Induction of Apoptosis and Micronuclei by Bleomycin Sulfate in Different Cell Cycle Stages of Human Lymphocytes

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Abstract

Introduction: Bleomycin sulfate is a DNA damaging agent used in cancer chemotherapy. The effect of this drug on various cell cycle stages might be different, thus inducing different modes of death (apoptotic or mitotic death). The aim of this investigation was to study the effects of bleomycin on human peripheral blood lymphocytes at various cell cycle stages by two different end points (induction of apoptosis or micronuclei).

Material and Methods: Human peripheral blood lymphocytes were treated with various doses of bleomycin at G0, G1, and G2 phases of the cell cycle and the percentages of apoptosis (AP) and micronuclei (MN) were determined. The peripheral lymphocytes were isolated by ficoll hypaque and suspended in RPMI-1640 containing 15 % fetal calf serum. The isolated lymphocytes were stimulated by phytohemagglutinin (PHA), cultured again in RPMI-1640, harvested after 64 hrs and 96 hrs, and stained with acridine orange and ethidium bromide to determine the percentage of apoptotic cells. MN assay was done according to the standard in vitro micronucleus assay.

Results: The results showed that the percentages of apoptotic cells and MN at G2 stage were significantly higher than those of G0 and G1 stages. At higher doses, MN formation and apoptotic cells were increased; however with increasing time, the percentage of MN decreased while the percentage of apoptotic cells generally increased in all the cell cycle stages.

Conclusion: The results indicate that bleomycin is a potent inducer of both micronuclei and apoptosis. The incidence of apoptotic cells following bleomycin treatment in G0 and G1 was much higher than the incidence of micronucleated cells at the two sampling times. The percentage of AP cells following bleomycin treatment remained constant across cell cycle stages.

Key words: Bleomycin, Micronucleus, Apoptosis, G0, G1, G2- Lymphocytes.

Introduction

The cancer chemotherapy drug, bleomycin (BLM) is a potent inducer of genetic damage in a wide variety of assays. Because of its extensive use in clinical chemotherapy, its clastogenic effects have been studied extensively (1, 2). Bleomycin is a cytotoxic glycopeptide isolated from Streptomyces verticillies (3). It is an antibiotic that has antitumor activity against squamous cell carcinoma and malignant lymphomas (4). The activity of BLM is due to the effects on DNA synthesis (5, 6) and its cytotoxicity is related to DNA damage, chromosome breakage and inhibition of protein synthesis (7, 8). As well as being cytotoxic, BLM interferes with cell progression when applied to G2 cells (9, 10). It has been shown that BLM induces molecular and cytological effects similar to ionizing radiation (11, 12) such as induction of DNA strand breaks, G2 delay and chromosome aberrations (1, 13). The chemical structure of BLM consists of two domains, a DNA binding domain and an oxygen activating domain (14). BLM is a potent inducer of sister chromatid exchange (7, 15) and also MN, in vivo and in vitro (16, 17). BLM has also been shown to produce a positive response in the comet assay in human lymphocytes. BLM induces AP in many cell lines such as pancreatic carcinoma cells, mouse leukemia L1210 cells, and others (18, 19).

There is evidence that apoptosis induction by BLM may involve the formation of reactive oxygen species (ROS) (18, 20, 21). The physico-chemical information indicates that the beta- aminalaninamide- pyrimidine- beta- hydroxyhistidine region of BLM forms an iron complex to activate the oxygen (20, 14). ROS may cause mitochondrial dysfunction, which results in release of cytochrome C and activation of the caspase cascades (22). DNA binding compounds can result in chromosomal aberration that may cause either MN formation or AP (16, 23). MN which is due to acentric chromosomal fragments or lagging chromosomes during mitosis can be seen as discrete bodies in the cytoplasm of proliferating nucleated cells following exposure to chemical or physical clastogens.

It is clear that bleomycin may exercise its cytotoxic effect through two mechanisms that result in AP and MN. It is also recognised that some chemotheropeutic agents induce their effect at specific stages of the cell cycle (24,25) Therefore the evaluation of BLM activity during different phases of the cell cycle seems essential.

The purpose of this study was to investigate the magnitude of MN formation and AP induction by BLM in normal human peripheral lymphocytes at G0, G1, and G2 phases of the cell cycle.

Material and Methods

Three phases of cell cycle (G0, G1 and G2), three doses of BLM for each phase, and totally 9 groups were used in this study. The cells in each group were subdivided into those used for evaluation of AP and MN with two different time schedules (64 and 96 hrs). For each subgroup, a non-treated control was used. In G0 and G1 groups, the doses of bleomycin were 100, 200 and 300 (g/ml, while for G2 phase lymphocytes they were 50, 100 and 200 (g/ml. All experiments were repeated twice.

* *Micronuclei assay*

The MN assays were done according to the standard in vitro micronucleus test. Blood samples were collected from a healthy normal individual with no history of smoking, exposure to diagnostic X-rays or use of antibiotics for at least three months. 0.5 ml of whole blood was cultured in 4.5 ml RPMI-1640 (Sigma) supplemented with 15% fetal calf serum (FCS), L-glutamine (Sigma), penicillin (100 iu/ml) and streptomycin (100 (g/ml), and PHA-M form (0.1 ml) (Gibco). The method described by Fenech and Morely (26) for MN assay, in which the MN can be scored in cytokinesis blocked lymphocytes at first mitosis, was not used because the experimental design used here required a longer incubation time so that the outcome in terms of MN could be compared with the apoptosis outcome.

For the G0 phase study, the cells were incubated in a CO2 incubator at 37°C and treated with BLM for 4 hrs. BLM was added at the beginning of culture. After treatment, the cells were washed twice with Hank's solution by centrifugation at 1200 rpm for 6 min. Fresh

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complete medium containing PHA for stimulation of non-cycling lymphocytes was added to the culture vessels. The cells were cultured until the scheduled evaluation.

For the G1 phase study, cells were incubated with BLM for 4 hrs after stimulation with PHA, then washed by centrifugation and resuspended in complete fresh medium.

For the G2 phase study, PHA stimulated lymphocytes were treated with BLM 4 hrs before harvesting.

The control cultures were treated according to the above schedules without BLM addition. After either 64 or 96 hrs, the cultures were centrifuged at 1200 rpm for 6 min. The supernatant was removed and cells were exposed to a hypotonic solution (KCl, 0.075 M) for 6 min. at room temperature. After centrifugation and KCl removal, lymphocytes were fixed in Carnoy's fixative consisting of methanol and glacial acetic acid (3:1 v/v) for 20 min., washed 3 times in fixative and slides were prepared. The slides were stained in 10 % Giemsa for 15 min. 1000 cells were analyzed for the presence or absence of micronuclei for each sample.
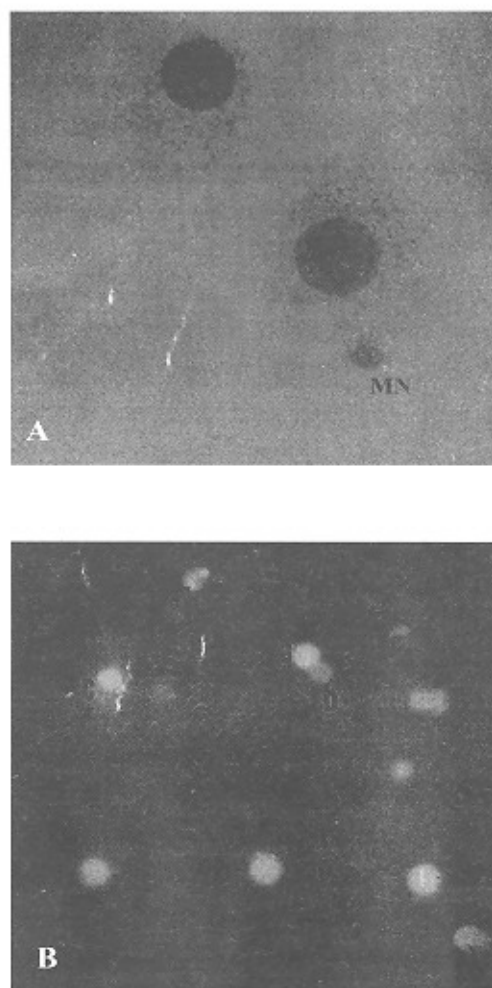
* Apoptotic cell assay

The apoptotic cell assay was done according to Liegler et al.(27). The lymphocytes were isolated from peripheral blood using Ficoll hypaque (provided by Iran blood transfusion organization). Five ml of blood was diluted with RPMI-1640 medium (1:1). To five ml of the diluted blood 3 ml of Ficoll was added and the cells were centrifuged for 20 min at 2000 rpm. The lymphocytes were isolated and washed twice by centrifugation with 2 ml of RPMI 1640 for 7 min. at 1000 rpm. The cells were resuspended in 1 ml RPMI-1640 and cultured. For BLM treatment one million lymphocytes were mixed with 2 ml RPMI-1640 with 15 % FCS and other components mentioned above. Cell cycle phase preparations, BLM treatments and time schedule were as for the MN assay.

Acridine orange/Ethidium bromide double staining was used for fluorescence microscopy of the cells. The stain consisted of 100 μg/ml acridine orange (Sigma) and 100 μg/ml ethidium bromide (Sigma) in phosphate buffer solution (pH=7.2 7.4).1μl of the stain was mixed with 25 μl of cell suspension, transferred to a slide and examined under a fluorescence microscope. A recent comparative study has found this double staining technique to be as reliable as other methods for detecting apoptotic cells (28) and has been used by many investigators (eg. 28-32). In this study 200 cells were analyzed to determine the percentage of apoptotic cells following BLM treatment at different cell cycle stages.

Figure 1 shows a photomicrograph of micronuclei in lymphocytes and apoptotic cells. Data analysis was performed using statistical correlations and the Kruskal-Wallis test.

Figure 1: Photomicrographs showing micronucleated (A) and apoptotic (B) lymphocytes. In figure A; (MN) indicates micronuclei and in figure B; (a) indicates Normal, (b) necrotic and (c) apoptotic cells. Magnification of both photomicrographs is x 600.

Results

Figures 2A and 2B show the frequency of micronuclei induced by each dose of BLM in different cell cycle stages after 64 and 96 hrs respectively. As seen, at 64 hr sampling time there is a small but not statistically significant increase in the frequency of MN with increasing dose of BLM for lymphocytes treated at the G0 and G1 phases of the cell cycle. At the same sampling time (64 h) there was a sharp significant ($p < 0.05$) increase in the frequency of micronuclei observed for lymphocytes treated with both doses of BLM at the G2 cell cycle stage compared with controls. Comparison of Figures 2A and 2B shows that the incidence of MN decreases with increasing post-treatment sampling time.

apoptotic cells following treatment at G0 and G1 cell cycle stages. The increase in AP was seen to be dose dependent for both G0 and G1 treated cells ($p < 0.05$) when harvested at 64 hrs post BLM treatment (Fig 3A). There was not a significant difference between the observed AP cells in G0 and G1 cells treated with similar BLM doses. Frequency of AP cells also increased in G2 cells treated with BLM in a dose dependent manner after 64 hrs sampling time. A similar trend of AP formation was observed for all cell cycle stages studied at 96 hours after BLM treatment (Fig 3B), although the percentage of AP formation was slightly higher in 96 h sampling time compared to 64 h sampling time.
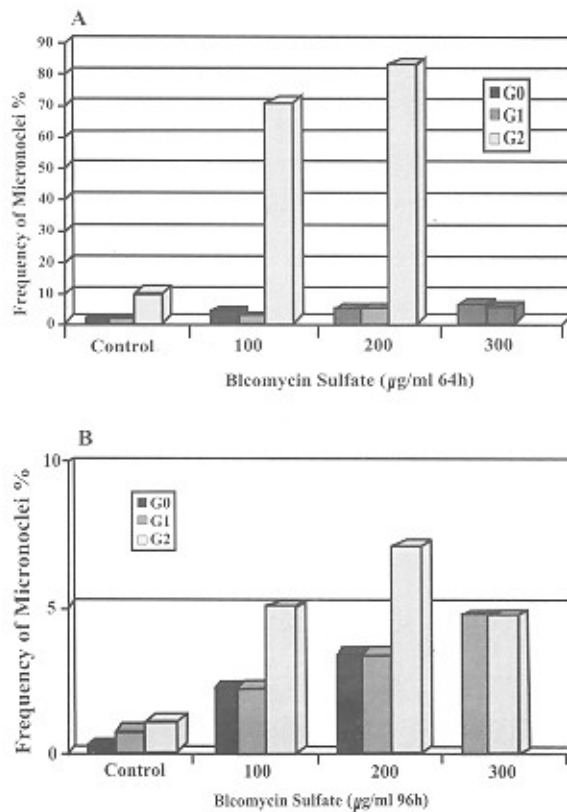

Figure 2: Frequency of micronuclei induced by various doses of bleomycin sulfate in different cell cycle stage. Cells were harvested either at 64 hrs (A) or 96 hrs (B) after BLM treatment. G2 cells weretreated for four hours prior to harvesting.
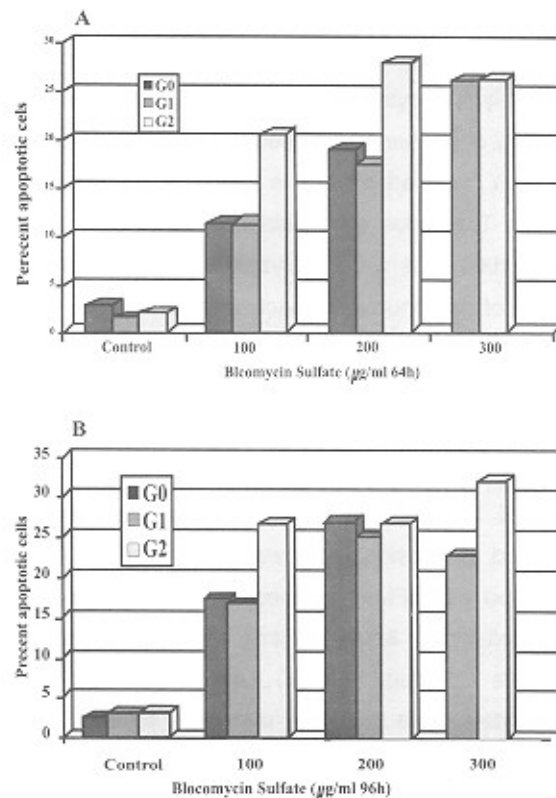

Figure 3: Comparison of the frequency of apoptosis induced by various doses of bleomycin sulfate in different cell cycle stages. Cells were harvested either at 64 hrs (A) or 96 hrs (B) after treatment. G2 cells were treated for four hours before harvesting.

Figures 3A and 3B show the incidence of apoptosis of lymphocytes treated with different doses of BLM at different cell cycle stages after 64 and 96 hrs respectively. BLM induced a high frequency of

However, at the 96 hr sampling time, there was not a significant difference between 100 and 200 µg/ml BLM in the frequency of AP for cells treated at the G2 cell cycle stage or between 100 and 300 µg/ml BLM for cells treated at the G0 and G1 phases of the cell cycle.

The statistical comparisons between the controls and the treatment groups in both schedules and all the phases, were significantly different (p<0.05).

Comparison of Figs 3A and 3B show that there was a small increase in the frequency of AP induction when the sampling time was increased from 64 to 96 hrs. This is in contrast to the effect of sampling time on the incidence of MN.

Discussion

The results show that BLM causes apoptosis and MN formation at 64 and 96 hrs in all the cell cycle stages used in this study (G0, G1 and G2) and are consistent with other studies (16-19) and the report by Logan et al. (33) that manipulation of the cell cycle can enhance the sensitivity to cytotoxic chemicals. The interesting finding that in G2 phase, the cells were more sensitive to bleomycin induced chromosomal damage with MN formation is consistent with the results of other investigators (1, 12, 34). Sikic (35) reported the selectivity of bleomycin on G2 stage of the cell cycle. The lower and similar sensitivity G0 and G1 cells, shown by similar frequency of MN formation, is consistent with another report (36). Bleomycin also induces a higher percentage of apoptosis at G2 phase. Tounekti et al (37) reported fewer cells underwent apoptosis at G0/G1 phases which is consistent with results of this investigation where the percentage of apoptotic cells was lower at both G0 and G1. However, the susceptibility of the cell to the effect of bleomycin at this phase with resultant apoptosis and MN needs to be elucidated particularly in the light of a report by Ishida and Takahashi (38) of the synergistic effect of bleomycin and free radicals on DNA breakage. The reduction in the effect of bleomycin at 96 hrs may be due to activation of recovery mechanisms such as repair enzyme in the case of MN or free radical scavenging in the case of apoptosis (39, 40).

The pattern of dose dependency in apoptosis of this study is consistent with the results of other investigators (2, 14, 41, 42, 43). This may be related to an increase in generation of free radicals with increasing dose of BLM. ROS toxicity to cells is well documented (13, 20, 42). The increase in ROS can induce mitochondrial dysfunction, which causes a shift in the inner mitochondrial trans-membrane potential (44, 45) resulting in release of cytochrome C which activates the caspase pathway (44, 46). Significant increases in the apoptotic cells at G0, G1 and G2 phases were also apparent, and generally are dose dependent. This result is consistent with other investigators who reported that there is apoptosis at G0 (47), G1 (48) and G2 (49). The results of this investigation show that BLM can induce MN and AP simultaneously during the same phases of the cell cycle.

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References

1. Miller K: Clastogenic effects of bleomycin, cyclophosphamide and ethylmethane sulfonate on resting and proliphereting human B- and T- lymphocytes. Mutation Res 1991; 25: 241-251

2. Erexson GL, Bryant MF, Kwanyuen P, Kligerman AD: Bleomycin sulfate-induced micronuclei in human, rat, and mouse peripheral blood lymphocytes. Environ Mol Mutagen 1995; 25: 31-36

3. Umezawa H, Maeda K, Takeuchi T, Okami Y: New antibiotics, bleomycin A & B. Journal of Antibiotics 1966; 19: 200-209

4. Umezawa H: Bleomycin. In: Corcoanand W, Hahn FE (eds.), Antibiotics, vol. 3, Berlin, Springer-Verlag, 1975; 21-33

5. Terazima T, Takabe Y, Katsumata T, Watanabe M, Umezawa H: Effects of bleomycin on mammalian cell survival.

J Natl Cancer Inst 1972; 49: 1093

6. Watanabe M, Tobnika Y, Katsumata T, Terazima T: The effects of bleomycin on progression through the cell cycle of mouse L- cells. Cancer Res 1974; 55: 121-145

7. Vig, BK, Lewis R: Genetic toxicology of bleomycin. Mutation Res 1978; 55: 121-145

8. Aghamohammadi SZ, Henderson L, Cole RJ: The human lymphocytes micronuclei assay, response of cord blood lymphocytes to gamma irradiation and bleomycin. Mutation Res 1984; 30: 395-401

9. Barranco SC, Humphrey RM: The effect of bleomycin on survival and cell progression in Chinese hamster cells in vitro. Cancer Res 1971; 31: 1218-1223

10. Tobby RA: Arrest of Chinese hamster cells in G2 following treatment with the antitumor drug bleomycin. J Cell Physiol

1972; 79: 259-266

11. Byfield JE, Lee YC, Tu L, Kulhunian F: Molecular interactions of the combined effects of bleomycin and X-rays on mammalian cell survival. Cancer Res 1976; 36: 1138-1143

12. Povirk LF: Bleomycin. In: Neidle S, Waring M (eds), Molecular aspects of Anticancer Drug Action. Berlin, Springer-Verlag. 1983; 157-181

13. Kligerman AD, Bryan MF, Doerr CL, Halperin EC, Kwanyuen P, Sontag MR, Erexon GL: Interspecies cytogenetic comparison; studies with X-irradiation and bleomycin sulfate. Environ. Mol Mutagen 1992; 19: 235-243

14. Suginaka R, Izui R, Inoue J-I, Muraoka, Y, Yamaguchi K, Otsuka M, Umezawa K: Induction of apoptosis in human pancreatic carcinoma cells by synthetic bleomycin-like ligand. Jpn J Cancer Res 1998; 89: 947-953

15. Platti PC, Tanzarella C, Cozzi R, Ricord R, Vitaglisno E, Fiore M: Comparison of the frequencies of SCES induced by chemical mutagens in bone marrow, spleen and spermatogonial cell in mice Mutation Res 1982; 103: 191-195

16. Doerr CL, Harrington-Brock K, Moore MM: Micronucleus, chromosome aberration and small-colony tk mutant analysis to quantitate chromosomal damage in L5178y mouse lymphoma cells. Mutation Res 1989; 222: 191-203

17. Mozdarani H, Monfared AS: Laser thermia enhances the clastogenic effects of antineoplastic agents in aerobic and chronically hypoxic Hela cells in vitro. Cancer Lett 2001; 167: 17-24

18. Umezawa K, Otsuka M: Molecular design of apoptosis inducing agents derived from bleomycin. Gan To Kagaku Ryoho 1997; 24: 412-417

19. Araki T, Enokido Y, Inamura N, Aizawa S, Reed J C, Hatanaka H: Changes in c-Jun but not Bcl-2 family proteins in p53-dependent apoptosis of mouse cerebellar granule neurons induced by DNA damaging agent bleomycin. Brain Res 1998; 794: 239-247

20. Otsuka M, Satake H, Sugiura Y: Restructuring of the bleomycin metal core. Novel oxygen-activating ligands with symmetrized structure. Tetrahedron lett 1993; 34: 8497-8500

21. Hug H, Strand S, Grambihler A, Galle J, Hack V, Stremmel W, Krammer PH, Galle PR: Reactive oxygen intermediates are involved in the induction of CD95 ligand mRNA expression by cytostatic drugs in hepatoma cells. J Biol Chem 1997; 272: 28191-28193

22. Geske FJ, Nelson AC, Lieberman R, Strange R, Sun T, Gerschenson LE: DNA repair is activated in early stages of p53-induced apoptosis. Cell Death Diff 2000; 7: 393-401

23. Mesner PW Jr, Budihardjo II, Kaufmann SH, Chemotherapy-induced apoptoses. Adv Pharmacol 1997; 41:

461-499

24. Kaufmann WK, Kies PE: DNA signals for G2 checkpoint response in diploid human fibroblasts. Mutat Res 1998; 400: 153-167

25. Koldamova RP, Lefterov IM: Synergistic effect of CCNU and bleomycin on human lymphocytes exposed at late G1 and G2 stages of the cell cycle. Mutat Res 1991; 260: 265-269

26. Fenech M, Morely AA: Measurement of micronuclei in lymphocytes. Mutation Res 1995; 147: 29-36

27. Liegler TJ, Hyun W, Yen TS: Stites DP, Detection and quantification of live, apoptotic, and necrotic human peripheral lymphocytes by single-laser flow cytometry. Clin Diagn Lab Immunol 1995; 2: 369-376

28. Leite M, Quinta-Costa M, Leite PS, Guimaraes JE: Critical evaluation of techniques to detect and measure cell death-study in a model of UV radiation of the leukemic cell line HL60. Anal Cell Pathol 1999; 19: 139-151

29. Gasiorowski K, Brokos B, Kulma A, Ogorzalek A, Skorkowska K: Impact of four antimutagens on apoptosis in genotoxically damaged lymphocytes in vitro. Cell Mol Biol. Lett 2001; 6: 649-675

30. Gasiorowski K, Brokos B, Kulma A, Ogorzalek A, Skorkowska K: A comparison of the methods applied to detect apoptosis in genotoxically damaged lymphocytes cultured in the presence of four antimutagens. Cell Mol Biol Lett 2001; 6: 141-159

31. Lecureur V, Thottassery JV, Sun D, Schuetz EG, Lahti J, Zambetti GP, Schuetz JD: Mdr1b facilitates p53 mediated cell death and p53 is required for Mdr1b upregulation in vivo. Oncogene 2001; 20: 303-313

32. Mukhopadhyay D, Sundereshan S, Rao C, Karande AA: Placental protein 14 induces apoptosis in T cells but not in monocytes. J Biol Chem 2001; 276: 28268-28273

33. Logan TJ, Evans DL, Mercer WE, Bjornsti MA, Hall DJ: Expression of a deletion mutant of the E2F1 transcription factor in fibroblasts lengthens S phase and increases sensitivity to S phase-specific toxins. Cancer Res 1995; 55: 2883-28891

34. MacLeod RA, Voges M, Bryant PE, Drexler HG: Chromatid aberration dose responses and dispersal in human G2 lymphocytes treated with bleomycin: comparison with equivalent X-irradiation reveals formation of a novel class of heavily damaged cells. Mutat Res 1994; 309: 73-81

35. Sikic BI: Biochemical and cellular determinants of bleomycin cytotoxicity. Cancer Surv 1986; 5: 81-91

36. Hoffmann GR, Colyer SP, Littlefield LG: Induction of micronuclei by bleomycin in G0 human lymphocytes: II. Potentiation by radioprotectors. Environ Mol Mutagen 1993;

169

21: 136-43

37. Tounekti O, Belehradek J Jr, Mir LM: Relationships between DNA fragmentation, chromatin condensation, and changes in flow cytometry profiles detected during apoptosis. Exp Cell Res 1995; 217: 506-16

38. Ishida R, Takahashi T: Increased DNA chain breakage by combined action of bleomycin and superoxide radical. Biochem Biophys Res Commun 1975; 66: 1432-1438

39. Bianchi L, Tateo F, Pizzala R, Stivala LA, Grazia-Verri M, Melli R, Santamaria L: Carotenoids reduce the chromosomal damage induced by bleomycin in human cultured lymphocytes. Anticancer Res 1993; 13: 1007-1010

40. D'Agnano I, Antonelli A, Bucci B, Marcucci L, Petrinelli P, Ambra R, Zupi G, Elli R: Effects of poly(ADP-ribose) polymerase inhibition on cell death and chromosome damage induced by VP16 and bleomycin. Environ Mol Mutagen 1998; 32: 56-63

41. Christensen JG, Gonzales AJ, Cattley RC, Goldsworthy TL: Regulation of apoptosis in mouse hepatocytes and alteration of apoptosis by nongenotoxic carcinogens. Cell Growth Diff 1998; 9: 815-25

42. Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, Hara N: Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. Am J Resp Cell Mol Biol 1997; 16: 91-101

43. Kato M, Ioritani N, Suzuki T, Kambe M, Inaba Y, Watanabe R, Sasano H, Orikasa S: Mechanism of anti-tumor effect of combination of bleomycin and shock waves. Jpn J Cancer Res 91; 2000: 1065-1072

44. Cook SA, Sugden PH, Clerk A: Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential. Circ Res 1999; 85: 940-949

45. Green DR, Reed JC: Mitochondria and apoptosis. Science 1998; 281: 1309-1312

46. Priault M, Chaudhuri B, Clow A, Camougrand N, Manon S: Investigation of bax-induced release of cytochrome c from yeast mitochondria permeability of mitochondrial membranes, role of VDAC and ATP requirement. Eur J Biochem 1999; 260: 684-691

47. Guedez L, Zucali J: Bleomycin-induced differentiation of bcl-2-transfected U937 leukemia cells. Cell Growth Diff 1996; 7: 1625-1631

48. Nelson WG, Kastan MB: DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 1994; 14: 1815-1823

49. Morris SM, Domon OE, McGarrity LJ, Chen JJ, Manjanatha MG, Andrews AM, Aidoo A, Casciano DA: A role for apoptosis in the toxicity and mutagenicity of bleomycin in AHH-1 tk+/- human lymphoblastoid cells. Mutat Res 1996; 357: 143-165

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