) (Serva, Germany) was added at a final concentration of 4 μg/ml. After 94 h, colchicine (Serva, Germany) was added for 2 h at a final concentration of 0.5 μg/ml, and cultures were harvested at the end of the incubation period. Cultures were kept in the dark to prevent or minimize photolysis of 5-BrdU. Chromosome preparations were stained by a modified fluorescence plus Giemsa technique [25].

For establishing the proliferation rate index (PRI) in each case, 200–300 cells were counted according to the formula: $PRI = (M_1 + 2M_2 + 3M_{3+})/N$, where M_1 is the percentage of cells in the first division, M_2 in the second and M_{3+} in the third and subsequent divisions, whereas N is the total number of metaphases. Furthermore, evaluation of MI for all cultures was performed by counting 3000 activated interphase nuclei. To determine the synergistic action of the combined treatments, the expected value (EV) was evaluated because the drugs were acting independently and additively, according to the following formula, for example: $EV_{CPZ+MMC+CAF} = (OV_{CPZ} - OV_{control}) + (OV_{MMC} - OV_{control}) + (OV_{CAF} - OV_{control}) + OV_{control,}$ or better, $EV_{CPZ+MMC+CAF} = (OV_{CPZ} + OV_{MMC} + OV_{CAF}) - 2^* OV_{control}$, where OV is the observed value for each treatment.

In-vivo experiments

Two to three months old male Balb/c mice with an average weight of 25–35 g were used for this study. The animals were kept at a standard pellet diet with water *ad libitum*. EAT cells were originally provided from the Theagenion Cancer Institute, Thessaloniki, Greece, and preserved by inoculation to host mice under aseptic conditions every 7 days. Cells were originally diluted in 0.9% NaCl solution so as to achieve a concentration of 10^6 cells/ml. Inoculation of the diluted EAT cells (0.2 ml) to host mice was achieved by an intraperitoneal (i.p.) injection [20].

Evaluation of sister chromatid exchanges and mitotic index in Ehrilch ascites tumor cells

Mice were divided in four groups of two mice each, and were marked accordingly to ensure positive identification. Animals were treated on the fourth day after tumor inoculation with an i.p. injection of 0.2 ml of the appropriate agent (MMC and/or CPZ) or 0.9% NaCl, according to the protocol. The drug concentrations used were determined by adjusting the respective treatments from our in-vitro experiments. One hour later, animals were given i.p. injections of 1 ml of BrdU-activated charcoal suspension at a BrdU concentration of 1 mg/g body weight. Before harvesting, mice were treated with 0.1 ml of colchicine (1 mg/ml) for 2 h. Animals were killed by ether narcosis after 48 h of drug administration. The differential staining of sister chromatids was accomplished using a modification of the fluorescence plus Giemsa technique [20]. In these in-vivo experiments, the genotoxic and cytotoxic effects of CPZ, in combination with MMC on EAT cells, were evaluated by SCE induction and cell cycle delays.

Evaluation of antitumour activity

Mice were divided into four groups of 16 mice each, and were appropriately marked. Mice were treated on the second, fourth, and sixth day after tumor inoculation with an i.p. injection of 0.2 ml of the appropriate agents (MMC and/or CPZ) or 0.9% NaCl, according to the protocol. Animals were weighed every day at the same time and the results were recorded and evaluated [20]. Animals bearing ascites tumors were kept alive, and the survival time of each mouse was recorded. In these in-vivo experiments, we examined the antineoplastic effects of MMC alone or in combination with CPZ. For this purpose, we observed and analyzed: (i) the mean life span of mice for each group and compared it with the control group and (ii) the mean body weight increase of mice for each experimental group and compared it with the control group. It can be hypothesized that the mean body weight increase of mice is equal to the weight of the tumor. The percentage of increase in life span for each group was estimated on the basis of the increase in mean survival of the groups concerned, compared with the mean survival time of the control group.

Statistical analysis

To achieve a proper comparison of the diverse treatments, logarithmic transformation of SCEs was performed using the one-way analysis of variance, and the Duncan's test was used for pairwise comparisons. The evaluation of MI and PRI was based on the χ^2 test. Correlations

among SCEs, MI, and PRI were also determined. Differences in survival time and body weight increase were evaluated by using the Wilcoxon's test [16,20].

Results and discussion

The concentrations of CPZ (8–25 μmol/l) used in this basic study were in agreement with previous in-vivo and in-vitro experiments; nevertheless, they were higher than the observed CPZ plasma concentrations in humans [1,26,27]. The results obtained from our in-vitro studies on human lymphocytes are illustrated in Tables 1 and 2. Both CPZ treatments did not have a cytogenetic effect on human lymphocytes. CAF acting as a DNA repair inhibitor was added to cultures to further expose the cytogenetic effects of CPZ on normal lymphocytes. Thus, the selected concentration was higher than normal CAF plasma concentrations (toxic levels > 30 μg/ml) [28]. Although CPZ, at both concentrations used, acted synergistically with CAF in inducing genotoxicity, SCE level induction was not statistically significant compared with control cultures. High CPZ concentration, though combined with CAF, showed significant cytostaticity ($P < 0.01$). The simultaneous administration of 3 μg/ml CPZ and 10 ng/ml MMC significantly induced SCE levels in a synergistic manner ($P < 0.05$). Although administration of 9 μg/ml CPZ and 2 ng/ml MMC did not show any significant genotoxicity, it was proven to be cytostatic ($P < 0.01$). The additional *in vitro* use of CAF (used as a DNA repair inhibitor), aimed to unravel further the genotoxicity of the combined administration of CPZ and MMC. The presence of CPZ, at both concentrations, in cultures treated with CAF and MMC (both concentrations), induced synergistically genotoxic, cytotoxic, and cytostatic effects on human lymphocytes. In Table 1, where 9 μg/ml CPZ and 2 ng/ml MMC were used, genotoxicity was positively correlated with cytotoxicity (MI suppression) ($r = -0.829$, $P < 0.05$). In Table 2, where 3 μg/ml CPZ and 10 ng/ml MMC were used, genotoxicity was positively correlated with cytostaticity (PRI suppression) ($r = -0.833$, $P < 0.05$), whereas the latter was positively correlated with cytotoxicity ($r = +0.817$, $P < 0.05$). Our findings illustrated above could be attributed to the fact that the action of CPZ is concentration dependent [10]. The genotoxic effect of CPZ is ambiguous [29]. Conflicting reports state that CPZ either enhances or reduces SCE levels [14,29]. This variation has been attributed to a possible reflection of individual differences in cell permeability, uptake, and metabolism of the compound, or differences in DNA repair mechanisms [29]. The previous observations

Table 1 Enhancement of cytogenetic damage by CPZ in cultured human lymphocytes exposed to MMC and/or CAF

Agent and concentration	Number of replicate cultures	Mean SCEs ± SEM (range)	PRI	MI (‰)
Control	3	7.98 ± 0.48 (1–16)	2.38	32.5
CPZ 9 μg/ml	3	8.22 ± 0.55 (2–15)	1.98	28.0
CAF 120 μg/ml	3	8.40 ± 0.576 (2–16)	2.37	33.5
CPZ 9 μg/ml + CAF 120 μg/ml	3	10.45 ± 0.62 (2–21)	1.88*	21.0
MMC 2 ng/ml	3	13.47 ± 0.75* (2–20)	2.44	25.5
MMC 2 ng/ml + CPZ 9 μg/ml	3	9.15 ± 0.82 (3–19)	1.88*	21.5
MMC 2 ng/ml + CAF 120 μg/ml	3	12.85 ± 0.84 (1–22)	2.42	16.5†
MMC 2 ng/ml + CPZ 9 μg/ml + CAF 120 μg/ml	3	18.71 ± 1.23** (3–40) (*EV* = 14.13)	1.52***	10.5***

SCE frequency was based on 60 seconds-division metaphases; for PRI evaluation, 200–300 cells were counted, and for MI, 3000 activated lymphocytes were counted. In Tables 1 and 2, the results were based on three experiments from three different donors, and in each experiment, eight cultures were performed.
CAF, caffeine; CPZ, chlorpromazine; EV, expected value if CPZ, CAF and MMC were acting independently and additively; MI, mitotic index; MMC, mitomycin C; PRI, proliferation rate index; SCEs, sister chromatid exchanges; SEM, standard error of mean.
*$P < 0.01$ versus line 1.
**$P < 0.05$ versus lines 5–7 and $P < 0.01$ versus lines 1–4 and 6.
***$P < 0.01$ versus all lines.
†$P < 0.05$ versus lines 1–6.

Table 2 Enhancement of cytogenetic damage by CPZ in cultured human lymphocytes exposed to MMC and/or CAF

Agent and concentration	Number of replicate cultures	Mean SCEs ± SEM (range)	PRI	MI (‰)
Control	3	8.27 ± 0.42 (4–16)	2.26	29.0
CPZ 3 μg/ml	3	7.95 ± 0.52 (1–15)	2.28	26.0
CAF 120 μg/ml	3	10.08 ± 0.85 (4–28)	2.15	27.0
CPZ 3 μg/ml + CAF 120 μg/ml	3	11.05 ± 1.22 (5–25)	1.94	25.5
MMC 10 ng/ml	3	24.32 ± 1.45* (8–39)	2.10	29.5
MMC 10 ng/ml + CPZ 3 μg/ml	3	25.75 ± 1.68* (10–45)	1.93	24.0
MMC 10 ng/ml + CAF 120 μg/ml	3	28.79 ± 1.80* (11–38)	1.90***	25.0
MMC 10 ng/ml + CPZ 3 μg/ml + CAF 120 μg/ml	3	35.42 ± 2.04** (14–59) (*EV* = 25.81)	1.70***	19.5†

CAF, caffeine; CPZ, chlorpromazine; MI, mitotic index; MMC, mitomycin C; PRI, proliferation rate index.
*$P < 0.05$ versus lines 1–4.
**$P < 0.01$ versus all lines.
***$P < 0.01$ versus lines 1, 3, and 5.
†$P < 0.05$ versus all lines.

Table 3 Effects of CPZ on SCEs and cell division delays in Ehrlich ascites tumor cells treated with MMC *in vivo*

Treatment duration	Mean SCEs ± SEM (range)	PRI	Percentage of cells in 1st, 2nd, 3rd+ divisions			MI (‰)
			1st	2nd	3rd+	
Control	12.07 ± 1.04* (3–31)	1.82	21.5	75.0	3.5	45.0
CPZ 5 μg/g bw	18.22 ± 0.9** (5–36)	1.73	30.0	66.7	3.3	40.5
MMC 10 ng/g bw	30.08 ± 2.55 (15–56)	1.78	25.6	71.3	3.1	47.5
MMC 10 ng/g bw + CPZ 5 μg/g bw	27.91 ± 2.29 (13–75)	1.51* (*EV* = 1.69)	52.0	45.0	3.0	15.0*

SCE frequency was based on 30–40 seconds-division metaphases; for PRI evaluation, 100–200 cells were counted and 2000–4000 cells were counted for MI. The results were based on two experiments from four different animals and four treatments were performed in each experiment.
bw, body weight; CAF, caffeine; CPZ, chlorpromazine; MI, mitotic index; MMC, mitomycin C; PRI, proliferation rate index; SCE, sister chromatid exchange.
*$P < 0.01$ versus all lines.
**$P < 0.01$ versus lines 3 and 4.

Table 4 Effects of CPZ on the survival time and on the ascitic volume of mice inoculated with Ehrlich ascites tumor cells and treated with MMC

Treatment and dosage[a]	No. of mice	Ascitic volume (g)[b]	Survival time (days)[b]	Percentage increase in life span
Control	16	22.8 ± 2.0	20.0 ± 0.7	–
CPZ 2.5 μg/g bw	16	13.0 ± 2.2*	21.7 ± 1.0	+8.5
MMC 5 ng/g bw	16	18.4 ± 2.3	22.1 ± 0.9	+10.5
MMC 5 ng/g bw + CPZ 2.5 μg/g bw	16	8.1 ± 1.6*,**	24.5 ± 0.8*,*** (*EV* = 23.8)	+22.5

bw, body weight; CAF, caffeine; CPZ, chlorpromazine; ÅV, expected value if CPZ, CAF and MMC were acting independently and additively; MMC, mitomycin C.
[a]CPZ was injected intraperitoneally on the second, fourth, and sixth day after tumor inoculation, whereas MMC was injected intraperitoneally on the second day and 6 h after CPZ injection.
[b]Mean value ± SEM.
*$P < 0.01$ versus line 1.
**$P < 0.05$ versus line 3.
***$P < 0.05$ versus line 2.

could also explain the differences in the correlations found in Tables 1 and 2. Furthermore, according to our findings, CPZ showed different cytogenetic effects in normal human lymphocytes and EAT cancer cells, which is of great importance. Similar findings have been reported in a study on cultured leukemic cells and normal human lymphocytes [26].

CPZ dose levels used for our in-vivo experiments were lower or in agreement with previous reports [27,30,31]. Administration of 5 μg/g body weight CPZ alone elicited a significant SCE induction ($P < 0.01$) (Table 3). Genotoxic effects were also induced in EAT cells, cotreated with CPZ and MMC ($P < 0.01$). Furthermore, the presence of CPZ in EAT cells treated with MMC *in vivo*, caused statistically significant cell cycle delays and MI suppression ($P < 0.01$). A positive correlation was found between PRI and MI ($r = +0.98$, $P < 0.01$).

As illustrated in Table 4, CPZ alone had a slight nonsignificant effect on the survival time of mice inoculated with EAT cells, whereas it significantly reduced the ascitic volume ($P < 0.01$). In the presence of MMC, a nonsignificant partial inhibition of tumor growth and an increase of survival, in comparison with control, were observed. Finally, in mice treated with MMC plus CPZ, a significant enhancement of survival time ($P < 0.01$ compared with control, and $P < 0.05$ compared with CPZ treatment alone) was accompanied by a significant inhibition of tumor growth ($P < 0.01$ compared with control, and $P < 0.05$ compared with MMC treatment alone). Zhelev *et al.* [26] established that phenothiazines possessed not only cytotoxic but also antiproliferative activity against leukemic cells. According to our findings, these two activities are also positively correlated. In a study designed to evaluate the potential chemopreventive and antitumor effect of phenothiazines *in vivo*, Azuine *et al.* [32] reported that several phenothiazines effectively inhibited tumor growth in mouse cancer models, which comes into agreement with our results.

The effectiveness in SCE induction by antitumor agents *in vitro* can be correlated with the in-vivo tumor response to these agents. The SCE assay can be used to predict both the sensitivity of human tumor cells to therapeutic agents and the heterogeneity of drug sensitivity within individual tumors [20,23,24]. As unrepaired DNA damage was expressed in SCEs in EAT cells *in vivo*, this reflects unrepaired DNA damage in human cancer cells, because both cell types have similar DNA repair mechanisms.

Apart from the antiproliferative and cytotoxic effects of phenothiazines, the effect of CPZ and phenothiazines, in general, on the activities of the antioxidant enzymes have been established [33,34]. This means that CPZ is

expected to exhibit a cytoprotective activity in the presence of prooxidants *in vivo* or *in vitro*, by reducing the degree of DNA damage caused by such agents through its ability to stimulate antioxidant enzyme activity [34]. In contrast, cancer cells show higher resistance to reactive oxygen species (ROS) than normal cells, because of adaptations in their antioxidant defence system [26]. Moreover, MMC used in this study is not a prooxidant, explaining the differentiation in the activity of CPZ when combined with this agent. Nevertheless, it was found that CPZ, alone, had a genotoxic effect only in EAT cells, which was followed by tumor growth inhibition. Phenothiazines inhibit Ca^{2+}-calmodulin binding proteins, which associate with DNA polymerase isoenzymes, thus suppressing DNA synthesis [35,36]. The efficacy of phenothiazines in suppressing cell growth in cancer cells has also been attributed to their ability to inhibit the P-glycoprotein efflux transporter [11].

In the quest of reasoning the antiproliferative and anti-apoptotic activities, of phenothiazines and, thus, CPZ researchers have suggested a phenothiazine-dependent induction of apoptosis in leukemic cells through a 'nonclassical' pathway, including suppression of mitochondrial homeostasis [26]. It has been reported that phenothiazines are localized predominantly in the mitochondria of normal and cancer cells [37,38]. The continued consumption of oxygen by mitochondria routinely leads to the generation of different types of ROS [39]. It has been established that ROS lead to SCE level induction, unless properly eradicated through cellular antioxidant mechanisms. This indirect increase in ROS by phenothiazines could explain the genotoxicity observed in EAT cells. This phenomenon might not be observed in normal human lymphocytes because of the fact that cellular antioxidant mechanisms work more efficiently in normal cells than in cancer cells [11].

Phenothiazines potentiate the cytotoxicity of conventional cancer chemotherapeutics [26,40,41]. They are thought to be effective in improving the action of certain antineoplastic compounds, such as melphalan and others [42,43], particularly in the presence of CAF [14,15,17]. In our in-vivo experiments, administration of CAF in combination with MMC and/or CPZ proved to be highly toxic.

CPZ-containing regimens are nowadays administered in cancer patients to manage the symptoms of agitation, delirium, psychosis, and nausea [11,44,45]. This study, in agreement with previous reports, illustrated that chromosomes or cells treated with CPZ are more sensitive to MMC in comparison with chromosomes or cells with no previous CPZ treatment. It is a basic study that places the foundation for further research to take place in this particular area. CPZ concentrations within the observed human plasma concentration range need to be tested along with antineoplastic agents *in vitro* for its synergistic action so as to evaluate a potential clinical application. The next step is to use other phenothiazines and antipsychotic drugs as well as other antineoplastic agents to study which combination is most effective. Apart from that, other cancer models have to be studied, as well as other cancer cell lines to identify the most appropriate dosages, combinations, and the particular types of cancer where the combined administration is more effective. Further experimentation concerning nude mice and other biological systems, such as P388 lymphocyte leukemia cells, and L1210 lymphoid leukemia cells, is required.

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