Cancer Chemother Pharmacol (1999) 44: 362–366

ORIGINAL ARTICLE

Michael D. Story · Raymond E. Meyn

Modulation of apoptosis and enhancement of chemosensitivity by decreasing cellular thiols in a mouse B-cell lymphoma cell line that overexpresses bcl-2

Received: 22 September 1998 / Accepted: 9 February 1999

Abstract *Purpose*: To determine whether the difference in the apoptosis and clonogenic survival responses to radiation observed between the murine lymphoma cell lines LY-ar, which expresses bcl-2, and LY-as, which does not, was also evident after treatment with chemotherapy agents; and to determine whether clonogenic survival after chemotherapy agent exposure could be diminished by enhancing apoptosis through a decrease in cellular thiols. *Methods*: Cells were treated with cisplatin, VP-16, or Adriamycin, and apoptosis was determined using a DNA fragmentation assay. Cellular survival was quantified by limiting dilution assay. Intracellular thiols were decreased by maintaining LY-ar cells in cystine/methionine-free medium (CMF medium) for 7 h after drug treatment. *Results*: LY-as cells were approximately four times more likely to undergo apoptosis than LY-ar cells, having differences in apoptosis of 80% and 20%, respectively, for the agents used. LY-as cells were also more sensitive as measured by cellular survival, with a dose-modifying factor of about 1.8 measured at a 10% survival level. Incubation of LY-ar cells in CMF medium after drug treatment increased apoptosis and reduced clonogenic survival to the levels seen in LY-as cells, except after treatment with VP-16, where the reduction in cell survival was more modest. *Conclusions*: Decreasing intracellular thiols enhances apoptosis and cell killing in lymphoma cells after exposure to a variety of chemotherapy agents. This may be especially true for tumor cells that overexpress bcl-2, a gene that modifies cellular thiol status and conveys resistance to apoptosis. In this case, decreasing cellular thiols allows killing independent of the expression of bcl-2.

M.D. Story (✉) · R.E. Meyn
Department of Experimental Radiation Oncology,
The University of Texas M.D. Anderson Cancer Center,
1515 Holcombe Blvd., Houston, TX 77030, USA
e-mail: mstory@mdanderson.org
Tel.: +1-713-792-3424; Fax: +1-713-794-5369

Key words Apoptosis · Bcl-2 · Thiols · Cisplatin · Adriamycin

Introduction

Chemotherapy agents are known to induce apoptosis in tumor cells and the protein products of certain specific genes correlate with the extent of apoptosis. One such protein is bcl-2. Cell lines derived from tumors that express more bcl-2 protein are often more resistant to chemotherapy agents than cell lines of the same tumor type that do not express bcl-2 [6, 21]. In contrast, cell lines in which bax, a bcl-2 protein family member, is upregulated, are likely to undergo more apoptosis and be relatively more sensitive than those that do not [16, 21]. Transfection of these genes and the use of antisense message to downregulate their expression has produced corresponding results [8, 32, 33]. Clinical correlations between expression of bcl-2, bax, or bcl-X with chemotherapeutic resistance or sensitivity have predicted treatment outcome in some cases [13, 15] but not in others [4, 5, 7, 11].

In our own studies, we have described a mouse B cell lymphoma cell system composed of two cell lines that differ in the regulation of bcl-2 and glutathione [18]. The bcl-2-expressing LY-ar cells, which were derived from the non-bcl-2-expressing, radiosensitive, apoptotically permissive LY-as cell line, have gained resistance to ionizing radiation and have lost the propensity to undergo apoptosis [27]. In addition, by maintaining LY-ar cells in cystine/methionine-free medium (CMF medium) after irradiation, we have shown previously that apoptosis and radiosensitivity increase to levels similar to those in LY-as cells despite the fact that bcl-2 levels remain constant [18].

The prevailing lesion produced by ionizing radiation is the DNA strand break, and the unrejoined double strand break (DSB) is considered the predominant lethal lesion [3, 23]. For LY-as cells, DNA DSBs are far more effective in initiating the apoptotic cascade than single

strand breaks (SSBs) [27]. We were therefore interested to know: whether other agents that cause DNA damage would also initiate apoptosis; whether these two cell lines would still exhibit the same differences in apoptosis and, if so, whether they also differ in clonogenic sensitivity; and finally, whether apoptosis and clonogenic survival could be modulated in LY-ar cells by CMF medium in such a way as to sensitize them regardless of the overexpression of bcl-2. cisplatin (CP), etoposide (VP-16), and Adriamycin (ADR) were selected for this study because of the different types of DNA damage that they produce. CP produces predominantly DNA intra- and interstrand crosslinks, with strand scission due to repair via endonuclease digestion that occurs later [20, 28]. VP-16 is competitive inhibitor of topoisomerase II, which is involved in the breakage and resealing of duplex DNA during recombination and DNA replication, and, by competing for topoisomerase II, produces DNA DSBs [29]. ADR, an antibiotic, acts via the production of an ADR free radical which causes DNA strand breaks upon interaction with DNA [2]. LY-ar and LY-as cells were treated with various concentrations of these anticancer drugs followed by incubation in the presence or absence of CMF medium. Apoptosis and clonogenic cell survival were assessed.

Materials and methods

Cell culture

The murine lymphoma cell lines LY-ar and LY-as were maintained in RPMI medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma), 2 m*M* glutamine, penicillin, and streptomycin. Under these conditions at 37 °C in a 95% air/5% CO_2 atmosphere, both cell lines doubled approximately every 12 h.

Drug exposure

CP, VP-16, and ADR were dissolved and/or diluted in complete medium at appropriate concentrations. Cells (10^6) were incubated in medium with various concentrations of CP, VP-16, or ADR for 1 h at 37 °C. After drug treatment, the cells were pelleted out of the medium by centrifugation at 1000 *g* for 4 min and then placed into complete medium or CMF medium and held at 37 °C for the appropriate time. Post-treatment incubation times were chosen from a previous assessment of the maximum apoptotic response under these conditions [18].

DNA fragmentation

A DNA fragmentation assay, as described previously [26, 27], was used to quantify apoptosis. This method is a modification of that of Sellins and Cohen [24] and is dependent upon the solubility of small DNA fragments in solutions of low salt concentration. Before being used experimentally, cells were radiolabeled with ^{14}C-TdR at a final concentration of 1.11 kBq/ml over a 24-h period (two cell cycles). DNA fragmentation was defined as radioactive signal found in the supernatant fraction vs. the total radioactive signal of both supernatant and precipitable fractions from cell lysates. A previous study of apoptosis showed that for these cells apoptosis measured by DNA fragmentation is equivalent to that seen by morphological analysis [26].

Cellular survival

Survival of LY-ar and LY-as cells after drug treatment was measured using a modified serial dilution assay designed to estimate the 50% endpoint between live and dead cells [9]. Live cells were identified by positive staining for 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). After a 1-h exposure to various concentrations of drug, the cells were pelleted by centrifugation, the medium removed, and the cells washed once with PBS and placed in either complete medium or CMF medium. They were then diluted appropriately into the wells of a 96-well plate, where they remained for another 7 h. Then an equal volume of either regular medium or medium containing twice the normal concentration of cystine and methionine (for cells that had been maintained in CMF medium) was added to each well. The 96-well plates were left undisturbed for 5 days in an incubator at 37 °C. A 20-µl volume of a 500 µ*M* stock solution of MTT was then added to each well of the 96-well plate, and the plates were returned to the 37 °C incubator for an overnight incubation. The next day, wells that showed positive MTT staining were scored against those that did not in order to calculate cellular survival according to an adaptation of the method of Reed and Muench [19, 22].

Results

Chemosensitivity of the murine lymphoma cell lines to these three anticancer drugs was initially assessed on the basis of apoptosis induction. LY-ar and LY-as cells were exposed to CP, VP-16, and ADR for 1 h in regular growth medium. Apoptosis was assessed 3 h later using the DNA fragmentation assay. For all three agents, substantial differences in apoptosis were noted between LY-as and LY-ar cells, reaching as high as 80% and 20%, respectively, a 4 to 1 ratio (Fig. 1). Interestingly, increasing concentrations of VP-16 and ADR actually diminished apoptosis in both cell lines, particularly ADR which is very likely to be toxic at these higher

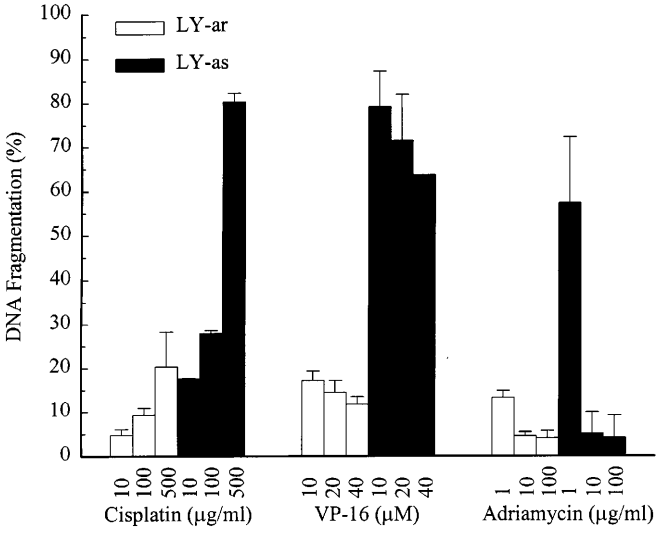

Fig. 1 Apoptosis in LY-as and LY-ar cells after exposure to CP, VP-16, and ADR. Drug exposure was for 1 h, and DNA fragmentation was determined 3 h after the removal of the drug. Error bars represent standard deviations from three independent experiments

concentrations. Others have described this effect as a poisoning of the apoptotic cascade by high drug or radiation doses [17].

We next tested a strategy for sensitizing the LY-ar cells by decreasing cellular thiols, which we had previously shown to be effective for irradiated cells. Modulation of the apoptotic potential of LY-ar cells after exposure to the same chemotherapy agents is shown in Fig. 2. Maintaining the cells in CMF medium for a 7-h period after drug removal markedly increased the extent of apoptosis. The extent of apoptosis was still dependent on drug concentration, as it was in the experiments shown in Fig. 1. Differences in apoptosis as a result of incubation in CMF medium were approximately 10-fold for CP, 3.5-fold for VP16, and 8-fold for ADR.

For these two cell lines, differences in apoptosis after irradiation were shown to be reflected in cellular survival differences [27], and this was also true after exposure to CP, VP-16, and ADR. In Fig. 3, clonogenic survival after drug exposure is shown for both cell lines. As has been shown for radiation [27], LY-as cells were more sensitive to the effects of drug exposure. At a survival level of 10%, the dose-modifying factors (DMF) for these agents were 1.83, 1.80, and 1.80 for CP, VP-16, and ADR, respectively, and compared well with the DMF of 2.40 for radiation [27].

The modulation of apoptotic potential by CMF medium was also reflected in cell survival as depicted by survival curve analysis. As seen in Fig. 3, in each case survival of LY-ar cells was diminished by CMF medium. For CP and ADR the survival of LY-ar cells in CMF medium was nearly identical to that of LY-as cells. For VP-16 the change in survival was less dramatic.

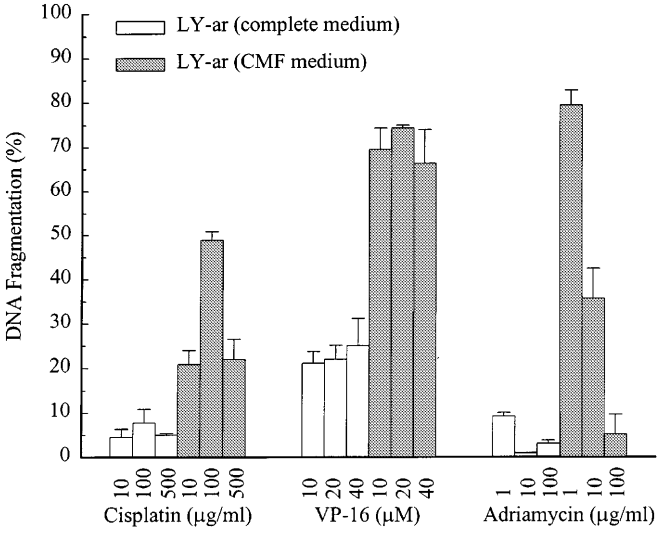

Fig. 2 Apoptosis in LY-ar cells after a 1-h exposure to CP, VP-16, or ADR followed by incubation in either regular culture medium or CMF medium for 7 h. Error bars are calculated as standard deviations from three independent experiments

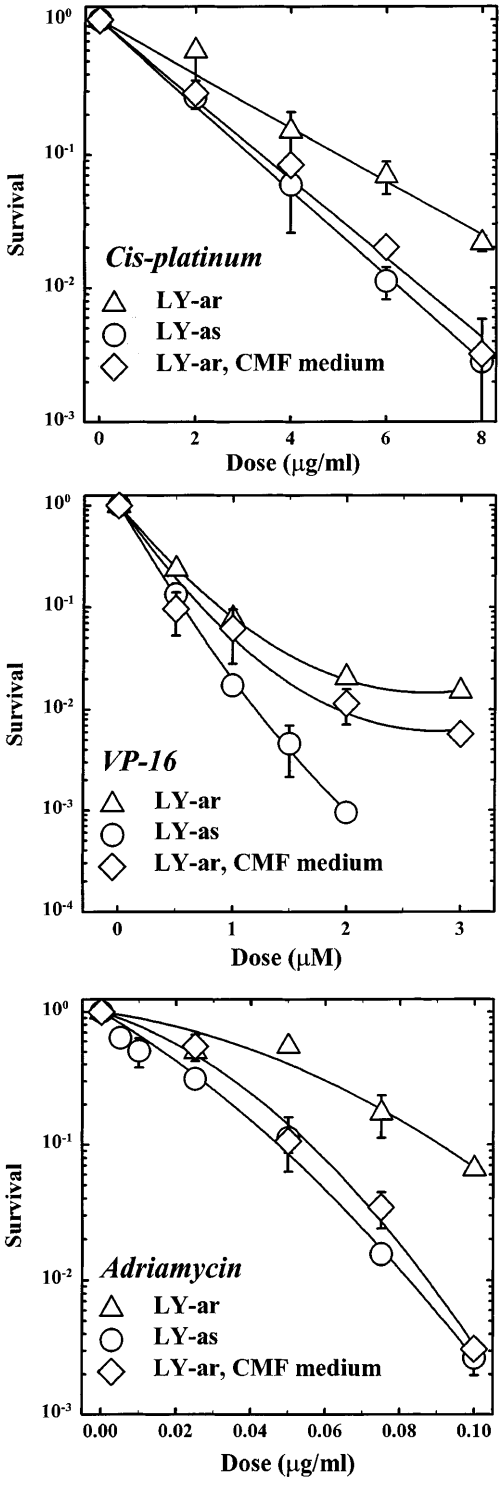

Fig. 3 Clonogenic survival, as measured by limiting dilution assay, of LY-ar (△) and LY-as (○) cells after exposure to CP, VP-16, or ADR. Drug exposure was for 1 h, after which the drug was removed and the cells maintained in regular medium. Modulation of cellular survival of LY-ar cells following alteration in redox status by their maintenance in CMF medium for 7 h after a 1 h exposure to CP, VP-16 or ADR is also shown (◇). Error bars represent standard deviations from two independent experiments, each with replicate points

Discussion

Apoptosis is a major mode of cell death in the response of tumor cells to cytotoxic cancer therapies. Loss of apoptotic potential, usually by the unregulated expression of particular genes such as bcl-2 or the loss of function of particular genes such as p53 or bax, has been linked to the chemoresistance of some tumor cell lines or the in poor treatment outcomes seen in some cancer patients. Because of this, determining the apoptotic response of tumors may provide insight into treatment efficacy or might be used to support alternative treatment strategies designed to circumvent the chemoresistance that is associated with the loss of apoptotic propensity.

DNA fragmentation is often used as a reliable marker for measuring apoptosis. However, inhibition of the DNA fragmentation associated with apoptosis does not necessarily lead to altered clonogenic survival [30]. Therefore, it was important for these studies to measure clonogenic survival to validate the notion that alterations in apoptosis would alter tumor cell chemosensitivity. In this study we examined the link between apoptotic propensity and chemosensitivity of two related mouse B cell lymphoma cell lines. The LY-ar cell line, which overexpresses bcl-2 protein and contains twice the content of reduced glutathione as LY-as cells, has been previously shown to be more resistant to radiation-induced apoptosis and more radioresistant [18, 27]. We subsequently showed that the apoptotic resistance can be modulated by CMF medium and that this enhances the radiosensitivity of these cells [18].

In the present study, we showed that these relationships extend to CP, VP-16, and ADR. However, the extent to which apoptotic death can affect clonogenic survival is likely dependent upon the drug used and the cell type. For example, after VP-16 exposure apoptosis increased dramatically in LY-ar cells that were incubated in CMF medium, yet clonogenic survival was altered only modestly. However, what may be more important is the type of cell being challenged by these chemotherapy agents. All three of these agents make strand breaks, albeit through different mechanisms. Both LY-ar and LY-as cells are prepared to undergo apoptosis in response to the appropriate stimulus. In LY-ar cells that signal transduction pathway is blocked and when that block is released, such as has occurred here by thiol depletion, the response is the same as in LY-as cells, that is, apoptosis. This means that regardless of the stimulus, be it DNA crosslinking, radical attack, or unrejoined DNA recombinational events, the result is the initiation of the apoptotic signaling cascade.

The mechanism by which bcl-2 inhibits apoptosis, appears to be quite complex [25]. A role for bcl-2 functioning in an antioxidant pathway was hypothesized several years ago [10, 12, 18], but until recently there has been little additional insight. We have shown that one of the functions of bcl-2 involves its redistribution of GSH into the nucleus via active transport, where it apparently blocks apoptosis by inhibiting caspase activity [31]. Thus, incubation in CMF medium may reverse this nuclear localization of GSH and thereby circumvent bcl-2's ability to block apoptosis. Whereas this requires further experimental verification, strategies designed to decrease cellular thiols would have to target nuclear thiol levels specifically to accomplish this sensitization. Nonetheless, such strategies may be ultimately useful in overcoming drug resistance due to bcl-2 expression. It is also important to point out that these strategies should be distinguished from those targeted to drug-resistance mechanisms whereby the drug activity is scavenged by intracellular thiols or thiol-mediated detoxification pathways [1, 14]. Here, the modification of thiols occurs after drug delivery, not prior to drug treatment, and we propose that this represents a previously unknown sensitization mechanism involving suppression of a thiol-mediated inhibition of apoptotic cell death.

In summary, the results of this investigation suggest that, in cases where resistance to chemotherapy agent-induced loss of clonogenic survival is associated with an overexpression of bcl-2, decreasing cellular thiols can alter clonogenic survival through a potentiation of apoptotic propensity. In a companion article we examine apoptosis and tumor growth delay in tumors generated in sygeneic mice using these cell lines for following treatment with the chemotherapeutic agents CP, VP-16, and ADR and with camptothecin, citarabine, and cyclophosphamide.

Acknowledgements This work was supported in part by NIH grants CA69003 (REM), CA62209 (MDS), and the institutional Core Grant CA16672 from the National Cancer Institute.

References

1. Asakura T, Takahashi N, Takada K, Inoue T, Ohkawa K (1997) Drug conjugate of doxorubicin with glutathione is a potent reverser of multidrug resistance in rate hepatoma cells. Anticancer Drugs 8: 199
2. Berlin V, Haseltine WA (1981) Reduction of Adriamycin to a semiquinone-free radical by NADPH cytochrome P-450 reductase produces DNA cleavage in a reaction mediated by molecular oxygen. J Biol Chem 256: 4747
3. Blöcher D, Pohlet W (1982) DNA double-strand breaks in Ehrlich ascites tumour cells at low doses of X-rays II. Can cell death be attributed to double-strand breaks? Int J Radiat Biol 42: 329
4. Campos L, Sabido O, Sebban C, Charrin C, Bertheas MF, Fiere D, Guyotat D (1996) Expression of BCL-2 proto-oncogene in adult acute lymphoblastic leukemia. Leukemia 10: 434
5. Carroll RS, Zhang J, Chauncey BW, Chantziara K, Frosch MP, Black PM (1997) Apoptosis in astrocytic neoplasms. Acta Neurochir 139: 845
6. Chresta CM, Arriola EL, Hickman JA (1996) Apoptosis and cancer chemotherapy. Behring Inst Mitt 97: 232
7. Coustan-Smith E, Kitanaka A, Pui CH, McNinch L, Evans WE, Raimondi SC, Behm FG, Arico M, Campana D (1996) Clinical relevance of BCL-2 overexpression in childhood acute lymphoblastic leukemia. Blood 87: 1140

8. Dole MG, Clarke MF, Holman P, Benedict M, Lu J, Jasty R, Eipers P, Thompson CB, Rode C, Bloch C, Nunez, Castle VP (1996) Bcl-X_S enhances adenoviral vector-induced apoptosis in neuroblastoma cells. Cancer Res 56: 5734
9. Dynlacht JD, Henthorne J, O'Nan C, Dunn ST, Story MD (1996) Flow cytometric analysis of nuclear matrix proteins: method and potential applications. Cytometry 24: 348
10. Ellerby LM (1996) Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2. J Neurochem 67: 1259
11. Herod JJ, Eliopoulos AG, Warwick J, Niedobitek G, Young LS, Kerr DJ (1996) The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. Cancer Res 56: 2178
12. Hockenberry D, Nunez G, Schreiber RD, Korsmeyer SJ (1990) *Bcl-2* is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348: 334
13. Kaiser U, Schilli M, Haag U, Neumann K, Kreipe H, Kogan E, Havemann K (1996) Expression of bcl-2-protein in small cell lung cancer. Lung Cancer 15: 31
14. Massart C, Gibassier J, Lucas C, Pourquier P, Robert J (1996) Expression of the MDRI gene in five human cell lines of medullary thyroid cancer and reversion of the resistance to doxorubicine by cyclosporin A and verapamil. Bull Cancer 83: 39
15. McConkey DJ, Chandra J, Wright S, Plunkett W, McDonnell TJ, Reed JC, Keating M (1996) Apoptosis sensitivity in chronic lymphocytic leukemia is determined by endogenous endonuclease content and relative expression of BCL-2 and BAX. J Immunol 156: 2624
16. McCurrach ME, Connor TM, Knudson CM, Korsmeyer SJ, Lowe SW (1997) Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proc Natl Acad Sci USA 94: 2345
17. Meyn RE, Stephens LC, Ang KK, Hunter NR, Milas L, Peters LJ (1993) Heterogeneity in apoptosis development among irradiated murine tumors of different histologies. Int J Radiat Biol 64: 583
18. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, Meyn RE (1997) Resistance to radiation-induced apoptosis in bcl-2-expressing cells is reversed by depleting cellular thiols. Oncogene 15: 1461
19. Nagasawa H, Chen DJ, Strniste GF (1991) Response of X-ray sensitive CHO cells to γ radiation. 1. Effects of low dose rates and the process of repair of potentially lethal damage in G_1 phase. Radiat Res 118: 559
20. Pascoe JM, Roberts JJ (1974) Interactions between mammalian cell DNA and inorganic platinum compounds. I. DNA interstrand cross-linking and cytotoxic properties of platinum (II) compounds. Biochem Pharmacol 23: 1359
21. Pepper C, Hoy T, Bentley DP (1997) Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. Br J Cancer 76: 935
22. Reed LJ, Muench H (1938) A simple method for estimating fifty per cent endpoints. Am J Hyg 27: 493
23. Ritter MA, Cleaver JE, Tobias CA (1977) High-LET radiations induce a large proportion of non-rejoining DNA breaks. Nature 266: 653
24. Sellins KS, Cohen JJ (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J Immunol 139: 3199
25. Story M, Kodym R (1998) Signal transduction during apoptosis; implications for cancer therapy. Front Biosci 3: d365
26. Story MD, Voehringer DW, Stephens LC, Meyn RE (1993) L-Asparaginase kills lymphoma cells by apoptosis. Cancer Chemother Pharmacol 32: 129
27. Story MD, Voehringer DW, Malone CG, Hobbs ML, Meyn RE (1994) Radiation-induced apoptosis in sensitive and resistant cells isolated from a mouse lymphoma. Int J Radiat Biol 66: 659
28. van den Berg HW, Roberts JJ (1975) Investigations into the mechanism of action of anti-tumor platinum compounds: time- and dose-dependent changes in the alkaline sucrose gradient sedimentation profiles of DNA from hamster cells treated with cis-platinum (II) diamminedichloride. Chem Biol Interact 11: 493
29. van Maanen JM, Retel J, deVries J, Pinedo HM (1988) Mechanism of action of antitumor drug etoposide: a review. J Natl Cancer Inst 80: 1526
30. Voehringer DW, Story MD, O'Neil RG, Meyn RE (1997) Modulating Ca^{2+} in radiation-induced apoptosis suppresses DNA fragmentation but does not enhance clonogenic survival. Int J Radiat Biol 71: 237
31. Voehringer DW, McConkey DJ, McDonnell TJ, Brisbay S, Meyn RE (1988) Bcl-2 expression causes redistribution of glutathione to the nucleus. Proc Natl Acad Sci USA 95: 2956
32. Wagener C, Bargou RC, Daniel PT, Bommert K, Mapara MY, Royer HD, Dorken B (1996) Induction of the death-promoting gene bax-alpha sensitizes cultured breast-cancer cells to drug-induced apoptosis. Int J Cancer 67: 138
33. Ziegler A, Luedke GH, Fabbro D, Altmann KH, Stahel RA, Zangemeister-Wittke U (1997) Induction of apoptosis in small-cell lung cancer cells by an antisense oligodeoxynucleotide targeting the Bcl-2 coding sequence. J Natl Cancer Inst 89: 1027