Drugs under

experimental and clinical

Research

Update on Ukrain

This work has been carried out under the auspices of the Austrian Ministry of Science and Research. Special thanks are due to Division II, Science and Technology, and to its Director Section-Chief Dr. Norbert Rozsenich.

Supplement to Vol. XXII, 1996

BIOSCIENCE EDIPRINT INC.

DRUGS EXPTL. CLIN. RES. XXII(Suppl.) 1-7 (1996)

INDUCTION OF BIMODAL PROGRAMMED CELL DEATH IN MALIGNANT CELLS BY THE DERIVATIVE UKRAIN (NSC-631570)

LIEPINS A.[1*], NOWICKY J.W.[2], BUSTAMANTE J.O.[3], LAM E.[1]

1) Faculty of Medicine, Memorial University, St. John's, Newfoundland, A1B 3V6, Canada.
2) Ukrainian Anti-Cancer Institute, Margaretenstrasse 7, 1040 Vienna, Austria.
3) Department of Physiology, University of Maryland School of Medicine, Baltimore, MD, 21201-1559, USA.

Summary: *Selective induction of malignant cell death is one of the major goals of effective and safe chemotherapy. Recent developments in the understanding of programmed cell death (PCD) or apoptosis are expected to provide new leads for a safer chemotherapy. The authors investigated whether the semisynthetic alkaloid thiophosphoric acid derivative Ukrain (NSC-631570) could induce PCD or apoptosis in human K562 leukaemia cells. Results showed that Ukrain induced two distinct modalities of cell death programmes. One modality corresponded morphologically to classical apoptosis or PCD characterized by blebbing and shedding of membrane vesicles with concomitant ^{51}Cr release; however, the Ukrain-induced apoptosis was not associated with the characteristic nuclear DNA fragmentation. Higher concentrations of Ukrain induced a second cell death programme characterized by cell surface blister formation, high specific ^{51}Cr release and extensive DNA polyploidy. These two cell death programmes are distinct from each other in that they are interphased by a silent period characterized by normal cell morphology and reduced specific ^{51}Cr release.*

Introduction

The discovery and development of drugs that could induce either selective lethal damage to malignant cells directly (chemotherapy) or via the activation of immune effector cells (BRM) are two of the major strategies in the development of new cancer therapies. Programmed cell death (PCD), or apoptosis, is a physiologically active cell process characterized by cell surface blebbing, changes in membrane permeability, elevated oxygen consumption rates and nuclear DNA condensation and fragmentation (1). The latter event, i.e., internucleosomal DNA fragmentation, may not occur in some experimental systems (2). Blebbing of the cell surface membrane has consistently been found during T-cell mediated tumour cell cytolysis (3), exposure to chemotherapeutic drugs (4), low temperature (5), anoxia (6), as well as during normal embryonic development (7). It is thought that the death of normal and malignant cells is under the control of intrinsic genetic mechanisms which, when properly executed,

* *Author to whom correspondence should be addressed.*

0378-6501/96/000001 + 7 $2.50/0

results in normal embryonic development (8), whereas failure to express the putative death genes may result in malignant cell growth (9). It is estimated that there are about 100 oncogenes that, when malfunctioning, stimulate cell growth; conversely there are at least six tumour suppressor genes (10). Thus the genetic options for malignant growth far outweigh the limited tumour suppressor genes.

From the practical point of cancer therapy, the ideal chemotherapeutic agent would be one that (a) triggers tumour cell death selectively, (b) does not activate the expression of the multidrug resistant genes, and c) when the multidrug resistance (MDR) phenotype is already expressed, i.e., P-glycoprotein, it would still exert its tumour selective cytolytic activity.

Ukrain (NSC-631570) was screened *in vitro* by the National Cancer Institute (NCI) Developmental Therapeutics Program (USA) and found to have cytotoxic effects on their 60 human tumour cell lines representing eight tumour types (11). Results of these assays showed that the mean molar concentration required to achieve 50% cell growth inhibition was $10^{-5.5}$ M; $10^{-4.8}$ M for total cell growth arrest and $10^{-4.17}$ M for 50% loss of the initial cell biomass (11). In view of this evidence, we set out to investigate whether Ukrain would induce programmed cell death (PCD) or apoptosis in human malignant cells. For this purpose we chose the K562 leukaemia cell line found to be relatively resistant to the cytostatic and cytotoxic effects of Ukrain in the NCI-Developmental Therapeutics *in vitro* tests. This leukaemia cell line was reported to have a GI-50 of 2.32×10^{-6}, a TGI of 1.85×10^{-5} and a LC_{50} of $> 3.83 \times 10^{-4}$ M.

Material and methods

The K562 erythroleukaemia cell line was purchased from the ATCC (Rockville, MD, USA) and maintained in culture according to the information accompanying cell shipment (RPMI-1640 + 10% FBS). Cells were passaged as necessary and maintained at 37°C, 5.0% CO_2. Ukrain (NSC 631570) $C_{66}H_{75}N_6O_{18}PS.6HCl$ was produced in pure crystallized form by J.W. Nowicky and dissolved in water at a concentration of 1.0 mg/ml. Further dilution, carried out shortly before *in vitro* use, was done in tissue culture medium (RPMI-1640 + 10% FBS).

Cell morphology: Ukrain was serially diluted in RPMI + 10% FBS to give a concentration range of 100 µg/ml to 0.78 µg/ml (8 dilutions) in flat bottom 96 well plates. Triplicate wells of each drug concentration and containing 50,000 cells per well were incubated at 37°C + 5.0% CO_2 for three hours. Morphological changes were monitored and photographed after four hours drug exposure.

Membrane permeability changes: Cell membrane permeability changes were evaluated by $Na_2{}^{51}CrO_4$ release assay (12). For this purpose cells were labelled with 200 µCi of $Na_2{}^{51}CrO_4$ for 1-2 h at 37°C, and washed to remove unincorporated $Na_2{}^{51}CrO_4$. In these experiments V-bottom 96-well plates were used. The effects of the various concentrations of Ukrain (100 µg/ml down to 0.78 µg/ml) were evaluated in triplicate wells at 20,000 cells per well after four hours incubation at 37°C in 5.0% CO_2. The percentage of ^{51}Cr release was calculated by the following formula:

$$\% \text{ specific } {}^{51}Cr \text{ released} = \frac{\{\text{cells + Ukrain}\} - \text{spontaneous release} \times 100}{\{\text{maximum 51 release}\} - \text{spontaneous release}}$$

Spontaneous ^{51}Cr release is the amount of ^{51}Cr released by cells in the absence of drug; maximum ^{51}Cr release is the ^{51}Cr released by exposing the cells to 1.0% of Triton x-100 detergent.

Cellular DNA content assays: The DNA content of K562 cells exposed to various concentrations of Ukrain were analysed using propidium iodide and flow cytometry. For this purpose 0.5×10^6 K562 cells per well were aliquoted in 12-well plates and exposed to eight serial dilutions of Ukrain ranging from 100 µg/ml down to 0.78 µg/ml for four hours at 37°C. After this time-period each well received 2.0 ml PBS buffer containing 2.0%

formaldehyde and cells were transferred to 12 x 75 mm tubes, pelleted by centrifugation and the medium removed. Cell pellets were treated with 200 µl of ice cold ethanol for 10 min and washed twice in PBS buffer and treated with RNAase (100 µg/ml) for 30 min at 37°C, followed by the addition of 200 µl of propidium iodide (50 µg/ml in 0.6% NP-40 detergent). The fluorescence intensity of cells was analysed in a Becton Dickinson flow cytometer.

Results

The morphology of K562 erythroleukaemia cells is illustrated in Fig. 1A. When these cells were exposed to various concentrations of Ukrain and periodically observed microscopically, it was found that at 2-4 h the classical morphology of PCD or apoptosis was manifest in ≥ 90% at concentrations of 6.25 or 12.5 µg/ml of drug (Fig. 1B). This morphology was manifest in at least two concentrations of Ukrain. Note that these cells displayed blebbing and membrane vesicles over the entire cell surface, consistent with current criteria of PCD or apoptosis. The unexpected morphological finding occurred at a higher drug concentration, i.e., 25 µg/ml, where the morphology of apoptosis was no longer manifest. We refer to this drug concentration interval as the silent period (SP) based on the fact that these cells displayed morphology similar to control cells not exposed to the drug (Fig. 1C). However, at higher concentrations of Ukrain (50-100 µg/ml) we discovered that >90% of these cells displayed predominantly one or two large membrane blebs or blisters (Fig. 1D). Thus, Ukrain induced the classical morphology of apoptosis followed by a SP and subsequent blistering of the cell surface (Fig. 1D).

In order to establish the relationship between cell morphology and the criteria of cell injury and death, we used the ^{51}Cr-release assay which is the standard and universal assay of immuno-effector cell lysis of tumour cells (12). Results of the ^{51}Cr release assays showed unexpected bimodal isotope release kinetics (Fig. 2). Significant ^{51}Cr release was detected at 6.25 and 12.5 µg/ml of Ukrain followed by a decline to background level at 25.0 µg/ml, i.e., silent period. These concentrations correspond to those that induce the classical morphology of apoptosis (Fig. 1B) and the silent period at 25 µg/ml of Ukrain (Fig. 1C). The following two-step increases of drug (50 and 100 µg/ml) produced a rapid rise in the specific ^{51}Cr release kinetics (Fig. 2) which corresponded to the drug concentrations that induced cell blister formation (Fig. 1D). Thus, concordance was found between the bimodal morphological changes (Fig. 1) and the bimodal specific ^{51}Cr release kinetics of Fig. 2.

DNA content of K562 cells treated with Ukrain was analysed by standard agarose gel electrophoresis, as previously used in our laboratory (13). Repeated attempts to find low molecular weight DNA, i.e., DNA fragmentation, were unsuccessful (data not shown). Consequently we used flow cytometry and propidium iodide staining of DNA to evaluate DNA integrity and content in cells treated with the various concentrations of Ukrain. Results showed that no significant changes in K562 cell nuclear DNA content could be detected within the concentration range of 0.78 through 25.0 µg/ml of Ukrain (Fig. 3). The cells did not show any morphological changes until the Ukrain concentration reached 3.12 and 12.5 µg/ml, when cells displayed the classical morphology of apoptosis or PCD characterized by cell surface blebbing and membrane vesicle formation (Fig. 1B). However, this cell morphology was not associated with significant changes in cell DNA content (Fig. 3). Moreover, cells exposed to 25.0 µg/ml of Ukrain showed no morphological changes detectable at the light microscope level (Fig. 1C), nor in nuclear DNA content (Fig. 3). Further doubling in drug concentrations to 50.0 and 100 µg/ml produced extensive increase in cell DNA content and hence polyploidy (Fig. 3), with concomitant changes in cell morphology which consisted in the formation of one or more cell surface blisters (Fig. 1D).

Discussion

Programmed cell death (PCD) or apoptosis has

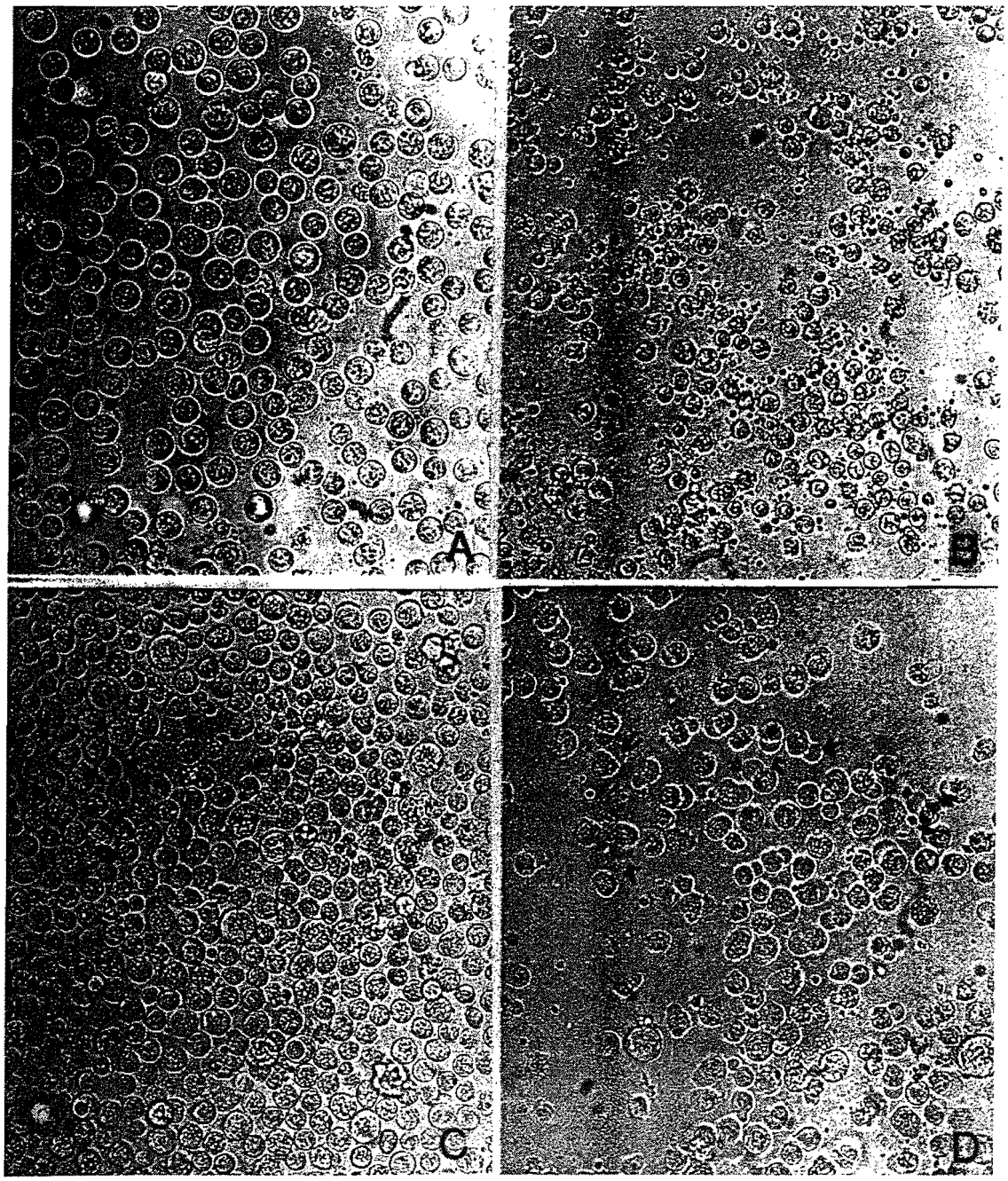

Fig. 1 Effects of Ukrain on the morphology of human K562 cells. A: control K562 cells cultured 4 h without drug. B: cells cultured 4 h in the presence of 6.25 µg/ml Ukrain display classical morphology of apoptosis or PCD, characterized by blebbing and shedding membrane vesicles from tumour cell surfaces. C: K562 cells treated with 25.0 µg/ml Ukrain enter the silent period characterized by normal cell morphology, which corresponds to low background levels of specific ^{51}Cr release (cf. Fig. 2). D: K562 cells treated with 50.0-100.0 µg/ml Ukrain display cell surface blister formation associated with high specific ^{51}Cr release (cf. Fig. 2).

generally been found to correlate with nuclear DNA fragmentation (13) and is considered to be the hallmark of apoptosis (14,15). This DNA fragmentation is thought to occur at the internucleosomal regions due to the activation of a specific endonuclease (16). Not all cells, however, manifest a strict correlation between the morphology of apoptosis and nuclear DNA fragmentation (17). To our knowledge there is only one report in the literature indicating that taxol induced a nominal degree of polyploidy in K562 cells treated with 10 µM of this drug for 24 h (18). At short time-intervals, i.e., four hours, no significant K562 cell ploidy could be detected whereas at 12 h an increase in cells at the G2 phase of the cell cycle could be detected (18).

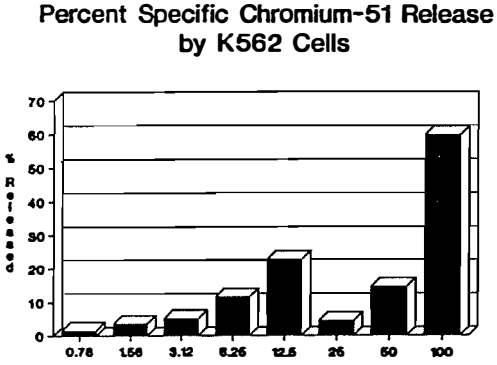

Fig. 2 Bimodal specific ^{51}Cr release by K562 cells treated with various concentrations of Ukrain for 4 h *in vitro*. Note that the first phase of ^{51}Cr release occurs at 6.25 and 12.5 µg/ml Ukrain, followed by background level of ^{51}Cr release at 25.0 µg/ml (silent period); high specific ^{51}Cr release occurs at 100.0 µg/ml which corresponds to cell surface blister formation (Fig. 1D).

Contrary to the aforementioned studies, our data showed that Ukrain was highly effective in inducing polyploidy in K562 cells within 3-4 h. As illustrated in Fig. 3, Ukrain at concentrations of 50.0 µg/ml induced disappearance of cells in the G1 phase by shifting the majority of them to a triploid and polyploid state of DNA content (Fig. 3), whereas at 100.0 µg/ml, Ukrain caused a higher degree of cell ploidy beyond the tetraploid G2 (4c) phase and even beyond octaploid DNA content (Fig. 3). It is significant that these high levels of DNA content correlated with the cells with their morphology at the blister stage (Fig. 1D). Moreover, these drug concentrations induced the second phase of high specific ^{51}Cr release (Fig. 2). Thus, these results demonstrate a high degree of concordance among cell morphological changes, high specific ^{51}Cr release and an extensive increase in DNA content, all of which occurred in four hours or less of exposure to Ukrain. To our knowledge there is nothing in the literature describing this second multiparameter mode of cell injury which we have denoted as blister cell death (BCD). This cell death modality was sensitive to cyclohexamide, indicating that protein synthesis was required for BCD to occur (data not shown). Moreover, fluorescence microscopy of propidium iodide-stained cells showed no multinucleation indicating that the extensive increase in DNA content was intranuclear.

Cell surface blister formation in hepatocytes induced by hypoxia have been reported to produce blebbing and/or blister formation in more than 70% of the cells (6). This morphology was found to be independent of intracellular Ca^{2+} fluxes, and the rupture of these large cell surface blebs was considered by the authors to represent the abrupt transition from reversible to irreversible cell injury. This interpretation of blister formation and eventual rupture is in agreement with out findings where such cell morphology correlates with the loss of clonogenic potential of cells exposed to Ukrain for only four hours. Thus, our findings are unique in that we have identified two distinct modalities of cell injury and potential death induced by Ukrain in short term culture conditions. The unique and intriguing finding that polyploidy is associated with the second cell death modality, i.e. BCD, with concomitant high levels of specific ^{51}Cr release, strongly suggests that these parameters define irreversible cell injury and hence cell death. On the other hand, the classical morphology of apoptosis, i.e., cell surface blebbing and shedding of membrane vesicles without significant changes in DNA content and only temporal changes in membrane permeability to ^{51}Cr,

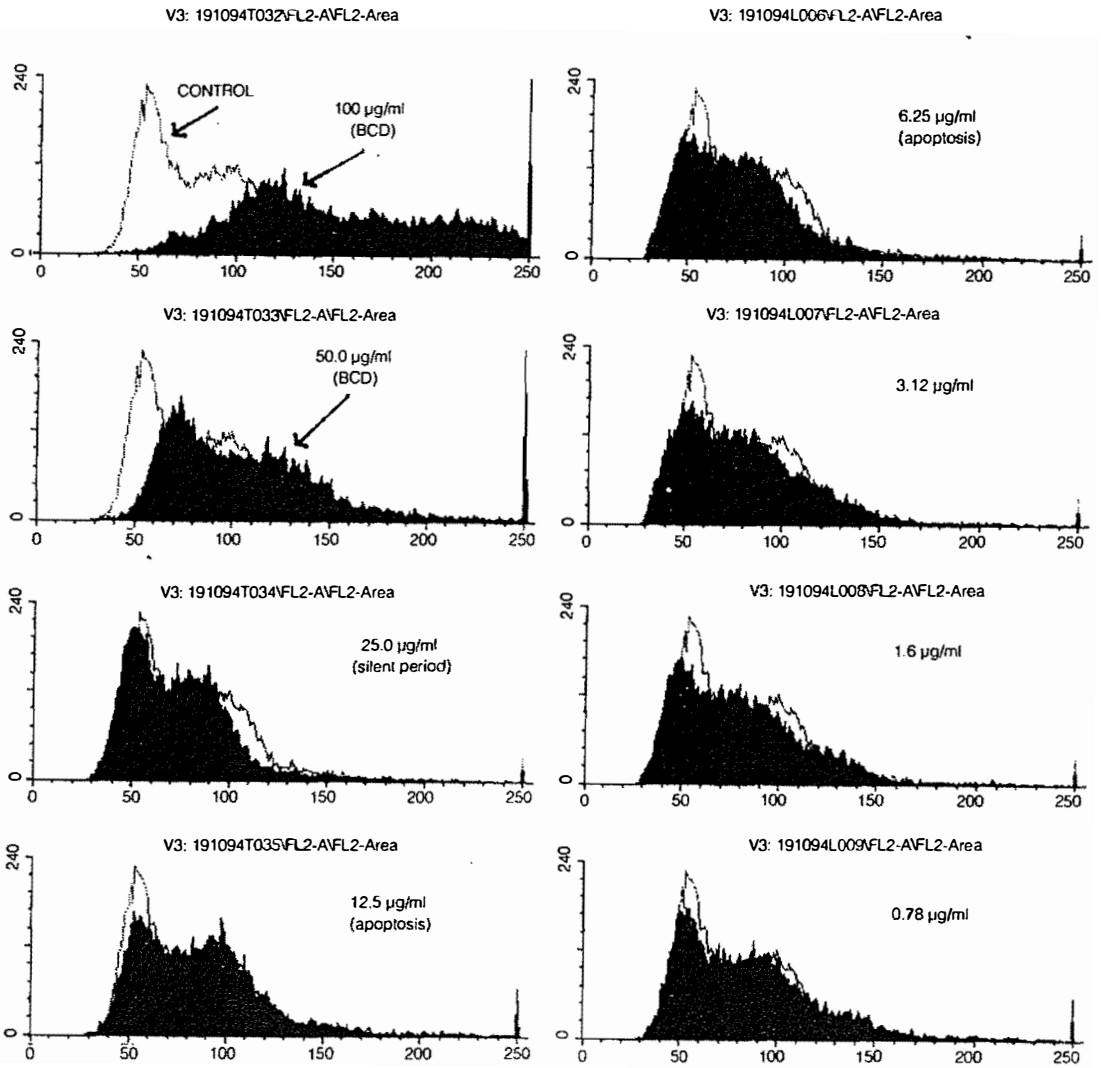

Fig. 3 Effects of Ukrain on K562 cell DNA content measured by propidium iodide staining and flow cytometry. Note that at 100.0 and 50.0 µg/ml Ukrain for 4 h, extensive DNA ploidy is induced. This increase in nuclear DNA content corresponds to cell surface blister formation (Fig. 1D) and high specific 51Cr release (Fig. 2). Serial dilutions of Ukrain showed cells with normal diploid DNA content (from 25.0 down to 0.78 µg/ml). Note the morphology of apoptosis or programmed cell death (PCD) stage, (Fig. 1B) in association with normal DNA content. BCD: blister cell death.

suggests that these parameters may represent reversible cell injury or the manifestation of a cell detoxification process similar to that of the induction of multidrug resistance phenotype.

We postulate that Ukrain induces bimodal cell death programmes, the first of which, apoptosis, is mediated by quinidine sensitive Ca^{2+}-dependent K^+ channels; the second modality, blister cell death, is mediated by preventing microtubule formation and thus inducing polyploidy.

References

(1) Liepins A. *Morphological, physiological and biochemical parameters associated with cell injury.* Immunopharmacol. Immunotoxicol., **11**, 539, 1989.

(2) Stewart B.W. *Mechanisms of apoptosis: integration of genetic, biochemical and cellular indicators.* J. Natl. Cancer Inst., **86**, 1286, 1994.

(3) Liepins A., Faanes R.B., Choi Y.S., de Harven E. *Lymphocyte mediated lysis of tumor cells in the presence of alloantiserum.* Cell Immunol., **36**, 331, 1978.

(4) Kaufman S.H. *Induction of endonucleotide DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note.* Cancer Res., **49**, 5870, 1989.

(5) Liepins A., Younghusband H.B. *Low temperature induced cell surface membrane vesicle shedding is associated with DNA fragmentation.* Exp. Cell Res., **161**, 525, 1985.

(6) Lemasters J.J., Di Guiseppi J., Nieminen A.L. *Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes.* Nature, **325**, 78, 1987.

(7) Yuan J.Y., Horvitz H.R. *The* Caenorhabditis elegans *genes ced-3 and ced-4 act autonomously to cause programmed cell death.* Dev. Biol., **138**, 33, 1990.

(8) Cotter T.G. et al. *Cell death via apoptosis and its relationship to growth, development and differentiation of both tumour and normal cells.* Anticancer Res., **10**, 1153, 1990.

(9) Vaux D.L., Haeker G., Strasser A. *An evolutionary perspective in apoptosis.* Cell, **76**, 777, 1994.

(10) Schwartz L.M., Osborne B.A. *Programmed cell death, apoptosis and killer genes.* Immunol. Today, **14**, 582, 1993.

(11) Nowicky J.W., Nowicky W., Liepins A. *Cytostatic and cytotoxic effects of Ukrain malignant cells.* J. Chemother., **5** (Suppl. 1), 797, 1993.

(12) Liepins A. *Possible role of microtubules in tumor cell surface membrane shedding, permeability and lympholysis.* Cell Immunol., **76**, 120, 1983.

(13) Liepins A., Younghusband H.B. *A possible role of K^+ channels in tumor cell injury: membrane vesicle shedding and nuclear DNA fragmentation.* Exp. Cell Res., **169**, 385, 1987.

(14) Arends M.J., Morris R.G., Wyllie A.H. *Apoptosis. The role of endonuclease.* Am. J. Pathol., **136**, 593, 1990.

(15) Compton M.M. *A biological hallmark of apoptosis: internucleosomal degradation of the genome.* Cancer Metastasis Rev., **11**, 105, 1992.

(16) Barry M.A., Eastman A. *Identification of deoxyribonuclease II as an endonuclease involved in apoptosis.* Arch. Biochem. Biophys., **300**, 440, 1993.

(17) Ucker D.S. et al. *Genome digestion is a dispensable consequence of physiological cell death indicated by cytotoxic T lymphocytes.* Mol. Cell. Biol., **12**, 3060, 1992.

(18) Roberts J.R., Allison D.C., Donehower R.C., Rowinsky E.K. *Development of polyploidization in taxol-resistant human leukemia cells* in vitro. Cancer Res., **50**, 710, 1990.