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Wortmannin enhances the induction of micronuclei by low and high LET radiation

N.G.Oliveira[1,2], M.Castro[2,3], A.S.Rodrigues[1,4], I.C.Gonçalves[5], O.M.Gil[1,5], A.P.Fernandes[5], J.M.Toscano-Rico[3] and J.Rueff[1,6]

[1]Department of Genetics, Faculty of Medical Sciences, New University of Lisbon, R. da Junqueira 96, P 1349-008 Lisbon, [2]Faculty of Pharmacy, University of Lisbon, Lisbon, [3]CFEC, Faculty of Medicine, University of Lisbon, Lisbon, [4]University Lusófona, Lisbon and [5]Nuclear and Technological Institute, Sacavém, Lisbon, Portugal

In mammalian cells, the repair of DNA double-strand breaks (DSBs) is mainly mediated by DNA non-homologous end joining. DNA-dependent protein kinase (DNA-PK), a nuclear serine-threonine kinase and a member of the phosphaditylinositol-3 kinase-related kinase family that is activated by DSBs, is a key component of this pathway. Wortmannin (WM) is known to be an irreversible and potent inhibitor of DNA-PK and has thus been proposed as an effective sensitizer for ionizing radiation and for radiomimetic compounds. The present study, using the cytokinesis block micronucleus assay, reports on the differential effect of WM on the repair of the DNA damage induced by low LET (^{60}Co γ-radiation) and high LET radiation by the boron neutron capture reaction (α and Li particles) in V79 Chinese hamster cells. Significant increases in the number of micronuclei per binucleated cell as well as in the frequency of micronucleated binucleated cells were observed in the presence of different concentrations of WM for high LET radiation from the boron neutron capture reaction. The increases observed reached a maximum of ~2-fold in comparison with the respective controls. WM, however, had a more pronounced effect on ^{60}Co γ-radiation-induced micronuclei, increasing the genotoxic damage from this radiation by ~3- to 4-fold. These results are in general in agreement with the concept that DSBs induced by high LET radiation are not a more suitable substrate for the end joining processes mediated by DNA-PK, yet they do not preclude a role for DNA-PK in high LET-induced damage repair.

Introduction

In mammalian cells the repair of DNA double-strand breaks (DSBs) is mainly mediated by the DNA non-homologous end joining pathway (NHEJ). This pathway is first mediated by DNA-dependent protein kinase (DNA-PK), a nuclear serine-threonine kinase and a member of the phosphaditylinositol-3 kinase-related kinase (PIKK) family. DNA-PK is a heterotrimeric enzyme complex containing a DNA end-binding Ku heterodimeric complex [Ku 70 (XRCC6) and Ku 80 (XRCC5)] that recruits and activates the kinase activity of a 465 kDa catalytic subunit [DNA-PK$_{cs}$ (XRCC7)] (Critchlow and Jackson, 1998). This crucial DNA repair pathway, which is activated by DSBs, is a highly conserved process by which two broken DNA ends rejoin without the requirement of extensive homology (for a review see Chu, 1997; Critchlow and Jackson, 1998; Featherstone and Jackson, 1999; Pfeiffer *et al.*, 2000).

The fungal metabolite wortmannin (WM) is known to be an irreversible inhibitor of DNA-PK$_{cs}$ and has thus been proposed as an effective radiosensitizer (Boulton *et al.*, 1996, 1999; Price and Youmell, 1996; Hosoi *et al.*, 1998; Okayasu *et al.*, 1998; Sarkaria *et al.*, 1998; Chernikova *et al.*, 1999, 2001; Kinashi *et al.*, 2001). Given the use of different types of radiation in radiotherapy, the present study, using the cytokinesis block micronucleus assay (for a review see Fenech, 2000), reports on the differential effect of WM on the repair of DNA damage inflicted in V79 Chinese hamster cells by high linear energy transfer (LET) radiation generated through the boron neutron capture reaction (BNC) and by low LET radiation from ^{60}Co γ-radiation.

The BNC reaction is based on the capture of thermal neutrons (n_{th}, low energy) by the minor stable isotope of boron (^{10}B), with the release of α and lithium particles and an average energy of 2.3 MeV. The ^{10}B is delivered to the cell using a boronated compound, e.g. *p*-borono-L-phenylalanine (BPA). Propagation of the α and lithium particles in biological tissues is characterized by short range and high LET, with remarkable destructive power (Hawthorne, 1993; Coderre and Morris, 1999; Oliveira *et al.*, 2001). The BNC reaction has been used in clinical trials (boron neutron capture therapy) to treat malignant glioma (Hatanaka and Nakagawa, 1994) and melanoma patients (Mishima *et al.*, 1989, 1997; Busse *et al.*, 1997).

The study of high LET radiation-induced DNA repair is justified by the pattern of induced DSBs, which is quite different from non-ionizing radiation, such as low LET ^{60}Co γ-radiation, with a sparsely-random distribution and the formation of multiply damaged sites (for a review see Prise *et al.*, 2001). Recently, the structural requirements for DNA-PK activation were elucidated, underlining the need to examine the role of DNA-PK in the recognition of complex strand breaks, as produced by high LET radiation (Martensson and Hammarsten, 2002). Moreover, the involvement of α-particles in carcinogenesis (e.g. the association radon and lung cancer) fully warrants a cytogenetic evaluation of its genotoxic effects in the abrogation of a key repair pathway. Zhao *et al.* (2001) and Hei *et al.* (2001), using a model of neoplastic transformation of human bronchial BEP2D cells exposed to α-particles, recently found down-regulation of the gene for DNA-PK (~2-fold), suggesting an important role for DNA-PK in α-particle-induced tumorigenesis.

Materials and methods

Chemicals and culture medium

Fetal calf serum, RPMI medium, WM and cytochalasin B (cyt-B) were purchased from Sigma (St Louis, MO). ^{10}B enriched BPA (>99%) was

[6]To whom correspondence should be addressed. Fax: 351 21 3622018; Email: rueff.gene@fcm.unl.pt

obtained from KatChem (Prague, Czech Republic). Methanol and Giemsa dye were obtained from Merck (Darmstadt, Germany). Trypsin was purchased from Difco Laboratories (Detroit, MI).

Cell culture

Wild-type V79 Chinese hamster cells (MZ) were kindly provided by Prof. H.R.Glatt (Mainz and Postdam). These cells were cultured in RPMI medium supplemented with 10% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) and incubated at 37°C under an atmosphere containing 5% carbon dioxide.

BNC reaction: BPA incubation

For BPA-treated cells, a BPA stock supplemented medium was prepared with a final BPA concentration of 2.4 mM (500 μg/ml, 24.0 p.p.m. ^{10}B). Cells were seeded (~1.0 $\times 10^5$) in 25 cm^2 tissue culture flasks (Greiner, Frickenhausen, Germany) and incubated with either 5 ml of BPA medium (2.4 mM) or BPA-free culture medium. The cells were grown as monolayers for 48 h and then irradiated with thermal neutrons (low energy neutrons, average value 0.025 eV). Two independent experiments were performed.

BNC reaction: thermal neutron irradiation

The irradiation of V79 cells took place at the vertical access of the thermal column of the Portuguese Research Reactor (RPI). Characterization of the radiation field and reduction of the background γ-radiation from the reactor were essential for these radiobiological experiments and have been described elsewhere (Gonçalves *et al.*, 1999; Oliveira *et al.*, 2001).

Thermal neutron irradiation of between 15 and 60 min was considered in this study, corresponding to average fluences (ϕ) of between 0.5×10^{11} and 2.1×10^{11} n_{th}/cm^2. The neutron flux of each irradiation was monitored using gold foil detectors.

The absorbed dose (expressed in Gy) for the α and Li particles (D_B) from the BNC reaction presented in Tables V and VI is given by the formula $D_B = 8.66\times10^{-8}[^{10}B]\phi$ (Charlton, 1991). Assuming a homogeneous distribution of ^{10}B, its concentration (24.0 p.p.m., corresponding to 2.4 mM BPA) is 24×10^{-6} w/w. The absorbed doses from α and Li particles for the fluences (ϕ) studied are thus in the range 0.1–0.4 Gy. The corresponding absorbed dose from photons released in the BNC reaction is considered to be negligible as calculated by Monte Carlo code MCNP4C.

BNC reaction controls

Controls included cells irradiated with thermal neutrons without BPA incubation (thermal neutron controls). Thermal neutron controls involved the genotoxicity of the reactor γ-ray background and also of nuclear reactions with other nuclides, namely hydrogen and nitrogen (Gupta *et al.,* 1994). These control cultures were exposed to a mixed field of high (protons) and low (γ-rays) LET radiation that could contribute to a small extent to the total dose. Subtraction of the genotoxicity of these neutron cultures from the BNC

Table I. Effect of WM (2.5–10 μM) on the number of micronuclei per cell induced by the BNC reaction (0.8×10^{11} n_{th}/cm^2, 2.4 mM BPA) in V79 cells

Experiment	Fluence ($\times 10^{11}$ n_{th}/cm^2)	BPA (mM)	WM (μM)	%BN cells	BN cells scored	Distribution of cells according to no. of MN					
						0	1	2	3	4	⩾5
Controls											
Background V79	0	0	0	63.5 ± 12.9	2000	1967	29	4	0	0	0
	0	0	2.5	35.2 ± 8.2	2000	1965	25	10	0	0	0
	0	0	10	23.6 ± 9.1	2000	1952	39	4	2	2	1
BPA	0	2.4	0	66.2 ± 2.0	2000	1969	26	5	0	0	0
	0	2.4	2.5	49.5 ± 3.0	2000	1965	32	3	0	0	0
	0	2.4	5.0	41.5 ± 0.1	2000	1957	41	2	0	0	0
	0	2.4	7.5	33.6 ± 6.8	2000	1959	37	3	1	0	0
	0	2.4	10	39.7 ± 6.6	2000	1965	32	3	0	0	0
Thermal neutrons	0.8	0	0	56.6 ± 6.8	2000	1936	57	6	1	0	0
	0.8	0	2.5	43.7 ± 8.9	2000	1915	76	8	1	0	0
	0.8	0	10	23.8 ± 4.2	2000	1871	120	8	1	0	0
BNC reaction											
	0.8	2.4	0	48.7 ± 2.1	2000	1779	191	26	4	0	0
	0.8	2.4	2.5	44.8 ± 0.0	2000	1656	286	53	3	2	0
	0.8	2.4	5.0	41.6 ± 5.1	2000	1575	345	61	14	3	2
	0.8	2.4	7.5	36.6 ± 5.9	2000	1607	323	53	11	6	0
	0.8	2.4	10	31.0 ± 10.5	2000	1586	350	55	7	2	0

Table II. Effect of WM (5.0 μM) on the number of micronuclei per cell induced by the BNC reaction (0.5, 1.2 and 2.1×10^{11} n_{th}/cm^2, 2.4 mM BPA) in V79 cells

Experiment	Fluence ($\times 10^{11}$ n_{th}/cm^2)	BPA (mM)	WM (μM)	%BN cells	BN cells scored	Distribution of cells according to no. of MN					
						0	1	2	3	4	⩾5
Controls											
Background V79	0	0	0	63.5 ± 3.3	6000	5894	101	4	1	0	0
	0	0	5.0	35.2 ± 5.6	6000	5875	110	13	2	0	0
BPA	0	2.4	0	70.8 ± 2.4	6000	5877	119	4	0	0	0
	0	2.4	5.0	44.8 ± 6.6	6000	5871	119	7	3	0	0
Thermal neutrons	0.5	0	0	61.3 ± 6.3	2000	1950	48	2	0	0	0
	0.5	0	5.0	32.7 ± 0.7	2000	1919	72	9	0	0	0
	1.2	0	0	66.3 ± 6.6	2000	1913	80	7	0	0	0
	1.2	0	5.0	33.2 ± 3.7	2000	1864	130	6	0	0	0
	2.1	0	0	55.0 ± 4.2	2000	1901	95	3	1	0	0
	2.1	0	5.0	30.1 ± 2.7	2000	1786	197	17	0	0	0
BNC reaction											
	0.5	0	0	65.2 ± 0.6	2000	1864	121	13	2	0	0
	0.5	0	5.0	37.1 ± 5.5	2000	1723	238	30	8	1	0
	1.2	0	0	59.7 ± 4.9	2000	1702	268	29	1	0	0
	1.2	0	5.0	44.3 ± 4.1	2000	1491	405	79	22	2	1
	2.1	0	0	60.0 ± 2.3	2000	1515	383	77	19	6	0
	2.1	0	5.0	25.5 ± 5.2	2000	1147	597	185	48	19	4

reaction-exposed cultures gives the genotoxicity of the α and Li particles (Tables V and VI). Other controls were cells incubated with BPA without thermal neutron irradiation (BPA controls) and unirradiated cells without BPA incubation (background V79 controls).

^{60}Co γ-radiation

V79 cells were cultured under the same experimental conditions as for the BNC experiments but BPA-free medium was used. After 48 h culture V79 cells were irradiated at the LMRIR-ITN with ^{60}Co γ-radiation using an Eldorado model from Atomic Energy of Canada. The cells were exposed to 0.5–3.0 Gy (room temperature) at a dose rate of 321 mGy/min. Controls included non-irradiated V79 cells. Two independent experiments were performed.

WM treatment

WM was added 3.5 h before the genotoxic treatment and remained for a further 6 h afterwards. A 5.0 mM stock solution of WM was prepared in DMSO and different concentrations ranging from 2.5 to 10 μM were studied. In the case of the BNC experiments, DMSO was added to negative, neutron and BPA controls and did not exceed 0.2% (v/v). For the ^{60}Co γ-irradiation DMSO was added to the negative controls and also did not exceed 0.2% (v/v).

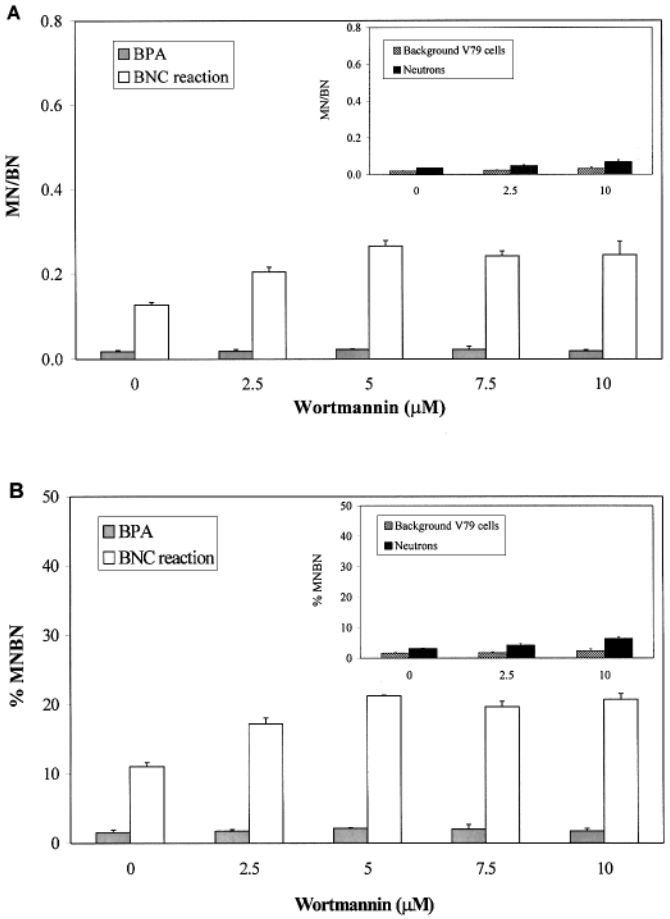

Fig. 1. Effect of different concentrations of WM on the induction of micronuclei by the BNC reaction in V79 cells (white bars). (**A**) MN/BN, number of micronuclei per binucleated cell. (**B**) %MNBN, percent micronucleated binucleated cells. The BNC reaction was performed using a final BPA concentration of 2.4 mM (corresponding to 24.0 p.p.m ^{10}B) and a thermal neutron fluence of 0.8×10^{11} n_{th}/cm^2. BPA alone (2.4 mM), without thermal neutron irradiation, was used as a control (grey bars). Results are average values (±SD) of two independent experiments. In each experiment 1000 binucleated cells were scored for identification of micronuclei. (Inset) Control with the effect of the same fluence of low energy thermal neutrons in V79 cells without BPA, with increasing concentrations of wortmannin (dark bars). Hatched bars represent the effect of WM alone in V79 cells. Results are average values (±SD) of two independent experiments. In each experiment 1000 binucleated cells were scored for identification of micronuclei.

CBMN assay and harvesting of binucleated cells

Six hours after the genotoxic treatment the cell culture medium was removed, the cells were washed and resuspended in fresh culture medium. Cyt-B was added at a final concentration of 6 μg/ml (Van Hummelen and Kirsh-Volders, 1990) and the cells were grown for a further 16 h for recovery of binucleated V79 cells. The cells were then harvested by trypsinization, rinsed and submitted to a mild hypotonic treatment as described elsewhere (Van Hummelen and Kirsh-Volders, 1990; Gil *et al*., 2000). The centrifuged cells were placed on dry slides and smears were made. After air drying the slides were fixed with cold methanol (30 min). One day later the slides were stained with Giemsa (4% v/v in 0.01 M phosphate buffer, pH 6.8) for 10 min.

Micronucleus identification and scoring

For each experimental point 1000 binucleated V79 cells (BN) with well-preserved cytoplasm were scored. Micronuclei were identified according to the criteria of Caria *et al.* (1995) using a 1250× magnification under a light microscope. Two indices were evaluated, MN/BN, which represents the average number of micronuclei per binucleated cell, and %MNBN, which represents the fraction of cytokinesis blocked (binucleated) cells with micronuclei, regardless of the number of micronuclei per BN cell (Oliveira *et al*., 2001).

The statistical analysis for the comparison of each WM-treated culture with the DMSO control was performed using the paired Student's *t*-test.

Cell proliferation

The decrease in cell proliferation in the experiments described above was assessed using the frequency of binucleated cells (%BN). For this index, 500 cells with well-preserved cytoplasm from two independent experiments were analyzed according to number of nuclei, using a 500× magnification.

Results

The sensitizing effect of WM on the induction of micronuclei by the BNC reaction in V79 cells is presented in Tables I and II and Figures 1 and 2. Table I represents the effect of different concentrations of WM (2.5–10 μM) on the distribution of the

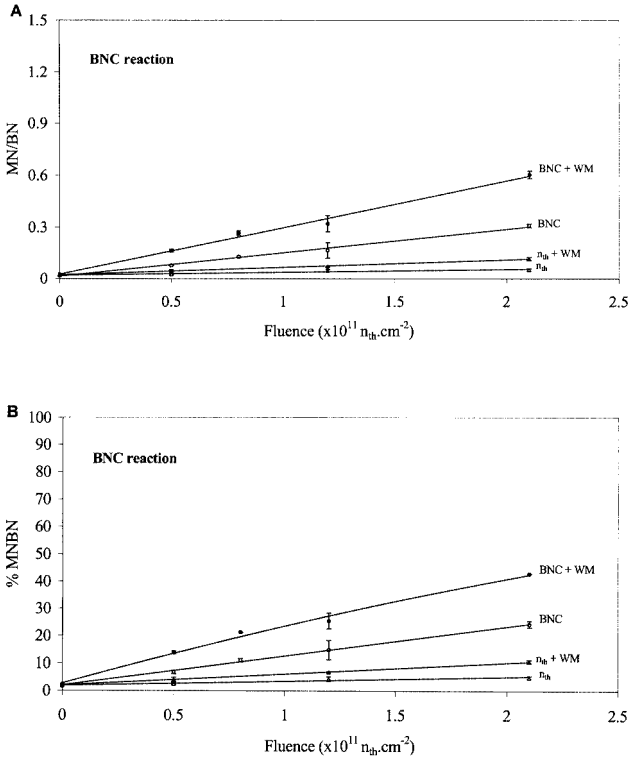

Fig. 2. Effect of a fixed concentration of WM (5 μM) on the induction of micronuclei by the BNC reaction in V79 cells. (**A**) MN/BN, number of micronuclei per binucleated cell. (**B**) %MNBN, percent micronucleated binucleated cells. A control for the effect of low energy thermal neutrons (n_{th}) is also presented for the same fluences studied. Results are average values (±SD) of two independent experiments. In each experiment 1000 binucleated cells were scored for identification of micronuclei.

Table III. Effect of WM (2.5–10 μM) on the number of micronuclei per cell induced by ^{60}Co-γ radiation (1.5 Gy) in V79 cells

Experiment	Absorbed dose (Gy)	WM (μM)	%BN cells	BN cell scored	Distribution of cells according to no. of MN					
					0	1	2	3	4	⩾5
Controls										
Background V79	0	0	66.0 ± 9.3	2000	1970	21	6	3	0	0
	0	2.5	42.9 ± 4.7	2000	1966	30	2	2	0	0
	0	5.0	34.0 ± 4.5	2000	1954	39	7	0	0	0
	0	7.5	35.0 ± 3.4	2000	1961	34	3	2	0	0
	0	10	31.1 ± 0.7	2000	1961	34	4	1	0	0
^{60}Co γ-radiation										
	1.5	0	58.0 ± 0.8	2000	1715	252	28	5	0	0
	1.5	2.5	45.5 ± 6.6	2000	1490	448	53	7	2	0
	1.5	5.0	38.4 ± 0.6	2000	1240	598	134	22	6	0
	1.5	7.5	28.5 ± 0.4	2000	1200	575	190	29	3	3
	1.5	10	26.4 ± 5.4	2000	1204	578	174	36	8	0

Table IV. Effect of WM (5.0 μM) on the number of micronuclei per cell induced by ^{60}Co-γ radiation (0.5, 1.0 and 3.0 Gy) in V79 cells

Experiment	Absorbed dose (Gy)	WM (μM)	%BN cells	BN cell scored	Distribution of cells according to no. of MN					
					0	1	2	3	4	⩾5
Controls										
Background V79	0	0	61.2 ± 4.0	2000	1976	21	2	1	0	0
	0	5.0	46.3 ± 4.4	2000	1966	33	1	0	0	0
^{60}Co γ-radiation										
	0.5	0	62.5 ± 5.5	2000	1894	102	4	0	0	0
	0.5	5.0	44.9 ± 2.4	2000	1627	321	50	2	0	0
	1.0	0	65.7 ± 7.2	2000	1826	162	11	1	0	0
	1.0	5.0	44.6 ± 0.6	2000	1338	531	115	12	2	2
	3.0	0	59.0 ± 2.0	2000	1459	435	90	14	0	2
	3.0	5.0	26.8 ± 0.3	2000	612	691	449	164	55	29

number of micronuclei per cell (0–4 and ⩾5) induced by the BNC reaction. The BNC reaction was carried out using a thermal neutron irradiation of 0.8×10^{11} n_{th}/cm^2 and a 2.4 mM concentration of BPA. The corresponding results for MN/BN (Figure 1A) and %MNBN (Figure 1B) are depicted in Figure 1. Both Table 1 and Figure 1 present data on the controls studied, i.e. background V79 cells, BPA-incubated cells and thermal neutron-irradiated cells. Table I also presents %BN cells (±SD) as an index of cell proliferation.

Table II presents the effect of a fixed concentration of WM (5.0 μM) on the distribution of MN per cell (0–4 and ⩾5) induced by the BNC reaction and the respective controls carried out with different thermal neutron fluences (0.5–2.1×10^{11} n_{th}/cm^2) and using the same BPA concentration of 2.4 mM. The corresponding results for MN/BN (Figure 2A) and %MNBN (Figure 2B) in the BNC reaction and neutron controls are depicted in Figure 2.

The sensitizing effect of WM on the induction of micronuclei by ^{60}Co γ-radiation in V79 cells is presented in Tables III and IV and Figures 3 and 4. Table III represents the effect of different concentrations of WM (2.5–10 μM) on the distribution of the number of micronuclei per cell (0–4 and ⩾5) induced by 1.5 Gy γ-radiation and the respective controls. Table IV presents the effect of a fixed concentration of WM (5.0 μM) on the distribution of the MN per cell induced by ^{60}Co γ-radiation (0.5–3.0 Gy). The corresponding results for MN/BN (Figures 3A and 4A) and %MNBN (Figures 3B and 4B) for γ-irradiated cultures and controls from Tables III and IV are depicted in Figures 3 and 4, respectively.

WM treatment of non-irradiated V79 cells did not particularly increase the induction of micronuclei (Tables I–IV and Figures 1–4). %MNBN for background V79 cells increased slightly in the presence of WM (5.0 μM), at $P < 0.05$ (Figure 4B). However, the increase observed was only ~0.7% and no dose–response effects were seen for increasing concentrations of WM either in background V79 cells (Figures 1 and 3) or in the BPA controls treated with WM (Figure 1). WM, however, decreased %BN cells in a dose-dependent manner (Tables I–IV) not only in the presence of the BNC reaction and γ-irradiation but also in the controls. Previous studies using higher doses of WM (20 and 40 μM) showed a decrease in %BN to levels of ~80% (data not shown).

Tables V and VI summarize the data on the induction of micronuclei by thermal neutrons, the BNC reaction, α + Li particles from the BNC reaction and γ-radiation in the presence or absence of WM. These tables present average values (±SD) of MN/BN and %MNBN and the fold increases observed for these biomarkers in the presence of WM and the respective statistical significances (*P* values). Table V shows the effect of different concentrations of WM in the genotoxicity of a standard dose of each type of radiation, while Table VI shows the effect of a fixed concentration of WM on the genotoxicity of different doses of each radiation type.

It is clear in Tables V and VI and Figures 1–4 that WM significantly increased the genotoxicity of the BNC reaction by a factor of ~2 and the genotoxicity of γ-radiation by a factor of 3–4. The fold increases were more pronounced for the MN/BN index than for %MNBN for both radiation types. In addition,

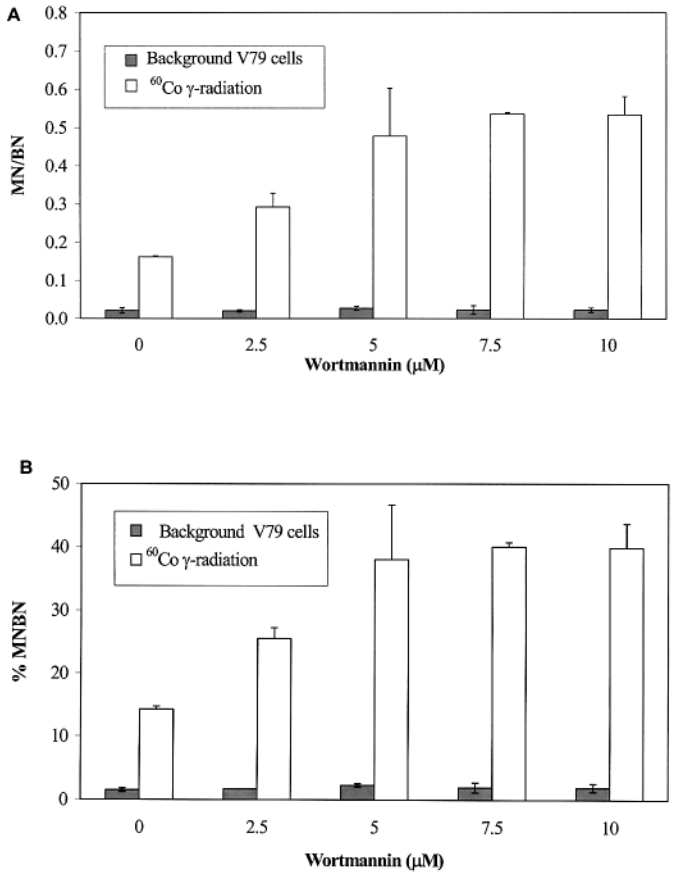

Fig. 3. Effect of different concentrations of WM on the induction of micronuclei by ^{60}Co γ-radiation (1.5 Gy) in V79 cells (white bars). (**A**) MN/BN, number of micronuclei per binucleated cell. (**B**) %MNBN, percent micronucleated binucleated cells. V79 cells without γ-irradiation were used as controls (grey bars). Results are average values (±SD) of two independent experiments. In each experiment 1000 binucleated cells were scored for identification of micronuclei.

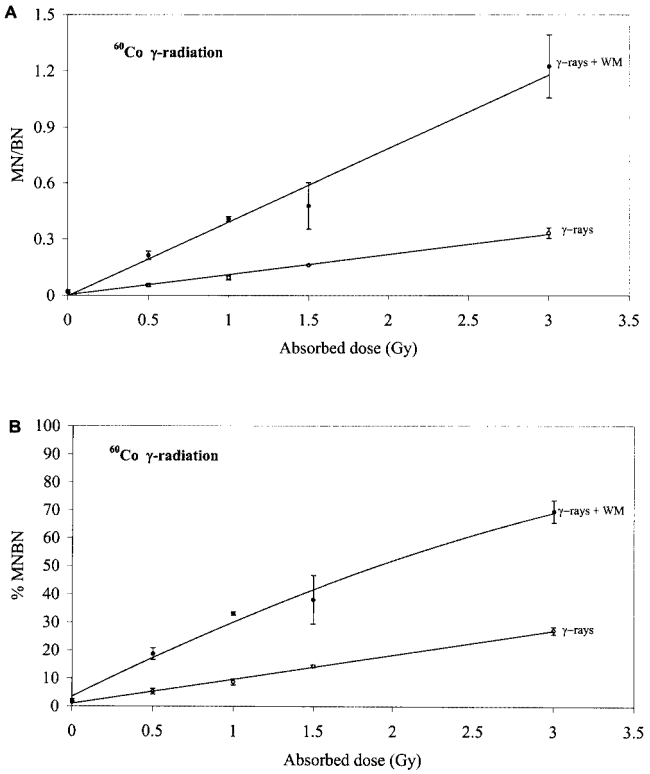

Fig. 4. Effect of WM (5 μM) on the induction of micronuclei by ^{60}Co γ-radiation in V79 cells. (**A**) MN/BN, number of micronuclei per binucleated cell. (**B**) %MNBN, percent of micronucleated binucleated cells. Results are average values (±SD) of two independent experiments. In each experiment 1000 binucleated cells were scored for identification of micronuclei.

WM incubation changed the pattern of distribution of the micronucleated cells for both the BNC reaction and γ-radiation (Tables II and IV), especially for the higher doses studied, showing an increase in the micronucleated cells with two or more micronuclei. The genotoxicity of low energy thermal neutrons was also included in our study as a control for the BNC reaction and involves contributions by different sources of potential DNA damage (namely γ-rays from the reactor and neutron capture reactions of hydrogen and nitrogen) that contribute to a small extent to the final BNC reaction effects. WM also had an effect of 1.5- to 2-fold on the repair of the lesions induced by thermal neutron irradiation (Figures 1 and 2 and Tables V and VI). This is in agreement with Kinashi *et al.* (2001), who studied the effect of WM on CHO cell survival after thermal neutron irradiation and the BNC reaction, although they found a more pronounced effect on survival with neutrons than with the BNC reaction.

Tables V and VI also show data on the genotoxicity of α + Li particles from the BNC reaction. The MN/BN and %MNBN presented correspond to subtraction of the genotoxicity of thermal neutrons from the genotoxicity of the BNC reaction. This was included in this study in order to emphasize that if the other components of the BNC reaction, other than the high LET α + Li particles, were disregarded, a clear effect of WM was maintained.

Discussion

The present work shows that treatment of V79 cells with WM significantly enhances the genotoxic burden caused by both high and low LET radiation. We studied the effect of different concentrations of WM (2.5–10 μM) on the genotoxicity of the BNC reaction (fluence 0.8×10^{11} n_{th}/cm^2) and ^{60}Co γ-radiation (1.5 Gy). The doses of radiation were clearly genotoxic, with a similar induction of micronuclei in the absence of WM (Tables I and III and Figures 1 and 3). In addition, these doses did not markedly decrease %BN cells (Tables I and III). The concentrations of WM used were moderate (2.5–10 μM) but clearly inhibitory for DNA-PK (Sarkaria *et al*., 1998; Davies *et al*., 2000). Higher concentrations of WM (20 and 40 μM) were not used for the assessment of micronucleated cells because they markedly diminished cell proliferation, thus resulting in a sparse number of binucleated cells (data not shown). Moreover, the use of high doses of WM may increase the possibility of a broader kinase activity inhibition.

The genotoxicity induced by the BNC reaction increased the frequency of %MNBN and MN/BN ~2-fold in the presence of WM, whereas an ~3-fold increase was found for γ-radiation. The increases were initially lower for 2.5 μM WM and then reached a plateau for 5.0 μM and higher concentrations of WM (Table V). These results suggest that some kind of saturation in the increment in genotoxic damage occurred for increasing concentrations of WM. One reason for this could be the anti-proliferative or anti-mitogenic properties of higher concentrations of WM, which blocked cell cycle progression as measured by the frequency of BN cells, increasing the number of non-dividing cells and thus interfering with

Table V. Summary of the results obtained from a dose–response curve for the effect of WM on the induction of micronuclei by different types of radiation in V79 cells

Radiation	WM (μM)	MN/BN			%MNBN		
		Mean ± SD	Fold increase	*P* value	Mean ± SD	Fold increase	*P* value
Thermal neutrons (n_{th}/cm^2)							
0.8×10^{11}	0	0.036 ± 0.001			3.2 ± 0.1		
0.8×10^{11}	2.5	0.048 ± 0.004	1.3	<0.05	4.3 ± 0.5	1.3	0.13
0.8×10^{11}	10	0.070 ± 0.008	1.9	<0.05	6.5 ± 0.5	2.0	<0.05
BNC reaction (n_{th}/cm^2)							
0.8×10^{11}	0	0.128 ± 0.006			11.1 ± 0.6		
0.8×10^{11}	2.5	0.205 ± 0.012	1.6	0.05	17.2 ± 0.8	1.6	0.05
0.8×10^{11}	5.0	0.266 ± 0.013	2.1	<0.05	21.3 ± 0.1	1.9	<0.05
0.8×10^{11}	7.5	0.243 ± 0.011	1.9	<0.05	19.7 ± 0.8	1.8	<0.01
0.8×10^{11}	10	0.245 ± 0.033	1.9	0.05	20.7 ± 0.8	1.9	<0.01
α + Li particles[a] (Gy)							
0.17 (0.8×10^{11})	0	0.092 ± 0.005			7.9 ± 0.8		
0.17 (0.8×10^{11})	2.5	0.157 ± 0.016	1.7	0.07	13.0 ± 1.3	1.6	0.09
0.17 (0.8×10^{11})	10	0.175 ± 0.025	1.9	0.05	14.3 ± 0.4	1.8	<0.05
^{60}Co γ-rays (Gy)							
1.5	0	0.162 ± 0.002			14.3 ± 0.5		
1.5	2.5	0.292 ± 0.036	1.8	0.06	25.5 ± 1.7	1.8	<0.05
1.5	5	0.478 ± 0.124	3.0	0.08	38.0 ± 8.6	2.7	0.08
1.5	7.5	0.535 ± 0.004	3.3	<0.01	40.0 ± 0.7	2.8	<0.01
1.5	10	0.533 ± 0.047	3.3	<0.05	39.8 ± 3.8	2.8	<0.05

[a]The absorbed dose of α + Li particles (0.17 Gy) was calculated according to Materials and methods. The corresponding values of MN/BN and %MNBN were calculated by subtracting the genotoxicity of the respective thermal neutron control from the genotoxicity of the BNC reaction.

Table VI. Summary of the results obtained from a dose–response curve for the effect of WM (5.0 μM) on the induction of micronuclei by different types of radiation in V79 cells

Radiation	WM (μM)	MN/BN			%MNBN		
		Mean ± SD	Fold increase	*P* value	Mean ± SD	Fold increase	*P* value
Thermal neutrons (n_{th}/cm^2)							
0.5×10^{11}	0	0.026 ± 0.003			2.5 ± 0.3		
0.5×10^{11}	5	0.045 ± 0.007	1.7	0.11	4.1 ± 0.8	1.6	0.14
1.2×10^{11}	0	0.047 ± 0.006			4.4 ± 0.8		
1.2×10^{11}	5	0.071 ± 0.004	1.5	<0.05	6.8 ± 0.3	1.5	<0.05
2.1×10^{11}	0	0.052 ± 0.007			5.0 ± 0.5		
2.1×10^{11}	5	0.116 ± 0.009	2.2	<0.01	10.7 ± 0.6	2.1	<0.01
BNC reaction (n_{th}/cm^2)							
0.5×10^{11}	0	0.077 ± 0.001			6.8 ± 0.6		
0.5×10^{11}	5	0.163 ± 0.008	2.1	<0.05	13.9 ± 0.5	2.0	<0.01
1.2×10^{11}	0	0.165 ± 0.045			14.9 ± 3.5		
1.2×10^{11}	5	0.321 ± 0.047	1.9	<0.01	25.5 ± 2.9	1.7	<0.05
2.1×10^{11}	0	0.309 ± 0.010			24.3 ± 1.2		
2.1×10^{11}	5	0.604 ± 0.022	2.0	<0.01	42.7 ± 0.1	1.8	<0.05
α + Li particles[a] (Gy)							
0.10 (0.5×10^{11})	0	0.051 ± 0.004			4.3 ± 0.8		
0.10 (0.5×10^{11})	5	0.118 ± 0.001	2.3	<0.01	9.8 ± 0.3	2.3	<0.05
0.25 (1.2×10^{11})	0	0.118 ± 0.039			10.6 ± 2.8		
0.25 (1.2×10^{11})	5	0.250 ± 0.042	2.1	<0.01	18.7 ± 2.6	1.8	<0.01
0.44 (2.1×10^{11})	0	0.257 ± 0.003			19.3 ± 0.7		
0.44 (2.1×10^{11})	5	0.488 ± 0.013	1.9	<0.01	32.0 ± 0.6	1.7	<0.05
^{60}Co γ-rays (Gy)							
0.5	0	0.055 ± 0.008			5.3 ± 1.0		
0.5	5	0.214 ± 0.022	3.9	<0.05	18.7 ± 2.1	3.5	<0.05
1.0	0	0.094 ± 0.011			8.7 ± 1.1		
1.0	5	0.408 ± 0.013	4.3	<0.05	33.1 ± 0.6	3.8	<0.01
3.0	0	0.334 ± 0.028			27.1 ± 1.3		
3.0	5	1.226 ± 0.168	3.7	<0.05	69.4 ± 4.0	2.6	<0.05

[a]The absorbed doses of α + Li particles (0.10, 0.25 and 0.44 Gy) were calculated according to Materials and methods. The corresponding values of MN/BN and %MNBN were calculated by subtracting the genotoxicity of the respective thermal neutron control from the genotoxicity of the BNC reaction.

the genotoxic effects of the challenge agents. Alternatively, one cannot exclude that, if a substantial inhibition of DNA-PK had occurred, no dose–effect relationship could be easily observed using this cytogenetic approach.

The effect of WM on cell cycle kinetics was studied by Rosenzweig *et al.* (1997) in SW480 cells (colon adenocarcinoma), using fluorescence-activated cell sorting analysis. WM alone increased the number of G_1 cells over the first 30 h, with a corresponding decline in S phase and without accumulation in the S or G_2 phases. These results indicate that WM does not block DNA synthesis or mitosis, and the authors concluded that G_1 accumulation might occur through inhibition of the p110α catalytic subunit of phosphaditylinositol-3 kinase [PI3K (p110)], which is required for cell cycle progression through G_1. In the presence of γ-radiation WM prolongs the G_2 checkpoint up to 50 h post-irradiation (Rosenzweig *et al.*, 1997).

Additionally, we studied the effect of a fixed concentration of WM on the genotoxicity of different doses of both the BNC reaction and γ-radiation. The concentration of WM used was 5.0 μM because this was the lowest concentration effective in increasing the yield of micronuclei. The radiation doses for both the BNC reaction and γ-radiation provided equigenotoxic responses in the absence of WM for both radiation types (Figures 3 and 4). All the doses studied confirm the effect of WM in enhancement of the genotoxicity of both γ-radiation (3- to 4-fold) and the BNC reaction (2-fold). WM had a more evident impact as assessed by the fold increase in the MN/BN index than in %MNBN (Table VI). In fact, the dose–response curves for both the BNC reaction and γ-radiation in the presence of WM is approximately linear for MN/BN, but has a slight shoulder for %MNBN. This could be explained by the fact that WM also changed the pattern of distribution of the number of micronuclei, especially for the higher doses studied (Tables III and IV), increasing the number of micronucleated cells with 2, 3 and more micronuclei. This alteration in pattern was more pronounced for γ-radiation than for the BNC reaction, showing once again that WM enhances the effects of low LET radiation more than high LET radiation.

The differential involvement of DNA-PK in the repair of DNA lesions induced by different types of radiation has usually been explained by the nature of the DSBs generated by each agent. DNA damage inflicted by high LET radiation is considered to have a non-random distribution of DSBs resulting in a clustered pattern (Goodhead, 1989). Clustering of the damage can occur at the level of the DNA helix, giving locally multiply damaged sites, or over larger regions related to chromatin structure, giving regionally multiply damaged sites (Prise *et al.*, 2001). This pattern of DNA damage may not be the most suitable substrate for the end joining processes mediated by DNA-PK (Britten *et al*., 2001). Some previous reports using cell lines deficient in one of the components of DNA-PK (xrs-5, xrs-6 and scid) suggested a limited role of DNA-PK in high LET radiation when compared with γ-radiation and X-rays (reviewed in Blakely and Kronenberg, 1998; Britten *et al*., 2001). The reports on xrs-5 (Shadley *et al.*, 1991) and xrs-6 cells (Nagasawa *et al*., 1991) and α-particle-induced damage showed that these cell lines are much more sensitive to γ-radiation than α-particles. In these reports, however, increases in the frequencies of G_2 chromatid aberrations as well as decreases in cell survival were also seen for DNA-PK-deficient cells exposed to α-particles. Other more recent reports showed normal sensitivity or even a decrease in the sensitivity of DNA-PK-deficient cells irradiated with α-particles (Lucke-Huhle, 1994; Schwartz and Hsie, 1997).

WM is not a specific inhibitor of DNA-PK. It can also irreversibly inhibit other members of the PIKK family (Sarkaria *et al.,* 1998), which share a high degree of homology in the active site of the p110α catalytic subunit of PI3K (Hartley *et al.,* 1995). Some of these enzymes, based on their roles in DNA damage responses, were suggested as potential targets for WM radiosensitizing effects. WM is a potent inhibitor of DNA-PK (IC_{50} = 16 nM), but it also inhibits the ataxia telangiectasia mutated (ATM) protein (IC_{50} = 150 nM) and, to a much lesser extent, the ataxia telangiectasia rad3-related protein (ATR) (IC_{50} = 1.8 μM) (Sarkaria *et al.*, 1998). It could thus be argued that the observed increase in the genotoxicity of both low and high LET radiation is not entirely due to DNA-PK. Although this possibility exists, Chernikova *et al.* (1999) have demonstrated, using mutants defective in DNA-PK (scid, irs-20, xrs-6 and XR-1) and ATM protein (AT cells) that ATM does not play a significant role in WM radiation sensitization. Other authors (DiBiase *et al.*, 2000), using two glioma cell lines, one lacking DNA-PK_{cs} and the other the isogenic proficient counterpart, also concluded that DNA-PK is the main target for WM radiation sensitization. These recent results reinforce other reports that emphasized the prime role of DNA-PK for WM radiosensitization (Boulton *et al.*, 1996; Okayasu *et al*., 1998).

In conclusion, our results show that WM is able to increase %MNBN and MN/BN induced by different types of radiation, suggesting an important role of the NHEJ pathway and of DNA-PK and that this role is relatively more important in the repair of DSB induced by ^{60}Co γ-radiation than by α + Li particles from the BNC reaction.

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