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**APOPTOSIS AND CLONOGENIC SURVIVAL IN THREE TUMOUR  
CELL LINES EXPOSED TO GAMMA RAYS OR CHEMICAL  
GENOTOXIC AGENTS**

SŁAWOMIR KUMALA<sup>1</sup>, PAWEŁ NIEMIEC<sup>2</sup>, MARIA WIDEL<sup>1</sup>, RONALD  
HANCOCK<sup>3</sup> and JOANNA RZESZOWSKA-WOLNY<sup>1\*</sup>

<sup>1</sup>Department of Experimental and Clinical Radiobiology, Center of Oncology,  
Wybrzeże AK 15, 44-100 Gliwice, Poland, <sup>2</sup>Department of Chemistry and  
General Biochemistry, Silesian Medical Academy, 40-752 Katowice, Poland,  
<sup>3</sup>Laval University Cancer Research Centre, 9 rue MacMahon, Quebec,  
Canada G1R 2J6

**Abstract:** We compared the extent to which apoptosis is induced and clonogenicity reduced in three tumour cell lines – the human melanoma Me45 and promyelocytic leukaemia HL-60, and the rat rhabdomyosarcoma R1 – after exposure to the anticancer drugs etoposide and cis-platinum or to gamma radiation; each induces different types of DNA damage. Cells which readily underwent apoptosis did not necessarily show a correlated loss of clonogenicity; for example, Me45 cells showed the highest sensitivity to all three agents in clonogenic assays but much lower levels of apoptotic cells than R1 or HL-60 cells. These results show that the efficiency of the eradication of clonogenic cells by genotoxic agents does not solely depend on the induction of apoptotic processes, and suggest that the induction of apoptosis and suppression of clonogenicity are independent processes.

**Key Words:** Apoptosis Induction, Clonogenic Survival, Micronuclei, cis-platinum, Etoposide, Gamma Rays

**INTRODUCTION**

The cytotoxic and genotoxic agents used in clinical therapy show variable effectiveness against different types of tumour; for example, tumours originating

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\* Corresponding author: tel: (+4832) 278-96-77, fax: (+4832) 231-35-12,  
e-mail: [jwolny@io.gliwice.pl](mailto:jwolny@io.gliwice.pl)  
Abbreviations used: VP-16 - etoposide; cDDp or cis-platinum - cis-diaminodichloro-platinum.

from lymphoid cells (lymphomas, myelomas, leukaemias) are generally highly sensitive to radiation therapy, while squamous cell carcinomas show intermediate sensitivity, and melanomas and gliomas are the most resistant [1]. Even tumours of the same histological type can show variable responses to the same type of treatment [2]. This differential sensitivity to particular treatments may depend on many factors, with the efficiency of DNA repair and the cell cycle control mechanisms probably being the most important.

Successful cancer therapy must ensure the eradication of all clonogenic tumour cells from the organism, because cancer cells undergo unlimited division and even a single cell may be the source of a repopulating clone. The clonogenic survival assay is a classical method for testing cellular sensitivity to different doses of cytotoxic and genotoxic anticancer agents *in vitro*. It is used to determine the fraction of cells able to form colonies [3-5]. This seems to be one of the best tests for predicting the responsiveness of a tumour to clinical treatment [3, 6, 7], but it is not clinically applicable due to its long duration. It can be misleading for cells with low plating efficiency, long doubling time, or an inability to form colonies [8]. Consequently, other assays that measure DNA damage or apoptosis in cells treated with an anticancer drug have been sought. The measurement of the induction of apoptosis in cancer cells is simpler than clonogenic assays and is a relatively short process. It appears logical that apoptosis should be inversely correlated with clonogenic survival, but data are accumulating which cast doubt on the importance of apoptotic processes in the eradication of clonogenic tumor cells [9-12]. In this study, we compare the induction of apoptosis and the loss of clonogenicity in cells of three tumor cell lines exposed to two chemical anticancer drugs or to ionizing radiation.

## MATERIALS AND METHODS

### Cell cultures

Two human and one rat tumor cell line were used in this study – the human skin melanoma line Me45 (established at the Institute of Oncology, Gliwice), the human promyelocytic leukaemia line HL-60 (donated by the Institute of Biochemistry, Lviv, Ukraine), and the rat rhabdomyosarcoma line R1 (from the Institute of Immunology, Wroclaw, Poland). Me45 cells were grown as monolayers in DMEM/F12 [1/1] medium (Sigma), R1 cells as monolayers in DMEM (Sigma), and HL-60 cells in suspension in RPMI (Sigma), all supplemented with 15% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, and 80 µg/ml gentamycin (Polfa, Poland). The medium was changed twice a week.

### Exposure to gamma radiation, cis-platinum or etoposide

Exponentially growing cells were irradiated in a Philips Gammatron with a <sup>60</sup>Co source with 2, 4, 6, 8 or 10 Gy at a dose rate of 1 Gy/min, or exposed to cis-diaminodichloroplatinum (cis-platinum) or etoposide (VP-16) at 1, 2, 5 or 10 µg/ml in growth medium for 2 hours at 37°C. The medium was then replaced

with fresh medium and the cells were incubated for an appropriate time before scoring apoptosis or micronuclei.

#### **Clonogenicity assays**

An appropriate number of cells were seeded into 6 cm dishes (Nunc) in 5 ml of growth medium, supplemented with agarose (Sigma) to a final concentration of 0.5% for HL-60 cells. After 16 hours, the cells in the dishes were exposed to radiation or to different concentrations of etoposide or cis-platinum for 2 h. Immediately after treatment, the medium was replaced and the cells were incubated at 37°C in humidified air plus 5% CO<sub>2</sub> for colony formation for a period of 14-17 days. On day eight, 2 ml of fresh medium was carefully added to the dishes. When the incubation was terminated, colonies were fixed with ethanol, washed with distilled water and stained with crystal violet. Colonies containing at least 30 cells were counted. The data presented in Fig. 3 indicate the mean values and the standard deviations of four determinations.

#### **Apoptosis and micronucleus assays**

At 24, 48 and 72h after gamma radiation or treatment with chemical agents, cells were trypsinized, centrifuged at 1000 rpm for 5 min, fixed in methanol:acetic acid (3:1), air dried on a microscope slide, and stained with DAPI (4',6'-diamidinophenylindole) (2 µg/ml in water). Apoptotic cells were identified via fluorescence microscopy using the criteria of marked chromatin condensation, fragmentation of nuclei, and bright staining with DAPI [13]. In each sample, 1000 cells were scored and each experiment was performed twice. Micronucleated cells were counted on the same slides using the criteria proposed by Falkvoll [14]. The data presented in Figs. 1, 2 and 3 indicate the mean values and the standard deviations from two experiments.

## **RESULTS**

#### **Induction of apoptosis**

First, we compared the induction of apoptosis by gamma radiation or by the widely-used anticancer drugs etoposide and cis-platinum in the human melanoma line Me45, the promyelocytic leukaemia line HL-60, and the rat rhabdomyosarcoma line R1. The cells were exposed to different concentrations of the drugs and apoptosis was assayed 72 h after drug removal. R1 cells showed a markedly higher level of apoptosis than Me45 and HL-60 cells after exposure to cis-platinum and etoposide (Fig. 1A, B); cis-platinum at 10 µg/ml induced apoptosis in more than 99% of the R1 cells, whereas in the other cell lines the level of apoptotic cells did not exceed 30%. Me45 cells were the most resistant and the level of apoptosis did not exceed 20% at drug concentrations of 1-10 µg/ml. Furthermore, melanoma cells were also resistant to apoptosis induction by gamma radiation (Fig. 1C). By contrast, HL-60 cells, which did not exhibit

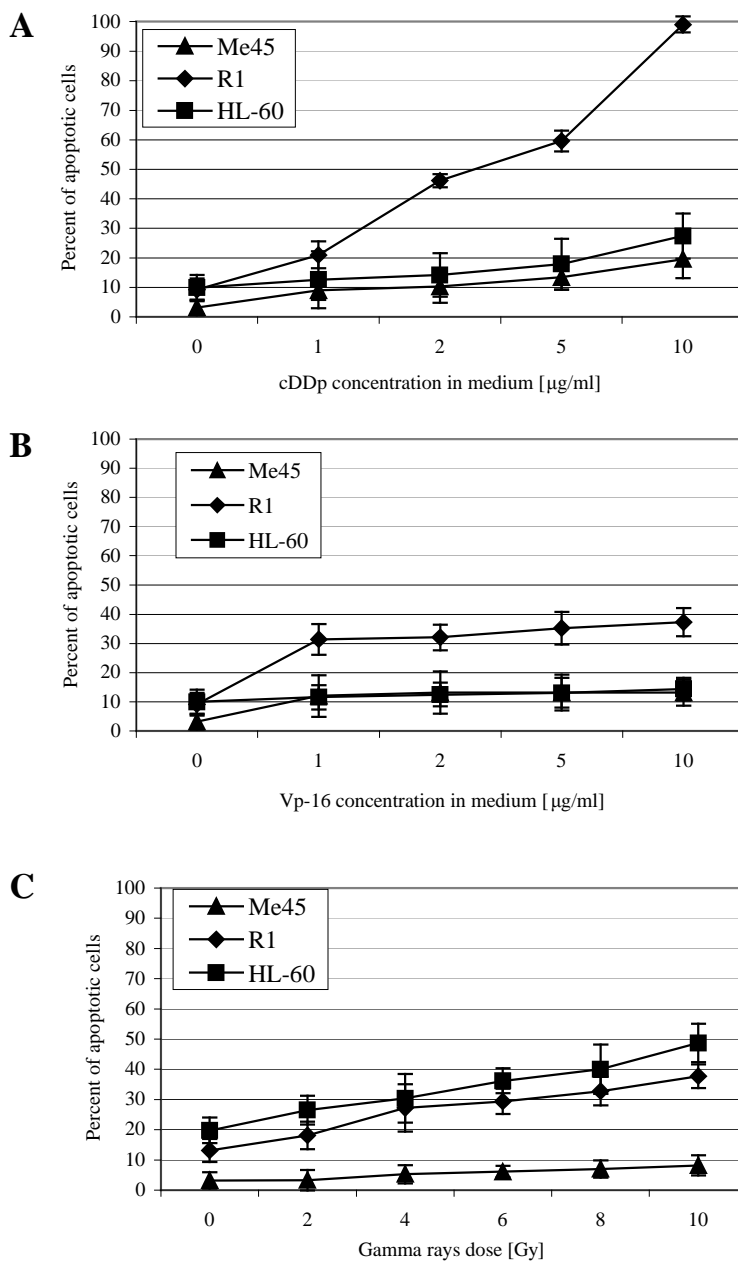


Fig. 1. The induction of apoptosis in R1, HL-60 and Me45 cell lines by different doses of cis-platinum (A), etoposide (B) and  $\gamma$ -rays (C).

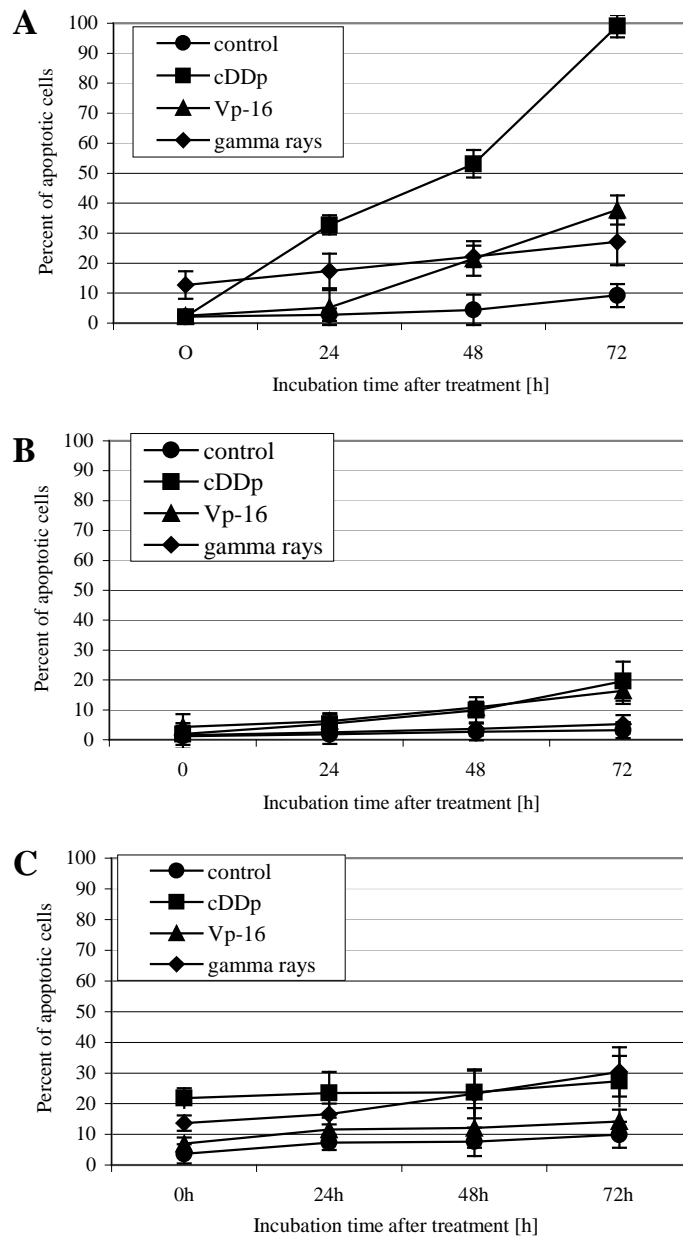


Fig. 2. The kinetics of the appearance of apoptotic cells after the exposure of R1(A), Me45(B) and HL-60(C) cells to cis-platinum (10  $\mu\text{g/ml}$ ), etoposide (10  $\mu\text{g/ml}$ ) or  $\gamma$ -rays (10 Gy).

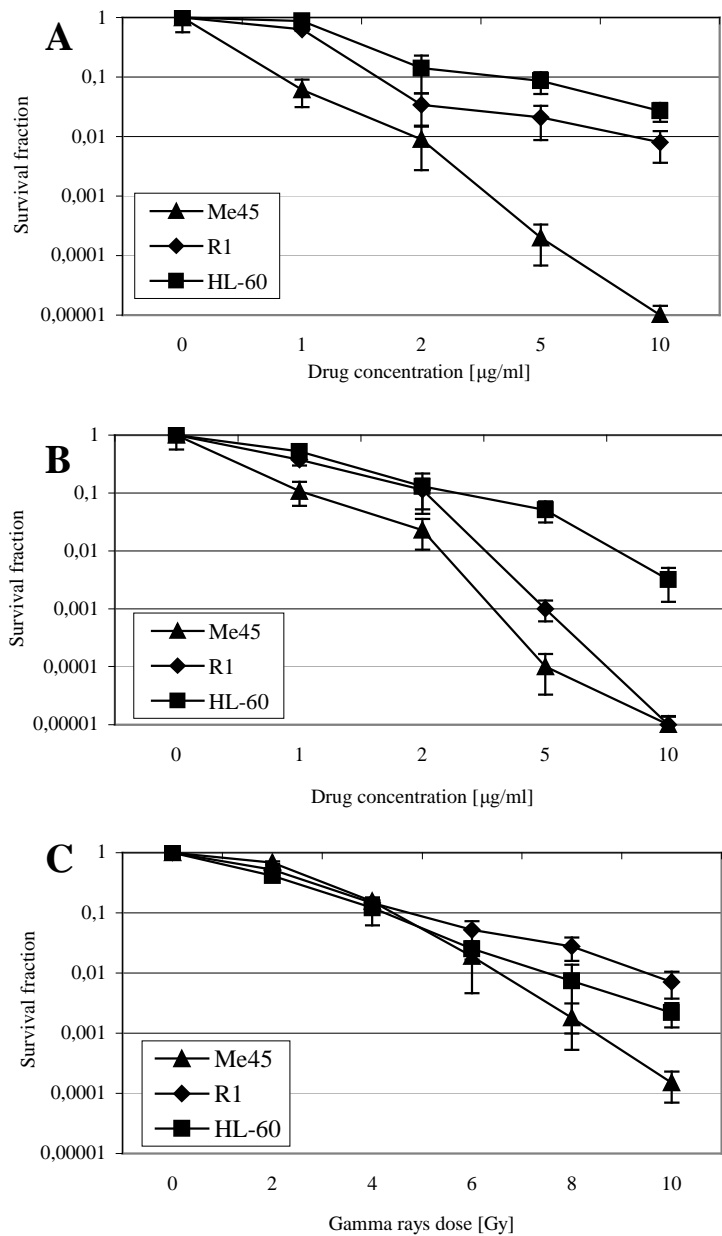


Fig. 3. The clonogenic survival of cells exposed to cis-platinum (A), etoposide (B) and  $\gamma$ -rays (C).

apoptosis after treatment with etoposide (Fig. 1B), were the most sensitive to the induction of apoptosis by gamma rays (Fig. 1C).

Kinetic studies showed that apoptotic R1 cells appeared with increasing frequency up to 72 h after exposure to drugs at 10  $\mu\text{g/ml}$ , and were detected earlier in cells exposed to cis-platinum than to etoposide (Fig. 2A). Gamma radiation induced a higher level of apoptosis immediately after the treatment of R1 cells (Fig. 2A). Me45 and HL-60 cells were markedly less sensitive to the induction of apoptosis by the chemical agents (Fig. 2B, C). By contrast, apoptotic HL-60 cells appeared immediately after exposure to genotoxic agents and showed a minor increase up to 72 h (Fig. 2C). Me45 cells did not show apoptosis after gamma radiation; cis-platinum and etoposide were more efficient but the level of apoptosis induced did not exceed 20% after 72 h (Fig. 2B).

### Clonogenic survival

We then studied the loss of clonogenicity of these cell lines after exposure to the same genotoxic conditions. Me45 cells were extremely sensitive to all three genotoxic agents (Fig. 3) in spite of their relatively low frequency of apoptosis; 10  $\mu\text{g/ml}$  of cis-platinum or etoposide reduced the clonogenic fraction by 5 orders of magnitude (Fig. 3A, B). In terms of clonogenic cell survival, HL-60 cells were less sensitive to both drugs, whereas R1 cells showed intermediate sensitivity. Survival curves after irradiation were similar for all three cell lines at lower doses; however, between 6 and 10 Gy their slopes differed and the surviving fraction for the most radiosensitive melanomas was nearly two orders of magnitude lower than that for the most resistant rhabdomyosarcoma.

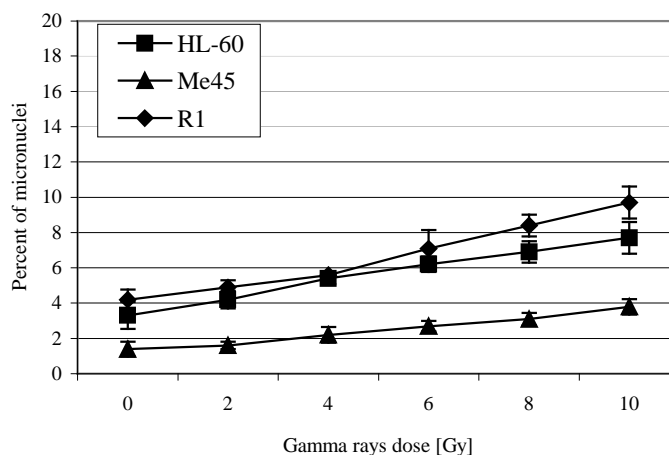


Fig. 4. The induction of micronucleus formation in R1, Me45 and HL-60 cells irradiated with  $\gamma$ -rays.

**The induction of micronucleus formation by gamma radiation**

Double-strand breaks in DNA may result in the creation of micronuclei. We examined the relative induction of micronuclei in the three cell lines by gamma radiation. R1 cells were most sensitive, Me45 cells the least sensitive, and HL-60 cells showed intermediate sensitivity (Fig. 4).

**DISCUSSION**

The aim of anticancer therapy is to eradicate all tumour cells which are potentially clonogenic; therefore, knowledge about a tumour's sensitivity to drugs or radiation prior to decisions on therapy could be very important for the outcome. Predictive tests based on the reduced clonogenicity of tumour cells in response to anticancer drugs are in many cases well correlated with the efficiency of clinical therapy [3, 6, 7], but clonogenicity testing is time consuming and sometimes misleading [8]. Other tests based on the induction of DNA damage and apoptosis or on DNA repair capacity are still under study. In the search for a simple test of individual tumour sensitivity to therapeutic agents, the measurement of the induction of apoptosis seems to be relatively rapid and promising, and it appears logical that the propensity to undergo apoptosis during treatment should be correlated with the loss of clonogenicity of the tumour cells. Several authors have reported such a correlation for irradiated cells [14, 15, 18]. However, the results described here indicate that cells which readily undergo apoptosis after exposure in culture to cytostatic drugs or gamma radiation do not necessarily show a correlated loss of clonogenicity. In fact, melanoma cells, which of those studied were the most resistant to the induction of apoptosis, showed the highest sensitivity in clonogenic assays. A similar lack of correlation between apoptosis and clonogenic survival after irradiation has been reported by others [9-12, 16, 17]. The same cells may show a very different reduction of clonogenicity in conditions where the induction of apoptosis is similar and *vice versa*. In R1 cells, 37-38% are apoptotic after 10 Gy of gamma radiation or 10 µg/ml of etoposide, but clonogenic cells are nearly completely eradicated by etoposide at the same concentration (survival fraction  $10^{-5}$ ), whereas gamma radiation at the dose of 10 Gy left the survival fraction close to  $10^{-2}$ . In HL-60 cells, the clonogenic fraction after 10 Gy gamma radiation or 10 µg/ml etoposide is similar, although there is a large difference in the level of apoptotic cells (14% and 50% respectively). Multivariate analysis of these data shows a negative correlation between the surviving cell fraction and the fraction of apoptotic and micronucleated cells, but the slopes of the correlation curves for different cell lines are very different; at 0.01 surviving fraction, about 50% of HL-60 and R1 cells but only 9% of Me45 cells are apoptotic (data not shown). These results strongly suggest that the reduction of clonogenicity does not solely depend on the induction of apoptotic processes, and that a high fraction of apoptotic cells does not necessarily lead to a reduction of clonogenicity. It has been shown that a correlation between reproductive cell death and radiosensitivity measured

separately by apoptosis or micronucleus yield can be improved by combining both assays [13, 19]. However, our study showed that Me45 cells, radiosensitive on the basis of clonogenic survival assays, express neither a high level of apoptosis nor of micronucleus formation. Thus, other factors such as mitotic failure or cell death through necrosis could contribute to the high radiosensitivity of these melanoma cells; however, necrosis was not assessed in this study. The answer to the very basic and important question for tumour therapy of why the level of apoptosis or inhibition of clonogenicity in response to different types of DNA damage can be so different in the same cells may lie in the processes of recognition of DNA damage. The agents studied here induce different types of DNA damage; gamma radiation induces various oxidative changes in DNA bases together with single- and double-strand breaks [20, 21]; cis-platinum introduces inter- and intra-strand crosslinks [22, 23], while etoposide causes strand breaks in the vicinity of sites where topoisomerase II acts [24, 25]. DNA adducts induced by cis-platinum and strand breaks or oxidative damage induced by gamma radiation are recognized and repaired by different systems [26], of which some also participate in signal transduction pathways [27]. The differential induction of apoptosis and inhibition of clonogenicity following exposure to different genotoxic agents is consistent with the idea that these processes are initiated by different proteins and depend on different signaling pathways and that the efficiency of cell cycle controls rather than induction of apoptosis may determine survival. Further study is required to establish what are the proteins and signal transduction pathways crucial for the inhibition of clonogenic survival.

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## REFERENCES

1. Fertil, B. and Malaise, E.P. Intrinsic radiosensitivity of human cell lines is correlated with radioresponsiveness of human tumors: Analysis of 101 published survival curves. **Int. J. Radiat. Oncol. Biol. Phys.** 11 (1985) 1699-1707.
2. Rofstad, E.K., Wahl, A. and Brustad, T. Radiation sensitivity *in vitro* of cells isolated from human tumor surgical specimens. **Cancer Res.** 47 (1987) 106-110.
3. Bjork-Eriksson, T., West, C., Karlsson, E. and Mercke, C. Tumor radiosensitivity (SF2) is a prognostic factor for local head and neck cancers. **Int. J. Radiat. Oncol. Biol. Phys.** 46 (2000) 13-19.
4. Budach, W., Gioioso, D., Taghian, A., Stuschke, M. and Suit, H.D. Repopulation capacity during fractionated irradiation of squamous cell

- carcinomas and glioblastomas in vitro. **Int. J. Radiat. Oncol. Biol. Phys.** 39 (1997) 743-750.
5. Geard, C.R. and Chen, C.Y. Micronuclei and clonogenicity following low- and high-dose-rate X irradiation of normal human fibroblasts. **Radiat. Res.** 124 (1990) Suppl. 56-61.
  6. Peters, L.J., Brock, W.A., Johnson, T., Meyn, R.E., Tofilon, P.J. and Milas, L. Potential methods for predicting tumor radiocurability. **Int. J. Radiat. Oncol. Biol. Phys.** 12 (1986) 459-467.
  7. West, C.M., Davidson, S.E., Roberts, S.A. and Hunter, R.D. The independence of intrinsic radiosensitivity as a prognostic factor for patient response to radiotherapy of carcinoma of the cervix. **Brit. J. Cancer** 76 (1997) 1184-1190.
  8. Brock, W.A., Baker, F.L., Wike, J.L., Sivon, S.L. and Peters, L. Cellular radiosensitivity of primary head and neck squamous cell carcinoma and tumor control. **Int. J. Radiat. Oncol. Biol. Phys.** 18 (1990) 1283-1286.
  9. Brunet, C.L., Gunby, R.H., Benson, R.S., Hickman, J.A., Watson, A.J. and Brady, G. Commitment to cell death measured by loss of clonogenicity is separable from the appearance of apoptosis markers. **Cell Death Differ.** 5 (1998) 107-115.
  10. Hopcia, K.L., McCarey, Y.L., Sylvester, F.C. and Held, K.D. Radiation induced apoptosis in HL 60 cells: oxygen effect, relationship between apoptosis and loss of clonogenicity, and dependence of time to apoptosis on radiation dose. **Radiat. Res.** 145 (1996) 315-323.
  11. Rudner, J., Belka, C., Marini, P., Wagner, R.J., Faltin, H., Lepple-Wienhues, A., Bamberg, M. and Budach, W. Radiation sensitivity and apoptosis in human lymphoma cells. **Int. J. Radiat. Biol.** 77 (2001) 1-11.
  12. Vavrova, J., Marekova, M. and Vokurkova, D. Radiation induced apoptosis and cell cycle progression in TP53-deficient human leukaemia cell line HL 60. **Neoplasma** 48 (2001) 26-33.
  13. Abend, M., Kehe, K., Kehe, K., Riedel, M. and Van Beuningen, D. Correlation of micronucleus and apoptosis assays with reproductive cell death can be improved by considering other modes of death, **Int. J. Radiat. Biol.** 76 (2000) 249-259.
  14. Falkvoll, K.H. The occurrence of apoptosis, abnormal mitoses, cells dying in mitosis and micronuclei in a human melanoma xenograft exposed to single dose irradiation. **Strahlenther. Onkol.** 166 (1990) 487-492.
  15. Radford, I.R., Murphy, T.K., Radley, J.M. and Ellis, S.L. Radiation response of mouse lymphoid and myeloid cell lines. Part II. Apoptotic death is shown by all lines examined. **Int. J. Radiat. Biol.** 65 (1994) 217-227.
  16. Olive, P.L., Banath, J.P. and Durand, R.E. Development of apoptosis and polyploidy in human lymphoblast cells as function of position in cell cycle at time of irradiation. **Radiat. Res.** 146 (1996) 595-602.
  17. Olive, P.L. and Durand, R.E. Apoptosis: an indicator of radiosensitivity in vitro? **Int. J. Radiat. Biol.** 71 (1997) 695-707.

18. Guo, M., Chen, C., Vidair, C., Marino, S., Dewey, W.C. and Ling, C.C. Characterization of radiation-induced apoptosis in rodent cell lines. **Radiat. Res.** 147 (1997) 295-303.
19. Guo, G.Z., Sasai, K., Oya, N., Takagi, T., Shibuya, K. and Hiraoka, M. Simultaneous evaluation of radiation – induced apoptosis and micronuclei in five cell lines. **Int. J. Radiat. Biol.** 73 (1998) 297-302.
20. Olive, P.L. The role of DNA single- and double strand breaks in cell killing by ionizing radiation. **Radiat. Res.** 150 (1998) 42-51.
21. Breen, A.P. and Murphy, J.A. Reactions of oxyl radicals with DNA **Free Radic. Biol. Med.** 18 (1995) 1033-1077.
22. Cohen, S.M. and Lippard S.J. Cisplatin: from DNA damage to cancer chemotherapy. **Prog. Nucleic Acid Res. Mol. Biol.** 67 (2001) 93-130.
23. Jordan, P. and Carmo-Fonseca, M. Molecular mechanisms involved in cisplatin cytotoxicity. **Cell. Mol. Life Sci.** 57 (2000) 1229-1235.
24. Beck, W.T., Mo, Y.Y. and Bhat, U.G. Cytotoxic signalling by inhibitors of DNA topoisomerase II. **Biochem. Soc. Trans.** 29 (2001) 702-703.
25. Kreuser E.D., Wadler S. and Thiel E. Biochemical modulation of cytotoxic drugs by cytokines: molecular mechanisms in experimental oncology. **Recent Results Cancer Res.** 139 (1995) 371-382.
26. Widłak, P. The DNA damage-induced cell cycle checkpoints. **J. Theor. Med.** 2 (2000) 237-243.
27. Łanuszewska, J., Cudak, A., Rzeszowska-Wolny, J. and Widłak, P. Detection of damage-recognition proteins from human lymphocytes. **Acta Biochim. Pol.** 47 (2000) 443-450.