Cytogenetic toxicity of cycloplatam in human lymphocytes: detection by the micronucleus test and fluorescence *in situ* hybridization

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Cycloplatam has been shown to be effective in the treatment of pleural mesothelioma, myeloma and ovarian carcinoma. Cycloplatam is not nephrotoxic with respect to the platinum-based anti-tumor agents. We have investigated the mechanism underlying the induction of micronuclei (MN) in human lymphocytes by cycloplatam compared to that by its parent drugs cisplatin and carboplatin. The cytokinesis-block micronucleus assay in human lymphocytes was applied in combination with fluorescence *in situ* hybridization (FISH) with an all-chromosome centromeric probe allowing discrimination between MN due to chromosomal fragments (centromere negative, C^-) and those containing whole chromosomes (centromere positive, C^+). A statistically significant increase of MN frequency ($P<0.001$) was detected for cisplatin, carboplatin and cycloplatam. However, cycloplatam was active at a much lower dose (0.1 μmol/l) than cisplatin or carboplatin (1 μmol/l). No significant increase in the frequency of C^+ or C^- MN was observed for cisplatin and carboplatin compared to the controls. A statistically significant ($P<0.001$) increase in the percentage of C^- MN was observed in cycloplatam-treated cells. The results obtained suggest different mechanisms for cytogenetic damage induced by platinum drugs. Cycloplatam induces one type of MN and it could be considered a clastogenic agent, whereas cisplatin and carboplatin appear to induce both chromosome breakage and numerical chromosomal abnormalities. *Anti-Cancer Drugs* 17:289–295 © 2006 Lippincott Williams & Wilkins.

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Introduction

Cycloplatam, a new platinum compound first synthesized in Russia [1], and patented in the UK, France, Italy, Switzerland, Finland, Japan and the USA, is effective in the treatment of pleural mesothelioma, myeloma, ovarian carcinoma and urinary bladder cancer [2], prostate cancer, and cervical carcinoma [3]. Cycloplatam is effective in the treatment of ovarian carcinoma as monotherapy in 60% of patients with stage III–IV disease [4], whereas cycloplatam–cyclophosphamide is almost as effective as cisplatin–cyclophosphamide, although less toxic [5]. Cycloplatam is not nephrotoxic with respect to the platinum-based anti-tumor agents due to the different dynamics of excretion from the kidney [6,7]. Experimental studies revealed that in-vitro cycloplatam showed a similar spectrum of anti-tumor activity as cisplatin [1,8,9]. In addition, studies in cisplatin-resistant cell lines showed a clear advantage for cycloplatam [8].

The mechanism of the anti-cancer properties of the platinum drugs is related to the cell cycle arrest they cause at the G_2 phase that can trigger apoptosis [10]. Cisplatin and carboplatin act similarly, causing a concomitant decrease in p53 mRNA and an increase in p53 protein level [11]. Cycloplatam induces apoptosis in a time- and dose-dependent manner, also revealing a higher efficiency with respect to cisplatin in human lymphoblastoid cell lines [12]. The anti-tumor activity of platinum drugs is mediated by interaction with DNA [13,14] through the formation of bifunctional adducts, crosslinking two adjacent guanines or adjacent adenine and guanine residues in the same DNA strand (*intra*-strand crosslinks) or in opposite strands (*inter*strand crosslinks). In addition to DNA binding, the platinum drugs interact with enzymes and proteins. DNA–protein crosslinks have been also described [15].

These reactions perturb the structure, transcription and replication of DNA, and result in clastogenic and aneugenic effects. The micronucleus (MN) test is a well-established assay for assessing chromosome damage in a wide number of experimental systems. The evidence of MN-induced activity of an anti-tumor drug suggests a potential risk for long-term survivors. The in-vitro MN

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test is currently being used as a screening assay in the early stages of pharmaceutical development [16]. The possibility has been discussed that the test could be used interchangeably with the in-vitro chromosomal aberration assay in the assessment of genotoxicity prior to investigational new drug submission of new pharmaceutical entities [17]. MN are formed from an acentric fragment or a whole chromosome that lags behind during cell division [18].

In a previous work we studied the MN-inducing effect of cytoplatam *in vivo* in murine bone marrow and in human lymphocyte cultures by the cytokinesis block assay [19,20]. Our results showed that cycloplatam is less toxic and MN inducing *in vivo* in murine bone marrow than cisplatin, but it produces a dose-dependent increase of MN in micronucleated binucleated (MNBN) cells beginning at very low doses [21].

The aim of the present study is the investigation of the mechanism underlying MN formation. We applied the cytokinesis block MN (CBMN) assay in its comprehensive mode [22,23] including markers of genotoxicity and cytotoxicity: MN as a marker of chromosomal breakage and or loss, nucleoplasmic bridges as a marker of chromosome rearrangement, nuclear buds as a marker of gene amplification, cellular necrosis and apoptosis.

In addition, the measurement of MN combined with fluorescence *in situ* hybridization (FISH) with a centromere-specific DNA probe allows discrimination between MN due to chromosomal fragments, as clastogenic damage, and MN containing whole chromosomes, as an aneugenic event, since the presence of a hybridization signal in a MN is a direct measure of the presence of a centromere [24–26].

Materials and methods

Drug preparation

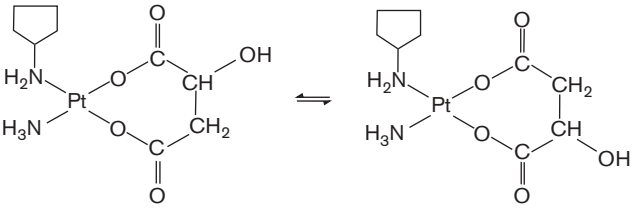
Cycloplatam [ammine(cyclopentylamine)-*S*-(–)-malatoplatinum(II)] (Fig. 1) was a kind gift from Dr L. Ayrapetyan (Department of Chemotherapy, Cancer Research Center, Yerevan, Armenia). Cisplatin and mitomycin C were obtained from Sigma (St Louis, Missouri, USA). Carboplatin was from Aldrich (Milwaukee, Wisconsin, USA).

Cytoplatam, cisplatin and carboplatin were dissolved in water immediately before use. Cytoplatam has a unique water solubility (more than 1 g/ml).

Cell culture and treatment

Venous blood samples from two healthy donors (male and female) were collected in sodium heparin tubes. Duplicate cultures were set up for each experimental point. Whole blood (0.4 ml) was added to 4.5 ml complete medium RPMI 1640 and 10% FCS (Gibco/BRL Life Technologies, Milan, Italy) with phytohemagglutinin 1% (Murex Biotech, Dartford, UK).

Fig. 1



Chemical structure of cycloplatam.

The drugs were tested at equitoxic doses. The final concentrations tested were: cycloplatam 0.5, 1.0 and 5.0 μmol/l, cisplatin 1.0, 2.0 and 2.5 μmol/l, and carboplatin 1.0, 10.0 and 20.0 μmol/l. The compounds were added to the cultures after 24 h. Mitomycin C was used as a positive control (0.12 μmol/l).

Lymphocytes were cultured for 72 h and cytochalasin B was added at 44 h to block cytokinesis. At the end of incubation, cells were harvested and treated with hypotonic solution (0.075 mol/l KCl). The cells were then fixed in methanol and acetic acid 3:1 for 30 min.

Microscope slides were stained with Giemsa 5% for 10 min or hybridized within 1 week of preparation.

FISH

Centromeric FISH was performed using an alphoid centromere-specific biotinylated probe for all centromeres (P5095-B.5; Oncor, Gaithersburg, Maryland, USA). This probe was previously tested on metaphase chromosomes for centromere-specific labeling.

The slides were allowed to age for at least 3 days and treated with 10% pepsin (Sigma) in HCl 10 mmol/l for 10 min at 37°C. The slides were then washed briefly in distilled water and PBS, and post-fixed for 10 min at room temperature with 1% formaldehyde (Prolabo, Fontenay s/Bois, France) in PBS. They were then washed with PBS and dehydrated in an 80–90–95% ethanol series. DNA denaturation was performed in 70% formamide (Sigma) in 2 × saline/sodium citrate buffer (SSC) at 70°C for 2 min and dehydrated in an ethanol series of increasing concentration.

The hybridization mixture containing the probe (2.5 μg/ml) and 500 μg/ml salmon sperm DNA (salmon testes DNA; Sigma) in 2 × SSC was denatured at 70°C for 5 min, followed by chilling on ice for 4 min. An aliquot of 50 μg per slide was

applied to the slides which were then covered with coverslips sealed with rubber cement.

Hybridization was performed for 16 h at 37°C in a moist chamber. After the incubation the slides were washed 2 times in 2 × SSC for 4 min and then in Tween-20 (Sigma) buffer for 5 min. The slides were then incubated with the blocking reagent (5% skimmed milk in 4 × SSC) at 37°C for 10 min. The slides were washed with 4 × SSC, covered with a 1:250 dilution of anti-biotin antibody (Sigma) in Immunological Buffer (0.5% skimmed milk in 4 × SSC) and incubated at 37°C for 30 min. After a wash in Tween-20, the slides were incubated in a 1:20 dilution of FITC-conjugated anti-mouse antibody (Boehringer Mannheim, Mannheim, Germany), followed by incubation with a 1:20 dilution of FITC-conjugated anti-sheep antibody (Sigma) for 30 min at 37°C. All the incubations were performed in a moist chamber and were followed by washes in Tween-20 buffer. After the last wash the slides were dehydrated with an increasing series of ethanol and then stained with propidium iodide (Sigma) 5 μg/ml in DABCO anti-fade solution (Sigma).

Slide scoring and statistical analysis

Giemsa-stained slides were coded and scored blind under a magnification of × 400. In total, 2000 binucleated lymphocytes (1000 per slide) were scored from each experimental point. The MN frequency was calculated as the number of MNBN cells; 500 lymphocytes were scored to determine the cells with one, two or more than two nuclei. The identification of MN and other nuclear abnormalities was performed according to the criteria described by Fenech *et al.* [22]. The nuclear division index (NDI), as a measure of cell cycle delay, was calculated according to the formula: $NDI = M_1 + 2M_2 + 3M_{multi}/n$, where M_1 to M_{multi} represent the number of cells with one to multiple (more than 2) nuclei and n is the number of cells scored.

For FISH analysis the slides were scored with a Leica DMRXA microscope with fluorescence equipment. The MN present in the binucleated lymphocytes with intact cytoplasm were examined for the presence of one or more centromeric spots and were classified as centromere positive (C^+) or centromere negative (C^-).

A total of at least 1000 binucleated lymphocytes were scored for each experimental point. Statistical analysis was performed by using the Mann–Whitney *U*-test and the χ^2-test.

Results

Table 1 and Fig. 2(a and b) show the frequency of MN and other nuclear abnormalities, associated with parameters for cell cycle delay and cytotoxicity in binucleated lymphocytes following the exposure of whole-blood cultures from healthy donors to different concentrations of cycloplatam, cisplatin and carboplatin. A statistically significant increase of MN for each platinum drug as compared with the control level was detected ($P < 0.001$) at all concentrations tested.

Cycloplatam was active at very low doses (0.1 μmol/l of cytoplatam induced 32.4 and 32.5 MNBN/1000 BN lymphocytes in donor 1 and 2, respectively). The highest concentration led to a 10-fold increase in the frequency of MNBN cells over the control.

The genotoxic activity of cisplatin and carboplatin is lower. Cisplatin and carboplatin treatment of whole blood from both the donors (male and female) induced a statistically significant increase in the MN frequency; a dose–response effect was less evident.

Mitomycin C as a positive control induced a high frequency of MN (more than 10 times the control level), confirming the results from the scientific literature.

Statistically significant differences in the spontaneous or mitomycin C-induced MN frequency between the two subjects were not observed. In contrast, MN frequencies caused by treatment with platinum drugs showed significant differences (up to $P < 0.05$), reflecting individual sensitivities of the lymphocytes from the two subjects to the applied drugs.

A significant increase of nucleoplasmic bridges and nuclear buds was observed for specific concentrations of cycloplatam only in subject 1. Concerning genotoxicity, in terms of cell cycle delay, both the NDI and percentage of binucleated cells were considered. A parallel decrease of both parameters was detected only in cycloplatam-treated lymphocytes, showing a negligible difference between the two donors. A cytotoxicity effect, revealed as an increase in the number of necrotic cells, was observed in cisplatin- and, to a higher extent, in carboplatin-treated lymphocytes.

Table 2 shows the characterization of MN after FISH with a pan-centromeric DNA probe. A comparable level of C^+ and C^- MN was observed for the control culture from both subjects. Mitomycin C, as a positive control for clastogenic agents, induced a significant increase in C^- MN (70% C^- MN versus 50% for the controls) in blood culture from both subjects, confirming the data from the scientific literature [27,28]. No statistically significant difference in the frequency of C^+ and C^- MN was observed for the platinum drugs tested, i.e. cisplatin and carboplatin, compared with the controls, with the exception of the highest dose for carboplatin. The statistically significant increase of C^- MN observed in carboplatin-treated cells from both donors could probably be attributed to indirect effects mediated by toxicity at

Table 1 Frequency of MNBN, NDI and nuclear abnormalities after treatment with cycloplatam, cisplatin and carboplatin

	Dose (μmol/l)	BN scored	BN (%)	NDI	MNBN	MNBN/1000 BN	Nuclear abnormalities/1000 cells			
							Nucleoplasmic bridges	Nuclear buds	Necrosis	Apoptosis
Donor 1 (male)										
control		1500	50.1	1.69	8	5.30	1	0	3	0
mitomycin C	0.12	1750	42.8	1.47	129	73.60[a]	1	2	5	2
cycloplatam	0.1	1050	24.4[a]	1.38[a]	34	32.40[a]	0	3[a]	6	1
	0.5	2300	12.9[a]	1.19[a]	77	33.40[a]	1	1	4	0
	1.0	1824	15.9[a]	1.18[a]	54	29.60[a]	3[a]	7[a]	3	0
	2.5	1200	21.0[a]	1.24[a]	40	32.00	2[a]	4[a]	3	0
	5.0	3196	19.3[a]	1.22[a]	191	59.76[a]	3[a]	6[a]	4	0
cisplatin	1.0	2000	46.7	1.60	48	24.00[a]	0	1	5	0
	2.0	1200	35.7[a]	1.48	29	24.20[a]	1	4[a]	6	0
	2.5	1300	48.3	1.60	42	32.30[a]	0	2	11	0
carboplatin	1.0	1200	44.2	1.58	29	24.10[a]	1	1	4	0
	10.0	2070	22.7[a]	1.30[a]	71	34.20[a]	1	4[a]	8	4
	20.0	2500	24.5[a]	1.34[a]	93	37.20[a]	1	2.5	5.5	2.5
Donor 2 (female)										
control		2200	38.8	1.49	14	6.40	0	0	4	0
mitomycin C	0.12	1500	32.6	1.36[a]	106	70.60[a]	2[a]	2[a]	7	3
cycloplatam	0.1	1200	41.6	1.60	39	32.50[a]	0	0	5	0
	0.5	1500	17.8[a]	1.24[a]	59	39.50[a]	0	2[a]	4	0
	1.0	1500	16.9[a]	1.26[a]	42	27.80[a]	0	0	7	1
	2.5	1200	27.6[a]	1.39[a]	41	34.20[a]	0	1	6	0.5
	5.0	3000	25.4[a]	1.32[a]	162	54.00[a]	1	3[a]	3	0.5
cisplatin	1.0	1020	50.6	1.70	20	19.60[a]	0	1	6	3
	2.0	1200	37.1	1.54	23	19.00[a]	3[a]	0	7	3
	2.5	1650	37.4	1.53	25	15.30[a]	0	2[a]	11	0
carboplatin	1.0	1000	40.6	1.42	21	21.00[a]	0	3[a]	3	0
	10.0	1000	42.4	1.48	42	42.00[a]	1	2[a]	2	0
	20.0	1287	12.1[a]	1.13[a]	55	42.74[a]	3.9[a]	0.8	10	0.8

[a]$P<0.001$ compared with negative control (Mann–Whitney test).

Table 2 Characterization of MN after FISH with a pan-centromeric DNA probe in binucleated lymphocytes treated with cycloplatam, cisplatin and carboplatin

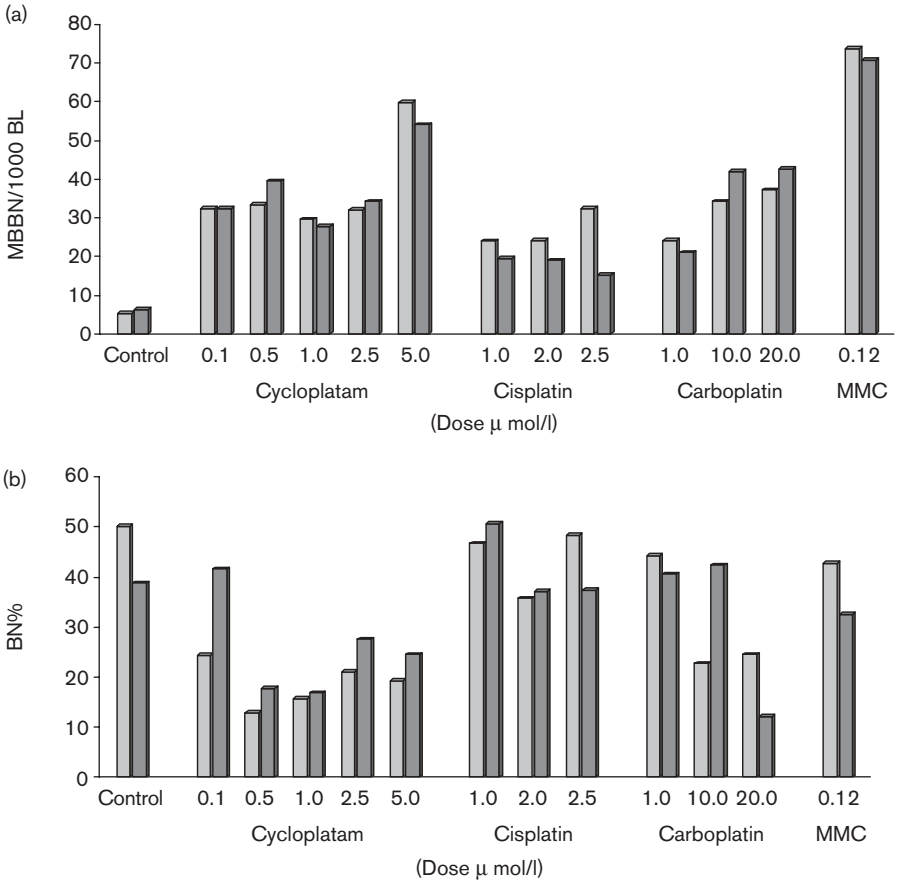
	Dose (μmol/l)	C^+MN (× 1000 cells)	C^-MN (× 1000 cells)	C^-MN (%)	P (χ^2)
Donor 1 (male)					
control		4	4	50.0	
mitomycin C	0.12	40	89	69.0	<0.001
cycloplatam	0.5	12	27	69.2	<0.001
	1	6	21	77.8	<0.001
	5	13.5	36.2	72.4	<0.001
cisplatin	1	29	20	40.9	NS
	2	18	13	41.9	NS
	2.5	27	21	43.8	NS
carboplatin	1	13	15	53.6	NS
	10	14	23.5	62.7	<0.001
	20	22	25	53.7	NS
Donor 2 (female)					
control		6	8	57.2	
mitomycin C	0.12	32	77	70.0	<0.001
cycloplatam	0.1	6	32	84.2	<0.001
	0.5	8	33	79.5	<0.001
	1	7	29	80.5	<0.001
	5	6.5	56.5	89.8	<0.001
cisplatin	1	8	12	60.0	NS
	2	9	18	68.0	NS
	2.5	11	16	60.0	NS
carboplatin	1	7	16	70.0	<0.001
	10	12	19	60.0	0.05
	20	toxic	–	–	–

the highest tested dose (20 μmol/l) of carboplatin; such toxicity prevented MN evaluation in donor 2. However, a statistically significant dose-dependent increase in C^- MN was observed in cycloplatam-treated cells, whereas C^+ MN remained almost unchanged, suggesting a clastogenic mechanism of action.

Discussion

The binding of platinum drugs to DNA is considered the critical step for their anti-tumor activity, but it is indicative also of a genotoxic hazard to the host. Cisplatin has been shown to be mutagenic in *in vitro* mutagenicity tests and carcinogenic in in-vivo models

Fig. 2



Frequency of MNBN/1000 binucleated lymphocytes from two donors before and after in-vitro treatment with cycloplatam, cisplatin and carboplatin, and mitomycin C as positive control.

[29]. Cytogenetic effects of cisplatin have been demonstrated in different experimental systems *in vivo* and *in vitro*. Significant increases of chromosomal aberrations, sister chromatid exchanges (SCEs) or MN frequencies have been observed in CHO cells [30,31] as well as in human lymphocytes [32]. The drug was also found to be highly clastogenic in bone marrow cells of mice [33,34] and rats [35].

Cisplatin chemotherapy resulted in a dose-related increase of chromosomal damage evaluated as MN frequency in peripheral blood lymphocytes of cancer patients. The increase in MN frequency in human lymphocytes after chemotherapy cycles correlated significantly with the cumulative dose of cisplatin [36]. In addition, increased aneuploidy has been detected in spermatozoa from testicular cancer patients after chemotherapy with cisplatin [37].

A number of analogs of cisplatin have been developed, and some of these, such as carboplatin, seem to be less toxic and mutagenic than the parent compound [38–40]. Cycloplatam is less cytotoxic and genotoxic in bacterial systems [38]. It shows less deleterious effects than cisplatin in patients. The ability of carboplatin to induce SCEs and chromosomal aberrations in peripheral lymphocytes after drug administration is weaker than that of cisplatin at the same peak plasma concentrations [39].

The evidence from the present study confirms the genotoxic hazard of the platinum drugs. Both cisplatin and carboplatin induced a statistical increase of chromosomal effects in peripheral blood lymphocytes, although cytotoxicity and MN induction by carboplatin were lower than with cisplatin, confirming the evidence from literature.

Cycloplatam is more genotoxic and less cytotoxic to human cells *in vitro* than cisplatin and carboplatin. It was active at a very low dose (0.1 μmol/l), inducing a 10 times increase of MN frequency with respect to the control level at a concentration of 5 μmol/l.

The MN assay combined with FISH with a pan-centromeric probe reveals the nature of the chromosomal damage. Our results show that the clastogenic compound mitomycin C, as a positive control, induced 69% C^- MN at 0.12 μmol/l, confirming the data from the literature.

Neither cisplatin nor carboplatin seem to induce a specific increase of C^+ or C^- MN. This evidence does not allow us to hypothesize about a prevalent mechanism of action.

Compared to the control level, cycloplatam induced a significant percentage of MN with no hybridization signal. The percentage of C^- MN increased in a dose-dependent fashion from 69 to 77 and 84 to 89% in donor 1 and 2, respectively, suggesting the induction of structural chromosomal aberrations.

The different mechanisms of the genotoxic action of these compounds could be related to a number of factors, including DNA binding and alteration, and the mechanisms and efficiency of the DNA repair pathway. All the platinum drugs initially bind in a monofunctional manner and this step occurs much faster for cisplatin than for carboplatin. The slow binding of carboplatin to DNA was observed in cultured cells *in vivo* and confirmed by *ex vivo* biomonitoring studies in buccal cells from patients [41,42].

The different kinetics of the interaction of platinum drugs with DNA could be associated with the nature of the leaving groups. The didentate ligand cyclobutyldicarboxylate in carboplatin instead of the two chlorides makes the compound more resistant to aquation and therefore less reactive toward DNA than cisplatin [41]. The mechanism of interaction of cycloplatam with DNA carrying an aminocyclopentylamine group and existing in equilibrium between two isomeric forms is still unknown.

Direct and indirect interaction of platinum drugs with proteins and enzymes contributes to the complex mechanism of apoptosis involved in the anti-tumor effect. The level and strength of the protein interaction and DNA–protein crosslinks are responsible for impairing DNA replication and for the dysfunction of the mitotic apparatus leading to aneugenic damage.

The present evidence relating to the different origin of MN induced by platinum drugs contributes to the understanding of mechanisms involved in their anti-tumor activity and might be helpful in predicting any genotoxic hazard in cancer patients.

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