

THE EFFECT OF PACLITAXEL ALONE AND IN COMBINATION WITH CYCLOHEXIMIDE ON THE FREQUENCY OF PREMATURE CENTROMERE DIVISION *IN VITRO*

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Abstract – Premature centromere division (PCD) can be viewed as a manifestation of chromosome instability. In order to evaluate the ability of Paclitaxel (Ptx) and Cycloheximide (Cy) to induce PCD we used a cytokinesis block micronucleus assay (CBMN), fluorescent *in situ* hybridization (FISH), and the chromosome aberration (CA) assay in human peripheral blood lymphocytes. Results showed that Ptx can induce PCD alone or in combination with Cy. These findings call us to pay more attention to PCD as a parameter of genotoxicity in the pre-clinical research of mono- and/or combinational therapies for cancer treatment.

Key words: Paclitaxel (Ptx), premature centromere division (PCD), cycloheximide; cytokinesis block micronucleus assay (CBMN), fluorescent *in situ* hybridization (FISH).

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INTRODUCTION

In a four dimensional cascade of cyclin regulated cell cycle kinetics, chromosomes possess an essential role in maintaining the stability of the fluctuating genome. Numerous data show that the spatial organization of chromosomes is accompanied by the temporal regulation of replication (Amiel et al., 1998; Litmanovitch et al., 1998; Reish et al., 2002) and the separation and segregation of these processes in dividing cells (Garcia-Orad et al., 2000; Mailhes et al., 2003; 2008). Centromere separation is a sequential/temporal process in which certain chromosomes tend to separate first. In humans the first chromosome to separate is chromosome 18. In an ordered sequence, chromosomes 17, 2, 10, and 12 follow the separation of chromosome 18 (Vig et al., 1989; Spremo-Potparevic and Bajic, 2000; Bajic et al., 2007).

This sequence of chromosome separation can be deregulated in ageing cells (Fitzgerald et al.,

1986; Zivkovic et al., 2006), Alzheimer's disease (Miglione et al., 1997; Spremo-Potparevic et al., 2004; 2008; Zivkovic et al., 2006; Bajic et al., 2008) various tumors and chromosome instability syndromes (Choo, 1997). Recent investigations have found that premature centromere division (PCD) yields are significantly higher in populations exposed to mixed chemicals, pesticides, crude oils and cytostatic drugs (Mailhes et al., 1999; Major et al., 1999; Vig and Hallet, 2000; Rodriguez et al., 2001; Bajic et al., 2007, 2009). Centromere instability has been recognized as a hallmark of human cancer caused by continuous mis-segregation during mitosis in which errors of centromere separation, particularly PCD, is seen to have a fundamental role (Vig and Sternes, 1991; Vig et al., 1993). PCD is today a well-recognized phenomenon associated with aneuploidy (Miglione et al., 1997; Mailhes et al., 1998; 2003, 2008; Vig and Hallet, 2000; Steuerwald et al., 2005; Tomonaga et al., 2005; Zivkovic et al., 2006).

The use of chemotherapy has become more extensive in recent years and has added to the success of treatments, but it has also permitted the assessment of survivors for the development of second malignant tumors (Pedersen-Bjergaard and Philip, 1988; Leone et al., 1999; Green et al., 2000).

Paclitaxel (Ptx) alone or in combination with other antitumor agents is widely used in a variety of tumor treatments, however, Ptx can induce chromosome damage and aneuploidy, thereby enhancing the possibility of survival of damaged cells (Bouchet et al., 2007). A number of studies have addressed the ability of Ptx to induce aneuploidy *in vivo* (Tinwell and Ashby, 1994; Mailhes et al., 1999) and *in vitro* (Jagetia and Adiga, 1995; Bouchet et al., 2007).

Tinwell and Ashby (1994) presented that Taxol (Taxol is the generic name for paclitaxel) induced an increase in the frequency of micronuclei (MN) in the mouse bone-marrow micronucleus assay. This data may indicate a carcinogenic potential of taxol for humans. Taxol showed a positive Comet test (single-strand breaks) on proliferating lymphocytes at doses of 5 and 7.5 nM but not in resting cells, even at 5 to 15 μ M (Digue et al., 1999). Also, Taxol may suppress centromere dynamics in osteosarcoma cells (Kelling et al., 2003).

Cycloheximide (Cy) does not exhibit a genotoxic potential in either the Comet assay (Henderson et al., 1998) or in the micronucleus assay (Bajic et al., 2007) *in vitro*, however it can induce centromere instability (Bajic et al., 2007), or premature centromere division. In this study we addressed the potential of Ptx to induce PCD, alone and with Cycloheximide, a known protein inhibitor, as well as the possible relationship of induced PCD to Ptx clastogenicity/genotoxicity.

Fluorescent *in situ* hybridization (FISH) was used in correlation with the cytokinesis block micronucleus assay (CBMN). CBMN is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity and is used as a reliable endpoint in the evaluation of the genotoxic

risk of various cytotoxic agents and xenobiotics (Bonassi et al., 2007; Fenech, 2007; Djelic et al., 2008). Detection of chromosome 18 was chosen because it is the first chromosome to separate, and thus segregate in the metaphase anaphase transition (Rodriguez et al., 2001; Steuerwald et al., 2005). The most prominent finding was that Cy induced a change in the pattern of PCD occurrence by Ptx which correlated with a significantly higher NDI suggesting the survival of cells that clearly show centromere instability. FISH for the α -centromeric probe of chromosome 18 detected dot-like signals in mononucleated, bi-nuclear and multilobulated nuclei induced by Ptx which clearly expresses PCD. Here we argue that increased levels of chromosomes affected by PCD induced by Ptx alone or in combination may lead to increased genome instability in normal peripheral blood lymphocytes.

MATERIAL AND METHODS

Material

For all experiments the material used was obtained from the human peripheral blood of ten healthy male subjects, 25-35 years of age who were non-smokers. Cycloheximide (CAS No. 66-81-9) and Paclitaxel (Taxol®) (CAS No. 33069-62-4), were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA.

Protocol for Cy combination with Ptx

Peripheral blood lymphocytes were exposed to Cy in a dose of 10 μ g/ml for an 18 h incubation time without adding phytohemagglutinin (PHA) (G_0 -phase). After 18 h of incubation with Cy, the Cy was rinsed out, phytohemagglutinin was added and after 48 h increasing doses of Ptx were added for 24 h. The same protocol was used for both the chromosome aberration (CA) assay and CBMN.

The CBMN

CBMN *in vitro* was done as described by Fenech (2000). Peripheral blood samples from 10 male subjects were collected in previously heparinized test tubes. Cells from lymphocyte rich plasma were

cultivated in a density of 5×10^5 cells/ml in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS) and a standard solution of antibiotics (penicillin and streptomycin, Galenika, Belgrade). Three groups were established. One of them was the control group or phosphate buffered saline (PBS)-treated group. The other two were the experimental group with cells exposed to Ptx alone and the experimental group with a combination of Cy and Ptx.

All groups were incubated for 18 h before adding PHA. After incubation the cells were rinsed and the remaining Cy was washed off with RPMI 1640. The lymphocytes of all the groups were stimulated by adding PHA (Murex Diagnostics Ltd, Dartford, England) at a final concentration of 5 $\mu\text{g/ml}$. 48 h after PHA stimulation, Ptx was supplemented to the cultures in a dose of 10, 50 and 200 nM. 44 h after PHA stimulation Cytochalasin B was added at a concentration of 6 $\mu\text{g/ml}$. After 24 h the cells were centrifuged for 10 min at 2000 rpm, treated with a hypotonic solution (0.56% KCl plus 0.9% NaCl mixed in equal volumes) and then fixed with the conventional 3:1 methanol:acetic acid fixative for 10 min. Cell suspensions after the last fixation were dropped onto clean grease-free slides. After air drying (24 h) the cells were stained with 2% Giemsa (Merck, Darmstadt, Germany) for 3 min. The blood samples were coded with an identification number and the slides were scored blindly. For MN analysis, at least 1000 binucleated (BN) cells were analyzed per culture and MN was accepted according to the criteria of Fenech (2000). The ability of the cells to proliferate *in vitro* was evaluated by counting the number of cells with one, two and four MN on the same slide using the nuclear division index (NDI), calculated according to the formula: $\text{NDI} = (M_I + 2M_{II} + 3M_{III} + 4M_{IV}) / N$, where M_I to M_{IV} represent the number of cells with one to four nuclei, respectively, and N is the number of cells scored.

The chromosome aberrations (CA) assay for the analysis of PCD

For the CA/PCD analysis, the cultures were processed in the same way as in the CBMN except

that the hypotonic treatment was performed with pre-warmed 0.075 M KCl for 15 min at 37°C. During the last two hours of incubation colcemid (Ciba, Basel, Switzerland) was added to the medium in a concentration of 0.05 $\mu\text{g/ml}$. The air dried slides were stained with 10% Giemsa at pH=6.8. Metaphase chromosome analysis for the detection of PCD was performed according to the conventional technique by Evans and O'Riordan (1975). Metaphase nuclei were screened for the presence of altered centromere dynamics, i.e. PCD. PCD was evaluated in 100 metaphase chromosomes based on the distribution pattern, i.e. presence of PCD on less than two chromosomes, more than two and on all chromosomes *per one* metaphase.

Fluorescent in situ hybridization (FISH) for chromosome 18

Probe preparation

Chromosomes were prepared in the same way as in the experiment for the CBMN and CA assay. The pL 1.84a repetitive DNA probe (Oncor) for the centromeric region of chromosome 18 was used. The probe was labeled with biotin-16 dUTP nucleoside-triphosphate in nick-translation reaction. For each slide approximately 50 ng of probe was mixed in 16 ml of hybridization buffer, consisting of 50% formamide, 10% dextran-sulfate, 1% SDS, 1 x Denhardt's solution, and 2 x SSC (1 x SSC:0.15 M NaCl and 0.15 M sodium citrate, pH 7.0).

Hybridization and detection

Hybridization and detection were performed as previously described (Wilkinson, 1995). The biotinylated probe was detected with avidin-DCS conjugated to yellow-green fluorophore FITC (fluorescein isothiocyanate; exc.max. 523 nm) (4 mg/ml) and biotinylated anti-avidin DCS (4 mg/ml, Vector Laboratories). The signals were amplified using three layers of avidin-FITC. The slides were mounted in 0.4 mg/ml 40-6-diamidino-2-phenylindole (DAPI) and propidium iodide counterstain in vectashield antifade buffer. The

slides were examined by using an Olympus RFL BH2 fluorescence microscope with an appropriate filter combination for detecting fluorescein, and propidium iodide (0-515 nm) was used for signal detection and evaluation. The bipartite centromere signal of chromosome 18 in interphase nuclei was evaluated as PCD, 18+ while the one dot-like signal was evaluated as PCD, 18-. The slides were photographed using Fuji 100 color film.

Statistical analysis

For statistical analysis we used the Mann-Whitney U-rank test performed by Windows compatible

program Statistica 5.0 for establishing the frequency of MN and the χ^2 -test for estimating the NDI. A p-value of ≤ 0.05 was considered as indicative of statistical significance for all tests used.

RESULTS

Our results show that Ptx can induce PCD in human peripheral blood lymphocytes alone or in combination with Cy. For PCD analysis we used the CA assay. Our results show that when compared to the control group, Ptx alone and in combination with Cy can induce a significant ($p < 0.001$)

Table 1. Comparative estimation of PCD in control and experimental groups treated with Cy and Ptx

Concentration	No of analyzed mitosis		PCD induced on less than two chromosomes		PCD induced on more than two chromosomes		PCD induced on all chromosomes		The overall number of mitosis with PCD	
	mean±S D	(%)	mean±S D	(%)	mean±S D	(%)	mean±S D	(%)	mean±S D	(%)
Negative control (K)	121 ± 9.7	100	/	/	/	/	2.0 ± 1.4	1.6	2.0 ± 1.4	1.6
10 nM Ptx (A)	123 ± 4.5	100	4.9 ± 1.9	4.0	65.3 ± 3.0	53.8	9.0 ± 1.2	7.3	79.2 ± 4.0	64.3
50 nM Ptx (B)	115 ± 9.5	100	1.3 ± 0.7	1.1	68.5 ± 2.3	59.5	15.0 ± 2.3	13.0	84.8 ± 3.0	73.6
200 nM Ptx (C)	98 ± 10.3	100	0.2 ± 0.4	0.0	35.4 ± 2.0	36.0	40.5 ± 3.7	41.3	75.9 ± 4.0	77.5
Cy/10 nM Ptx (D)	119 ± 7.7	100	4.0 ± 1.3	3.3	71.5 ± 3.2	60.0	16.9 ± 2.7	14.2	88.8 ± 4.0	75.0
Cy/50 nM Ptx (E)	122 ± 4.3	100	0.9 ± 0.5	0.7	68.5 ± 2.7	56.1	40.6 ± 2.6	33.0	110.0 ± 3.0	90.0
Cy/200 nM Ptx (F)	107±8.5	100	/	/	31.3±2.8	29.2	61.5±2.5	57.4	91.8±5.0	87.0

K:A=86.7**; K:B=151***; K:C=83**; K:D=96***; K:E=200***; K:F=70***; K:CX=29***

A:B=5***; B:C=7***; A:C=2,52*; D:E=10***; D:F=2.04 (NS)

E:F=5.4***; A:D=6.6***; B:E=25***; C:F=10***

df=38; $t_{0,05}=2,04$; $t_{0,01}=2,55$; $t_{0,001}=3,64$

NS=non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test for statistical analysis of the overall number of mitosis with PCD)

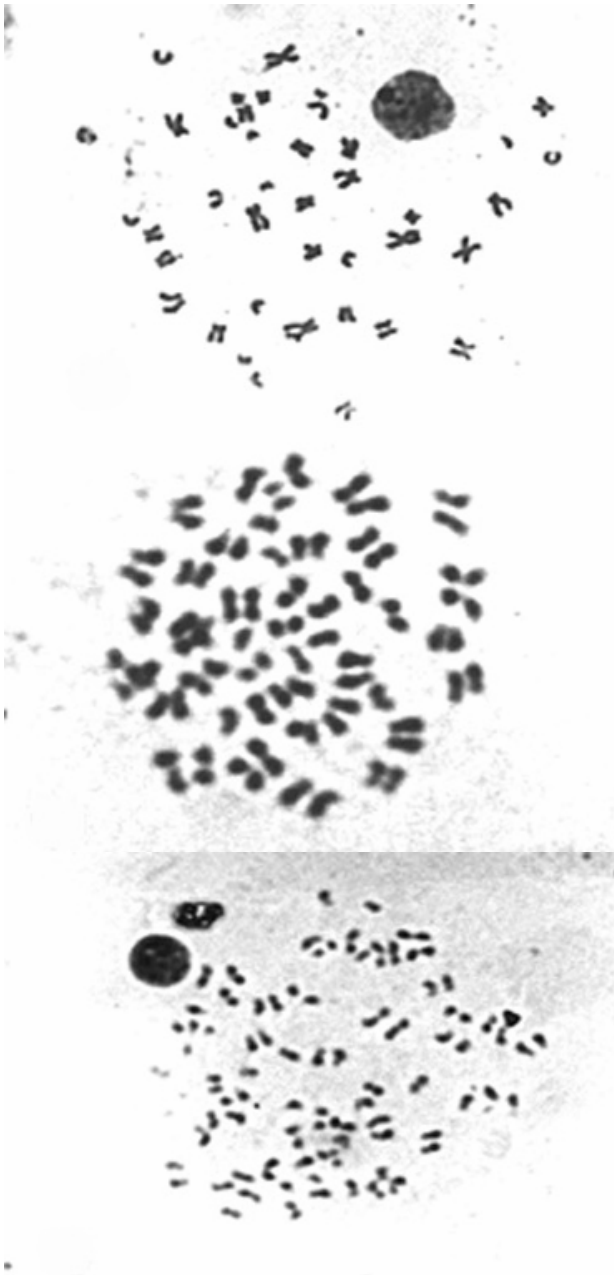


Figure 1. Metaphase nuclei showing increased occurrence of premature centromere division (PCD) and chromosome condensation induced by increasing doses of Paclitaxel (Ptx). Paclitaxel (Ptx) dose 10nM (a); Ptx dose 50nM (b); Ptx dose 200nM (c). From Fig. 1a to Fig. 1c we see increased instability of chromosomes. With increasing doses of Ptx, premature centromere division has shifted from a few chromosome that are affected (a) to all chromosomes (b & c). Also, we can see that chromosome condensation is more profound in higher doses.

increase in the overall percentages of PCD in all given doses (Table 1). When comparing experimental groups, Ptx alone and in a combination of Cy and Ptx, an interesting pattern is revealed, i.e. there is a significant ($p < 0.001$) increase in the overall percentages of PCD and a shift in the distribution of PCD occurrence. In the Ptx alone group, at a dose of 50 nM, the percentage of PCD occurring on all chromosomes was shifted from 13% to 33% in the group that was previously treated with Cy. This shift is seen in all respective doses with Ptx (Table 1, Figure 1). Here we see that incubation of cells with Cy in combination with Ptx induces a chromosome imbalance by expressing an increasing level of PCD with increasing doses of Ptx.

The same microscope slides used for observing micronuclei were also examined for cytotoxic and cell-cycle delay effects. The cytostatic effects of Ptx alone or in combination with Cy in the CBMN test were measured via the proportion of mono-, bi- and multinucleated (three and four nucleated) cells, i.e. the nuclear division index. The NDI shows the proliferative capability of peripheral blood lymphocytes. Comparing the doses in the experimental group with Ptx alone and the combinational group with increasing doses of Ptx (10, 50 and 200 nM), an increase of the NDI (1.112, 1.078 and 1.065) was observed, showing an increase in the possible cytoprotective effect of Cy (Table 2). By using the χ^2 -test we can see that the proliferative index shows a significant ($p < 0.01$) difference between the experimental group with Cy (F) and the experimental group with Ptx alone in a dose of 200nM (C) indicating that a cytoprotective effect may be at hand (Table 2). Of interest is that the proportion of bi-nuclear cells in the experimental groups are decreased in all experimental doses compared to the control group (Table 2). This difference is highly statistically significant ($p < 0.01$). Comparing the NDI and % of BNC we can conclude that cells that survive the first division (% BNC) may follow an increase in second divisions seen as an increase in the NDI in the combination experimental group (Table 2).

Table 2. Number of MN in peripheral blood bi-nuclear lymphocytes in control and experimental groups

Concentration	Average		No. MN in 10 ³ BNC		NDI	FISH analysis 18* + MN/1000cells
	No. of cells analyzed	% of BNC				
			mean	SD		
Control (K)	1028	55.7	5	3.0	1.707	/
10 nM Ptx (A)	1024	8.9	12.5	5.0	1.100**	1
50 nM Ptx (B)	1000	5.6	20.3	5.3	1.063***	2
200 nM Ptx (C)	1013	4.2	27.8	8.4	1.039***	1
†Cy/10 nM Ptx (D)	1022	9.1	14.5	4.3	1.112***	/
†Cy/50 nM Ptx (E)	1000	5.9	21.7	5.1	1.078***	1 ^{NS}
†Cy/200 nM Ptx (F)	1008	4.3	28.8	9.2	1.065***	2 ^{NS}

[~]BNC=binucleated cells; MN=micronuclei; [§]nuclear division index, [†]cycloheximide(10µg/ml); 18*-chromosome 18 signal

K:A, U=9; p<0.0002***

A:B, U=14; p<0.00651**

A:D, U= 37; p<0.325 (NS)

K:B,U=000; p< 0.000157***

A:C, U=5.5; p< 0.00076***

B:E, U=42; p<0.57 (NS)

K:C, U=000; p< 0.00015***

B:C, U=24.5; p< 0.054*

C:F, U=48; p<0.87 (NS)

K:D, U=3.5; p< 0.0044***

D:E, U=15; p<0.0081***

K:CX, U=47; p<0.85 (NS)

K:E, U= 1.5; p<0.0002***

D:F, U=11; p<0.0031**

K:F, U=000; p< 0.000157***

E:F, U=27; p< 0.082*

*statistical difference (p<0.05)

p<0.01, *p<0.01, NS= non-significant (Mann-Whitney U-test for CBMN and X² test for NDI)

Table 2 shows a significant ($p < 0.001$) difference in the number of binuclear cells with micronuclei (MN) between the negative control group (5.0 ± 3.0) and both experimental groups, combination of Ptx and Cy and Ptx alone in the following doses: 10 nM (12.5 ± 5.0 ; 14.5 ± 4.3 , respectively); 0.05 µM (20.3 ± 5.3 ; 21.7 ± 5.1 , respectively) and 200 nM Ptx (27.8 ± 8.4 ; 28.8 ± 9.2 , respectively). These results show that all doses of

Ptx, whether alone or in combination, are genotoxic when compared to the control group. When comparing both experimental groups our results show that there is no statistically significant difference between any of the used doses (Table 2). These results show that there is no correlation between the increase of chromosomes affected by PCD and Ptx aneugenic potential alone or in combination with Cy (Table 2).

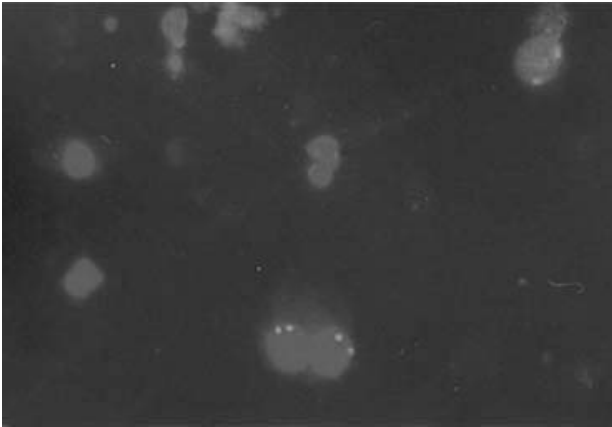


Figure 2. A representative binucleated (A) and mononucleated cell (B) with normal dot-like signals for chromosome 18.

The CBMN is readily used for FISH analysis. In our study we used FISH to detect if PCD occurs throughout the cell cycle, i.e. bi-nuclear cells that passed from the first division, and three nuclear and four nuclear that passed the second division. We also used the FISH of chromosome 18 to establish the possibility of signal detection in MN in both experimental groups.

Analysis of chromosome 18 by FISH

A small number of signals that were found in MN (Table 2) showed no statistical difference. Still, some features of the signals showing the PCD of chromosome 18 in bi-nuclear and multilobulated cells are addressed: (i) in Fig. 2 chromosome 18 is presented by 2 dot-like signals in every nuclei representing normal segregation of centromeres (PCD-); (ii) some nuclei are presented by 4 dot-like signals showing the presence of PCD of chromosome 18 (Fig. 3). These results indicate the complete segregation of chromosome 18 centromeres in the presented bi-nuclear cell. Both experimental groups show the same pattern of events; (iii) some nuclei and multilobulated nuclei show rod-like signals (A) suggesting the initiation of PCD (Fig. 4). The occurrence of PCD and normal signals in multilobulated nuclei show us that cells, even though impaired, show activity and

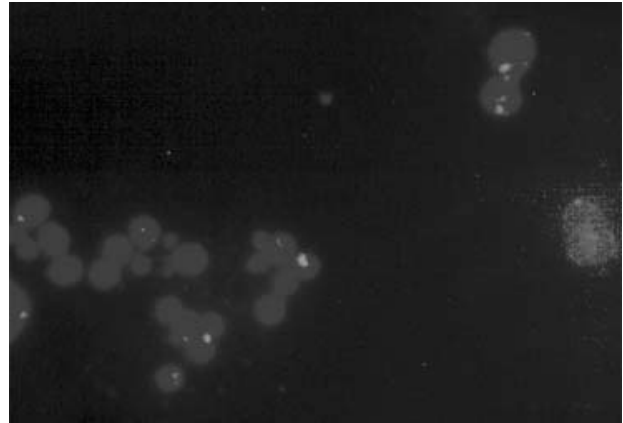


Figure 3. Representative multilobulated nucleus induced by a Paclitaxel (Ptx) dose 50nM in combination with Cycloheximide (Cy) and a bi-nuclear cell with dot like signals for chromosome 18 (PCD-).

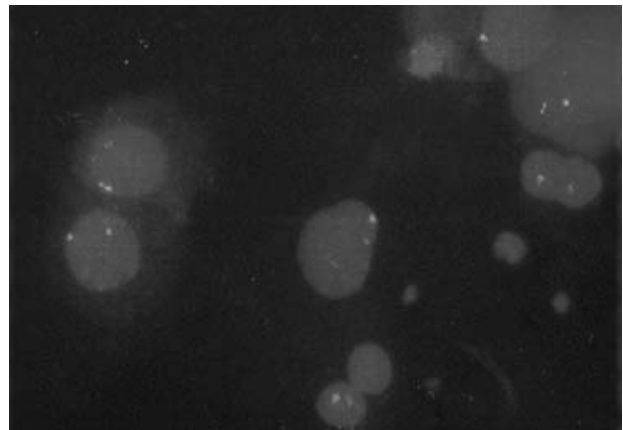


Figure 4. A representative binucleated cell with premature centromere division (PCD) of chromosome 18.

may have survived by abrogating the cell cycles checkpoint controls.

The results of FISH analysis clearly revealed that the properties of the investigated agent alone or in combination can induce the PCD of chromosome 18 in mononucleated cells, bi-nucleated cells and in multilobulated nuclei.

DISCUSSION

In cancer therapy cytotoxicity induced by the administered chemotherapeutic agents, whether

alone or in combination, is a desirable consequence of their action. Genotoxic damage by anticancer agents in tumor cells leads to cell death, a favorable property of cytostatics (Branch et al., 2000). However, increased genetic alterations may have adverse consequences if the affected cells are not malignant. In fact, it has been shown that genetic instability, characterized by an abnormal number of chromosomes, is associated with secondary malignancies. Thus, it is necessary to evaluate the potential aneugenicity of chemotherapy to humans before its clinical use.

A number of mechanisms have been proposed for aneuploidy induction (Mailhes et al., 1997) and the one of interest is that centromere instability has been correlated to increased aneuploidy from various xenobiotics. PCD is now generally recognized as a predisposition to aneuploidy (Fitzgerald et al., 1986; Mailhes et al., 1997, 1998, 1999; 2008; Major et al., 1999; Vig and Hallet, 2000; Steuerwald et al., 2005).

Our results showed that Ptx can induce centromere instability by separating centromeres before their time. This property can be induced by Ptx alone, or in combination with Cy. Moreover, the synergistic action of Cy and Ptx shows a change in the distribution pattern of PCD occurrence on chromosomes when compared to Ptx alone, i.e. PCD occurs on more than one chromosome showing increased chromosome instability (Fig. 1, Table 1). Nonetheless, Cy increased the measure of cell proliferation or the nuclear division index in the Cy and Ptx combinational experimental group when compared to Ptx alone. Elevated NDI but not the number of MN may show the survival effect of affected cells with PCD (Table 2) in the combination experimental group.

Other research has showed that a dose of 10 µg/ml Cy can induce cytoprotective action in various cell from various noxious stimuli (UV light, thermal exposure and toxic compounds) (Lee and Dewey, 1987; Lee et al., 1991; Liebmman et al., 1994; Lynch et al., 2008). Cy may antagonize the cytotoxicity of a variety of chemotherapeutic

agents, including actinomycin, doxorubicin, etoposid, vincristin and Taxol (Liebmman et al., 1994). In these studies different cell lines and protocols were used compared to our study. Thus in our study we argue that centromere instability which leads to PCD may be regarded as a marker of damaged cells which in turn show (elevated NDI and MI) a pseudo-cytoprotective effect.

The NDI gives us a more accurate measure of cytotoxicity vs cytoprotectivity than classical MI from the CA assay (data not shown), i.e. both experimental groups, Ptx alone or in combination with Cy, show a high statistical difference in the NDI when compared to the control group. Still, there is a difference in NDI when comparing the experimental groups. These results show that cells with an abnormal number of chromosomes with PCD survive at least one cell division (see Fig. 1, Table 2). The severity of PCD is based upon the chromosomes affected (Matsuura et al., 2000). Infants with cells that express PCD in 70-90% of their chromosomes develop a phenotype with developmental abnormalities accompanied by Wilm's tumor and consequently die at the age of three.

The FISH of chromosome 18 was used to detect PCD in all phases of the cell cycle by combining with the CBMN test. Chromosome 18 is the first chromosome to separate and thus segregate in the metaphase-anaphase transition and is one of the chromosomes that is most seen in other aneusomies, miscarriages, various tumors, syndromes and Alzheimer's disease (Choo, 1997; Zivkovic et al., 2006). Premature sister chromatids and centromere separation is a parameter of instability which may lead to mitotic checkpoint abrogation and aneuploidy (Vig and Sternes, 1991; Mailhes et al., 1998; Matsuura et al., 2000; Rodriguez et al., 2001; Steuerwald et al., 2005; Bajic et al., 2008). The property of chromosome 18 to divide first in a sequential manner was used to evaluate the possibility of preferential induction of chromosome 18 aneuploidy in micronuclei by Ptx and Ptx in combination with Cy. The notion of preferential aneugenicity was based on the fact that

chromosome 18 is the first chromosome to separate and thus segregate in the metaphase-anaphase transition of the cell cycle and by the work of Wuttke et al. (1997) who found the preferential occurrence of chromosome 7 aneuploidy induced by colchicine in MN using FISH.

Our results showed no statistical difference in the detection of chromosome 18 in MN between the experimental groups and the control group (Table 2). Still, PCD has been detected in mono-, bi-nucleated cells and multilobulated nuclei (Figs. 3 and 4) in cells exposed to Ptx alone or in combination with Cy. Our observations correspond to other studies that have demonstrated that Ptx arrested cells in mitosis and that prolonged exposure of cells to Ptx resulted in the development of multilobulated nuclei (Morse et al., 2005). The above results show that not only is PCD found in various stages of the cell cycle but it is also prominent in an altered nuclear phenotype, i.e. multilobulated nuclei, indicating chromosome/centromere instability which may lead to mitotic catastrophe.

A recent review about mitotic cell death showed that features of mitotic catastrophe induced by Taxol are not simply a representation of dying cells but are indicative of a switch to amitotic modes of cell survival that may provide additional mechanisms of genotoxic resistance (Theodoropoulos et al., 1999; Erenpreisa and Cragg, 2001; Morse et al., 2005). Our results are in agreement with the survival views even though the model used in our study is somewhat deficient, i.e. human peripheral blood lymphocytes are harvested after 72 h so that cells that show a survival pattern might go into apoptosis or some other form of cell death if left to proliferate for more than 72 h. Thus, the explanation of the possible survival of damaged cells observed in the experimental group with Cy and Ptx can be explained by the ability of Cy to synergistically act with Ptx in the induction of PCD, thereby acting on the centromere proteins that induce chromosome instability and possible alteration of the mitotic checkpoint control. Cy increased the number of chromosomes affected by

PCD when subsequently exposed to Ptx. Our results agree to some extent with Bouchet et al. (2007) who showed that Ptx can induce an aneuploidy prone phenotype and apoptosis resistance in normal epithelial mammary cells. This induced instability may be detrimental to normal cells leading to secondary tumors.

CONCLUSION

The instability of centromeres in normal cells should be taken into account when evaluating the genotoxic risk/benefit ratio of anti-tumor agents in pre-clinical trials. For now these investigations can help us to better assess the risk of combined therapies and therefore contribute to a more exact evaluation of the current therapeutic regimes and to the development of new pathways for targeting malignancy.

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**ЕФЕКАТ САМОГ ПАКЛИТАКСЕЛА, И У КОМБИНАЦИЈИ СА ЦИКЛОХЕКСИМИДОМ,
НА УЧЕСТАЛОСТ ПРЕВРЕМЕНЕ ЦЕНТРОМЕРНЕ ДЕОБЕ *IN VITRO***

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Превремена центромерна деоба (PCD) се може посматрати као манифестација хромозомске нестабилности. У циљу процене ефекта паклитаксела (Ptx) и циклохексимида (Cy) на индукцију PCD-а, користили смо микронуклеус тест (CBMN) уз флуоресцентну *in situ* хибридизацију (FISH), као и тест хромозомских абе-

рација (CA) на хуманим лимфоцитима периферне крви. Резултати су показали да Ptx сам, или у комбинацији са Cy може да индукује PCD. Ови налази указују да треба много више пажње обратити на појаву PCD-а као параметра генотоксичности у преклиничким испитивањима моно- и/или политерапија за лечење канцера.